Instituto Juan March
de Estudios e Investigaciones

98 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Telomeres and Telomerase: Cancer, Aging and Genetic Instability

Organized by

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Introduction

María A. Blasco

The extremists: telomeres, telomerase and associated partners

Incomplete duplication or aberrant segregation of chromosomes eventually compromises the proliferative potential of eukaryotic cells, limiting their life span. Increasing evidence indicates that the ends of chromosomes, or telomeres, have a fundamental role in both crucial moments of cell division. Telomere biology is essential to understand the molecular clocks underlying cell proliferation. Most importantly, proteins that act at the telomeres such as the enzyme telomerase and other telomere-associated proteins have become targets to control pathological aspects of cell division such as ageing or cancer. For these reasons, the structure and function of telomeres, as well as that of associated proteins, has become the focus of intense research during the last years. The role of telomerase components, telomerase-associated proteins and other telomere-binding proteins is being unravelled at the moment using both biochemical and genetic approaches.

In human cells, telomere length decreases proportionally to the number of times that a cell divides, presumably due to the incomplete replication of the DNA lagging strand. The rate of telomere shortening is 80 to 150 bps per cell. One of the key factors involved in regulating telomere is a reverse transcriptase called telomerase. Telomerase is a ribonucleoprotein DNA polymerase that is able to synthezise "de novo" telomeres onto chromosome ends. The construction and characterization of knock out strains for telomerase activity in different organisms has proven that telomerase is indeed essential to maintain telomeres and that the loss of telomeres is associated with genomic instability and the loss of cell viability. In mammals, telomere shortening has been associated to defects during embryonic development, in the germ line, and in the highly proliferative tissues of the adult. The precise regulation of telomerase during normal development tumor growth, however, remains unknown. The isolation of the different telomerase components together with the study of cell systems that lack telomerase will help to understand the regulation and function of this enzyme in mammals.

In yeast, deletion of the telomerase RNA gene leads to telomere shortening and, after a lag period of about 40 generations, to the loss of cell viability. It is possible to obtain "survivor" cells in yeast strains that have active recombination pathways and lack telomerase activity. In these survivors, telomeres are apparently lengthened via a recombination mediated gene conversion mechanism. Recent experiments suggest that a bypass mechanism for telomerase may also operate in mammalian cells. Telomerase-negative mammalian cell lines have been identified and in most cases the telomeres are elongated to 2 or 3 times their normal size. The identification of telomerase-independent telomere maintenance in mammalian cells is essential to understand telomere biology. The study of mouse strains deficient for telomerase activity and/or DNA recombination or DNA repair proteins will help to identify these mechanisms and to establish their physiological relevance.

All these different aspects of telomere biology have been the subject of the current March Foundation Workshop "Telomeres and telomerase: cancer, aging and genetic instability". This meeting has provided a forum were recent discoveries have been reported and discussed by the telomere and telomerase scientific community.

María A. Blasco

Session 1: Yeast telomeres

Chair: María A. Blasco

Regulation of telomerase in *Saccharomyces*: Zakian, VA, Teng, S.-C., and McCowan, B. Molecular Biology, Princeton University, Princeton NJ 08544 USA

Telomere length is tightly regulated in *Saccharomyces*. The Rif proteins are *in vivo* telomere binding proteins (Bourns et al., 1998) whose mutation is known to cause telomere lengthening (Hardy et al., 1992; Wotton and Shore, 1997). This lengthening is dependent on *TLC1*, which encodes the RNA component of telomerase but independent of *RAD52*, which encodes a protein required for most recombination events in mitotic yeast cells. Thus, Rif proteins restrict access of telomeres to telomerase. Most cells lacking yeast telomerase die but survivors arise by *RAD52* dependent events: the majority of these survivors have tandem duplications of the sub-telomeric Y' element (Lundblad and Blackburn, 1993). However, about 10% of the survivors have a different telomeric structure, very long tracts of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA, with some telomeres being as much as 12 kb longer than telomeres in wild type cells. This pattern of exceptionally long telomeres is similar to that seen in human cell lines and tumors that maintain telomeric DNA without expressing telomerase (Bryan et al., 1995; Bryan et al., 1997). The maintenance of these long telomeres requires the continuous presence of Rad52p. However, even in the presence of Rad52p, these long telomeres steadily shorten, suggesting that even in cells that maintain their telomeres by telomere-telomere recombination, recombinational mediated telomere lengthening is relatively rare. In addition to restricting access of telomeres to telomerase, Rif proteins appear to inhibit telomere-telomere recombination: most survivors arising in a *rif tlc1* strain have very long telomeres, rather than tandemly duplicated Y' DNA. Thus, telomere structural proteins can influence both telomerase dependent and telomerase independent pathways for maintenance of telomeric DNA.

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DYNAMICS OF TELOMERE LENGTH REGULATION IN YEAST

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The length of telomeric DNA reflects an equilibrium between its elongation and degradation. Telomeric repeats inserted in the vicinity of a telomere are taken into account by the length regulation mecanism. We took advantage of this to develop an experimental system to follow the elongation of a single yeast telomere.Two specific sites for the Flp1p recombinase were inserted between the internal and distal telomeric repeats and more internally downstream of a marker gene. Recombination by Flp1p deletes the internal telomeric repeats, leaving an abnormally short telomere of ~100-bp. This allows us to follow the kinetics of elongation of a telomere in a population of yeast cells. We observe that telomere elongation is restricted to a few base pairs per generation and that its rate decreases progressively with increasing telomere length. By contrast, in absence of telomerase or in presence of an over-elongated telomere, the degradation rate linked to the succession of generations appears to be a constant, that is independent of telomere length. Together, these results indicate that telomerase is gradually inhibited at its site of action by the elongating telomere.

We are currently determining how elongation proceeds at different cell cycle stages. We observe a lack of telomere elongation in stationary phase and during a cell cycle block in G1 or in G2/M. In a synchronous population of cells, telomere elongation can be detected in cells progressing through S-phase. The implications of these findings for the dynamics of telomere length regulation are discussed.

FACTORS AFFECTING TELOMERIC SILENCING, TELOMERE FORMATION, AND TELOMERE LENGTH REGULATION

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Telomere length in the yeast *S. cerevisiae* is controlled at least in part by the TG_{1-3} repeat binding protein Rap1 and two interacting factors Rif1 and Rif2. In the course of investigating the function of this regulatory system in the presence of mutations in other genes affecting telomere length, we noted that mutation of either the Ku70 or Ku80 genes (*HDF1* and *HDF2*) leads to a dramatic loss of telomere position effect (TPE). Evidence will be presented that this Ku mutant phenotype cannot be explained by telomere shortening, but instead appears to be due to a specific defect in the recruitment of the silencing factor Sir4 to the telomere. Yeast Ku protein probably plays a direct role in TPE since Gal4 DNA binding domain-Hdf1p hybrids can initiate silencing when targeted to either a telomere or a silent mating-type locus. Remarkably, the Ku mutant TPE defects can be suppressed by mutation of *RIF* genes. We interpret these observations in terms of a model in which Rif proteins compete with Sir4, and perhaps other silencing factors, for access to the telomere-binding proteins Rap1 and Ku70/80.

In a separate study we have investigated the *cis* requirements for telomere formation and proper telomere length regulation. In summary, telomere 'healing' (during transformation) can be obtained with linear molecules terminating in as few as 4 synthetic (non-TG repeat) Rap1p binding sites, but the efficiency of this reaction is improved dramatically by additional sites. Using this telomere healing assay we have been able to confirm an earlier prediction that telomere length regulation is correlated with the number of Rap1p binding sites at the chromosome end. However, the precise spacing of these sites is important for length regulation, for reasons still under investigation.

Interestingly, mis-oriented Rap1p binding sites can contribute to both the efficiency of telomere healing and telomere length regulation, despite the fact that they do not appear to serve as sites for telomerase addition. This fact has allowed us to generate artificial telomeres with unusually large numbers of Rap1p binding sites, most of which are mis-oriented. Consistent with our Rap1p 'counting' model for telomere length regulation, such telomeres are unstable. Using this property, we have devised a genetic screen to identify new mutations affecting telomere length regulation.

YEAST TELOMERASE: ANALYSIS OF PROTEIN SUBUNITS AND IN VIVO REGULATION.

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Our laboratory has previously used a genetic screen in *S. cerevisiae* to detect components and/or regulators of telomerase, which has led to the identification of three *EST* genes (*EST1*, *EST2* and *EST3*, for ever shorter telomeres) genes, as well as *CDC13*. *EST2* encodes a reverse transcriptase that comprises the catalytic subunit of telomerase and is required for core telomerase activity. In contrast, the Est1 and Est3 proteins are essential for telomerase function *in vivo* but dispensable for enzymatic activity *in vitro*. Therefore, we propose that these additional proteins function as *in vivo* regulators of enzyme action, either as regulatory subunits of the telomerase holoenzyme or alternatively as components of telomeric chromatin. Both Est1p and Est3p associate with telomerase, as evidenced by immunoprecipitation experiments, but interact differentially with the core enzyme: Est1 is present in a complex with TLC1 even in the absence of either Est2p or Est3p, whereas co-immunoprecipitation of Est3p and TLC1 depends on a functional Est2 protein. In order to more clearly define the mechanistic role of these two proteins, we have performed detailed site-directed mutational analysis, which has yielded several distinct classes of separation-of-function alleles for each gene. The results of genetic analysis of these mutants, as well as extragenic suppression screens, will be described.

Session 2: Mammalian telomeres

Chair: Virginia A. Zakian

The structure and function of the mammalian telomeric complex

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Mammalian telomeres are composed of duplex array of short tandem repeats, a 3' overhang of single-stranded telomeric DNA, and telomere specific proteins, including the telomeric repeat binding factors, TRF1 and TRF2. TRF1 is a regulator of telomere length and interacts with the telomere specific PARP tankyrase which may contribute to telomere length maintenance. New data indicate that TRF2 also contributes to telomere length regulation.

The most prominent function of TRF2 is in protection of chromosome ends. Inhibition of TRF2 results in immediate deprotection of telomeres as manifested by the loss of the 3' overhang, the activation of DNA damage checkpoints, apoptosis, and fusion of telomeres. However, the mechanism by which TRF2 prevents exposure of telomeres to cellular activities acting on DNA ends has remained unclear. We recently showed that TRF2 has the ability to sequester the telomere terminus in a large duplex loop, referred to as telomeric or t-loop, and provide evidence for the frequent presence of t-loops at telomeres in vivo.

Incubation of a model telomeric DNA with TRF2 *in vitro* was found to produce t-loops detectable by electron microscopy, prompting an examination of the *in vivo* structure of telomeres by electron microscopy of psoralen crosslinked, purified telomeric DNA. This revealed frequent t-loops containing telomeric repeat DNA with a size distribution consistent with their telomeric origin. A short bubble of single stranded DNA was demonstrable at the tail-loop junction consistent with invasion of the 3' overhang of telomeres into the duplex repeat array. We propose that the protection of natural chromosome ends critically depends on the sequestration of the telomere terminus in TRF2-induced t-loops. This model provides a general architectural solution for the problems posed by chromosome ends, including aspects of the maintenance of telomeric DNA.

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Telomere length dynamics in non-transformed human and murine cells

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The loss of telomere repeats has been causally linked to *in vitro* replicative senescence of human diploid fibroblasts (HDFs). In order to study the mechanism(s) by which telomere shortening signals cell senescence, we analyzed the telomere length at specific chromosome ends at cumulative population doublings in polyclonal and clonal HDFs by quantitative fluorescence in situ hybridization (Q-FISH). Replicative capacity was not proportional to the length of short telomeres present on chromosomes such as 17p, 19p or 20q. In contrast, significant correlations with the telomere length of chromosomes 1p, 5p and 22q were found. Despite the gradual decline of the overall telomere length with replication, the rate of telomere shortening varied between individual telomeres. In addition, rapid decreases in length as well as maintenance and occasional extension of short telomeres in subsequent passages of HDFs were observed. These results suggest that telomerase independent pathways are important for the maintenance of functional telomeres in normal diploid cells. Importantly, the results argue against induction of replicative senescence by DNA damage signals originating from the shortest telomere.

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Accelerated telomere shortening in the human inactive X chromosome?

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The length of the telomeres of the X chromosomes and the autosomes was measured in blood lymphocytes of new born[**] (n=5; umbilical cord), middle age (n=5; 34.6±4.8 years old), and elderly women (n=5, 63.4±4.0 years old) by combining X chromosome centromere specific fluorescence in situ hybridization (FISH) and quantitative FISH with a peptide nucleic acid telomeric probe[1,2]. The activation status of the X chromosomes was simultaneously visualized with antibodies against acetylated histone H4, a cytogenetic marker for gene expression[3,4]. We observed that the telomeres of the inactive X chromosome (Xi) were consistently shorter than the telomeres of all chromosomes, including the active X chromosome (Xa), in the middle age group (P=0.002) and in the elderly donors (P<0.00001) but not in new born individuals (P=0.8). This observation is especially true for the long arm of the Xi which, unlike the pseudoautosomic short arm, is completely heterochromatic, hypermethylated and composed of underacetylated histones. To our knowledge, this is the first evidence to show a differential rate of telomere shortening between and within homologous chromosomes in any species. Among the human chromosomes, the X chromosomes are known to be very prone to aneuploidy, especially in ageing women[3]. As recent evidences suggest a role of telomeres in chromosome segregation in mammalian cells[5,6], our data is consistent with a potential causative role of telomere shortening in increased X chromosome loss in humans. Considering the fact that the human Xi is a highly condensed looped chromosome with telomere association[7], our results also suggest that chromatin structure and nuclear architecture could modulate not only the length but also the rate of telomere shortening in human cells *in vivo*. Another interesting possibility is that X inactivation itself can lead to accelerated telomere shortening.

[*]These two authors contributed equally to this work.

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[**]Special thanks to Dr. Sergi Querol, Umbilical Cord Blood Bank Project, Cancer Research Institute, Spanish Eurocord Cord Blood Bank, Jose Carreras Foundation Cord Blood Program, and to Dr. F. Baro, Gynaecology and Obstetrics Service, Vall d'Hebro Hospital, Barcelona.

TELOMERASE AND TELOMERE LENGTH MAINTENANCE IN MAMMALIAN CELLS

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Telomerase independent mechanisms of telomere maintenance have been characterized in yeast and are also found in mammalian cells. In yeast lacking telomerase, telomeres shorten and viability is severely reduced, but survivors are generated spontaneously by a gene conversion mechanism. The generation of survivors is dependent on the *RAD52* gene. Mice in which the telomerase RNA has been deleted (mTR-/-) are viable and fertile and do not display gross abnormalities in early generations. To test whether recombination plays a role in telomere length maintenance, or on long term survival in the absence of telomerase in mice, we crossed the mTR-/- mice with mice deleted for the mRAD54 gene. *RAD54*, a member of the *RAD52* epistasis group, plays an important role in double strand break repair. Yeast null for both telomerase and *rad54* die more quickly than telomerase single mutants (1). Although the frequency of mRAD54-/- mTR-/- mice was significantly lower than the expected Mendelian ratio, these mice are also viable and fertile. The litter size of the double null mice was reduced compared to wildtype litters. In addition, the litter sizes of the double knock-out animals are progressively smaller with increasing generation. We are now examining the telomeres of these mice to determine how telomere length relates to the decreased litter size.

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Cellular Crisis and Cancer R. DePinho(1), L. Chin (1), S. Artandi(1), R. Greenberg(2), L. Rudolph(1), G. Gottlieb, R. Singer(2), C. Greider(3). (1) Dana Farber Cancer Institute of Harvard Medical School, Boston MA; (2) Albert Einstein College of Medicine, Bronx NY; (3) Johns Hopkins School of Medicine, Baltimore MD.

Telomere maintenance is thought to be required for the efficient proliferation of immortalized and transformed cells. The telomerase knockout mouse lacks the mTR gene encoding the RNA component of the telomerase holoenzyme. We have shown that critical telomere shortening induces apoptotic and growth arrest responses in highly proliferative organs in vivo. We now show that telomere dysfunction in late generation mTR-/- organs and cells activates and stabilizes p53. Furthermore, in late generation mTR-/- mice that have been rendered null for p53, these growth arrest and apoptotic checkpoints are abrogated, resulting in rescue of the male germ cell depletion and the MEF cell cycle blocks seen when p53 is intact. To determine the effect of telomere dysfunction on tumorigenesis, we generated mTR-/- mice that also lacked the INK4a tumor suppressor. Telomere dysfunction significantly impaired tumor formation in vivo in late generation mTR-/- INK4a-/- mice compared to early generation double null mice with ample telomere reserve. Telomere shortening also reduced the efficiency of transformation in MEFs from late generation mTR-/- INK4a-/- mice in the myc/RAS transfection assay and this reduction was rescued by restoring the mTR gene. The mTR-/- INK4a-/- mice harbor intact p53-DNA damage pathways which likely eliminate developing tumor cells with ongoing telomere dysfunction. To test this hypothesis, late generation mTR-/- p53-/- MEFs were transfected with myc/RAS/mTR or myc/RAS/vector. Restoring telomere function with mTR suppressed focus formation, indicating that telomere dysfunction accelerates carcinogenesis in p53-/- cells, but impairs carcinogenesis in p53+/+ cells. We define a p53-independent stage of late crisis, termed genetic catastrophe, in which telomere dysfunction and associated chromosomal instability facilitate cellular transformation.

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Telomere Maintenance in Human Cell Lines: Two "Alternatives"

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All immortalized human cell lines examined to date exhibit an active telomere maintenance mechanism. Most express telomerase, and the remainder have an alternative mechanism (ALT). In *in vitro* models of immortalization there is a very close temporal correlation between the immortalization event and activation of one of these telomere maintenance mechanisms. Our ongoing studies include analyses of alternative transcripts from the hTERT telomerase catalytic subunit gene, and of ALT.

Alternative hTERT transcripts. Alternative hTERT cDNAs (A. Kilian et al., Hum. Mol. Genet., 6: 2011-2019, 1997) identified to date include three insertions into the hTERT "consensus" sequence that introduce premature stop codons, one of these potentially encoding a substantial alternative C-terminus with an SH3 binding site. Other variants include a transcript (α) from which 36 bp has been deleted removing 12 amino acids from conserved reverse transcriptase domain A, and another (β) missing 182 bp resulting in a premature stop codon. The α deletion, β deletion and the alternative C-terminus (hTERT-C) cDNAs were each subcloned into a mammalian expression vector and transfected into telomerase-negative cells. The α and β variants did not induce telomerase activity, but the hTERT-C variant had activity equivalent to hTERT. Stable transfection experiments demonstrated that hTERT-C is able to maintain telomeres and allows SV40-transformed cells to bypass crisis and continue proliferating.

Alternative lengthening of telomeres (ALT). The development of anticancer therapies directed against telomere maintenance will require an understanding of the ALT mechanism and how it is controlled. When ALT cells are fused with normal cells, reimposition of a finite lifespan is accompanied by telomere shortening, but immortal segregants regain the typical ALT telomere pattern (highly heterogeneous terminal restriction fragment [TRF] length, ranging from short to abnormally long). ALT is thus repressible by factors contained within normal cells. When the GM847 (ALT) cell line was fused with telomerase-positive immortal lines from the same immortalization complementation group, the resulting hybrids continued to proliferate and were telomerase-positive. Southern analysis revealed: (a) an initial rapid shortening of long telomeres, followed by (b) a phase of less rapid shortening (at 37-77 bp/population doubling, which is similar to the rate seen in telomerase-negative normal cells), and (c) maintenance of telomeres at "normal" lengths. This shows that repressors of ALT also exist in telomerase positive cells. These data also demonstrate that rapid telomere loss may occur in mammalian cells. Further, the observation that telomere lengths decreased then stabilized in the continuing presence of telomerase is consistent with models based on yeast data in which an optimum telomere length is maintained by telomerase activity in a feedback regulatory loop.

To document the presence of ALT it is currently necessary to detect the typical TRF pattern by Southern blotting and to show by TRAP or other assay that there is no telomerase activity present. The activity assay requires fresh cell lysates or lysates of material that has been stored at -80°C or below. We have recently identified a morphologic marker that strongly correlates with the ALT mechanism. In interphase cells the presence of large nuclear bodies containing telomeric DNA, specific telomere binding proteins, and various other proteins, has been detected in 12/12 ALT cell lines, but not in 6/6 telomerase-positive cell lines or 5/5 mortal cell strains. Following transfection of cells with a GFP-hTRF-1 expression plasmid the fusion protein localized to these "telomere bodies" in ALT cells but not in telomerase-positive cells. The telomere bodies could also be detected in frozen or paraffin sections of ALT tumors. The availability of this morphologic marker of ALT may remove the need for fresh or deep-frozen tumor samples, and the ability to detect it in archival paraffin-embedded tumors will facilitate clinical studies of ALT.

Session 3: Telomeres and chromosomal instability

Chair: Titia de Lange

Role of fragile sites in mammalian gene amplification

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Gene amplification is a genetic alteration through which a cell gains additional copies of a small part of its genome. In mammalian cells, this phenomenon has been first characterized in cells selected *in vitro* to resist inhibitors of enzymes essential for cell growth. Resistance frequently results from an overproduction of the proteins inhibited by the drugs, which is often due to an increase in the copy number of the gene coding the target protein. Amplification was repeatedly correlated with the presence of abnormal chromosomal stuctures such as expanded chromosomal regions (HSR) and acentric extrachromosomal circular elements, called double-minutes (DMs). An increasing interest for gene amplification developped when a growing number of reports stressed the presence of amplified oncogenes on the same abnormal structures in a variety of human tumor cell lines and tumors. These mutations were shown to contribute importantly to tumor progression, and possibly to tumorigenesis (1, 6).

In cells recovered from advanced tumors, because of their high level of genome instability, the reconstitution of the mechanisms responsible for amplification is often obscured by a variety of secondary rearrangements. To overcome this difficulty, model systems of *in vitro* cultured cells were used to design experimental protocols allowing to recover and to analyse mutant cells a few generations after the initial event triggering the amplification process. Such studies were made possible by the development of the fluorescence in situ hybridization technique (FISH) which permits a cell by cell analysis of very small cell populations. These studies identified two segregative mechanisms of amplification at early steps of the process. The first one, the breakage-fusion-bridges (BFB) cycles, generates intrachromosomal amplification (2, 4, 7, 9, 10) The second one, DNA excision, leads to the accumulation of DMs (2, 9, 10). Nevertheless, factors governing the choice of either mechanism remain elusive.

To evaluate the contribution of DNA breaks to the initiation of amplification, we focused on multi-drug resistance 1 (*mdr1*) gene amplification which can be selected for with several drugs. We showed that only some clastogenic drugs induce mutants resistant through the intrachromosomal process. We strictly correlated triggering of BFB cycles to the induction of breaks at specific loci (2), called fragile sites (8). By studying several amplification model systems, we confirmed the key role of fragile sites both in the inititiation of BFB cycles and in the determination of the size of early amplified units (2). More recently, we were able to demonstrate that a single DNA break is sufficient to trigger BFB cycles (5). We also showed that fragile site activation is not involved in the initiation of extrachromosomal amplification but nevertheless governs the fusion of DMs and their targeted reintegration into chromosomal fragile sites (3).

However, the events we described in model systems also occur during the amplification of some oncogenes in tumor cells of untreated patients, strongly suggesting that some features of the tumor microenvironment activate fragile sites. We recently reported direct evidence for a causal relationship between hypoxia, an important parameter of tumor microenvironment, induction of fragile sites and gene amplification demonstrating a new role for hypoxia in tumor progression (3).

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The genetics of telomeric fusions in *Drosophila melanogaster*

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We have undertaken a systematic study of the genetic and molecular bases for telomeric associations, using *Drosophila melanogaster* as a model organism. We have found that mutants in the *Drosophila UbcD1* and *pendolino (peo)* genes display telomeric associations (TAs) that are not seen in wild-type. Mitotic metaphase figures from mutant larval brains contain polycentric linear and ring chromosomes indicative of interactions between both homologous and heterologous telomeres, as well as attachments between the ends of sister chromatids. Most of these telomeric attachments are resolved during anaphase, as very few cells with broken chromosomes or aneuploid chromosome complements are observed in mutant brains. Both *UbcD1* (Cenci et al., Genes Dev., 11: 863-875, 1997) and *peo* (our unpublished results) encode ubiquitin-conjugating (E2) enzymes, suggesting that the gene targets are telomeric proteins that are removed from chromosome ends via ubiquitin-mediated proteolysis, to prevent telomere stickiness.

In order to identify other mutations affecting telomere behavior in *Drosophila* somatic cells, we have cytologically screened more than 1200 EMS-induced lethals (generated in C. Zuker's laboratory, San Diego) that die at the larval/pupal boundary. This has led to the isolation of 14 mutants that exhibit elevated frequencies of TAs. Complementation analysis showed that these mutants identify 9 loci we call *beatoangelico (bao), capodimonte (cod), caravaggio (cav), michelangelo (mie), modigliani (moi), perugino (pru), tiziano (tiz), verrocchio (ver)* and *vesuvio (vu)*. These are names of Italian trains, in that the multicentric chromosomes observed in mutants resemble to little trains of chromosomes.

Cytological analysis has shown that most TAs observed in larval ganglia of *mie, moi, ver* and *vu* are resolved during anaphase, as occurs in *UbcD1* and *peo*. In contrast, many TAs present in *bao, cav, per* and *tiz* result in anaphase bridges causing extensive chromosome breakage. The latter cytological phenotype is very similar to that elicited by mutations in *Su (var)2-5* (Fanti et al., Mol. Cell, 2: 527-538, 1998), a well known dominant suppressor of position effect variegation (PEV). *Su (var)2-5* encodes HP1, an evolutionary conserved *Drosophila* protein enriched in both centric heterochromatin and telomeres.

To further characterize these 9 mutants displaying abnormal telomere behavior, we have focused on three parallel levels of research. We have begun mapping the above mutants over deficiency. We are analyzing whether the chromosomal distribution of HP1 is disrupted in the mutants. Finally, we are examining whether our mutants have the ability of modifying PEV. Results obtained to date along these three levels of observations will be presented.

A TELOMERIC RESPONSE TO DNA DAMAGE IN BUDDING YEAST

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The highly conserved Ku heterodimer is implicated in double strand break repair in many organisms. In addition, ku-deficient yeast strains have pronounced telomere specific phenotypes, including a significant shortening of the $TG_{1\text{-}3}$ repeat on chromosome ends, disruption of subtelomeric silencing, and dispersion of the silencing factors from telomeric foci. Intriguingly, the clustering of telomeres at the nuclear periphery is lost in ku mutants. By immunolabelling and *in vivo* crosslinking studies we show that the Ku heterodimer colocalizes with yeast telomeres, suggesting that it plays a direct role in telomere maintenance and their perinuclear positioning. Indeed, when Ku70 or Ku80 is targeted to an internal reporter gene, yKu is capable of nucleating SIR-mediated repression. When DNA damage is induced by bleomycin or MMS, yKu80p and components of repressed subtelomeric chromatin are displaced from telomere clusters. This displacement is partially curtailed in a *rad9* mutant. The displacement of SIR proteins and yKu coincides with a slight drop in telomere proximal repression, and a reduction in the ability of targeted yKu fusions to nucleate silencing. To see whether this is mediated by a signalling pathway, we created a single double-strand cut in an appropriate haploid strain, by inducing the HO endonuclease. This single cleavage also results in the redistribution of yKu and SIR complexes. *In vivo* crosslinking data confirm the immunofluorescence results. Work in progress aims to confirm whether or not SIR proteins play a direct role in the cellular response to DNA damage.

FUNCTIONAL LINKS BETWEEN THE DNA REPAIR MACHINERY AND THE TELOMERE MAINTENANCE APPARATUS IN YEAST AND MAMMALS

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The DNA double-strand break (DSB) is the principal lethal lesion induced by ionising radiation and by radiomimetic agents such as bleomycin. A single DNA DSB is capable of triggering cell-cycle arrest and, if left unrepaired, can lead to cell death or, in multicellular organisms, to cancer. Eukaryotic cells have therefore evolved elaborate and very efficient mechanisms to detect, signal the presence of and repair this form of DNA damage. Consistent with these mechanisms being of prime importance, recent work has established that they are highly conserved from yeast to man (for review, see (Critchlow and Jackson, 1998)). One of the key DNA DSB repair pathways is that of non-homologous end-joining (NHEJ), which employs the DNA end-binding protein Ku and a variety of other proteins (for recent reviews, see (Critchlow and Jackson, 1998)).

Telomeres are the natural ends of chromosomes. These ends do not generally serve as substrates for DNA NHEJ and do not trigger DNA damage responses and, for this reason, it has generally been considered unlikely that DNA DSB repair factors will play a role in maintaining proper telomere structure and function. Surprisingly, however, work in our laboratory and elsewhere has revealed that yeast Ku and several other NHEJ components play critical roles in controlling telomeric functions. For instance, yeasts lacking Ku have severely shortened telomeres and are debilitated in telomeric silencing, the process in which a gene placed close to a telomere is subject to transcriptional repression (for review, see (Shore, 1998)). Further evidence for linkages between telomeric silencing and NHEJ has come from the recent demonstration that the yeast silent information regulators (Sir) Sir2p, Sir3p and Sir4p, which are thought to bring about telomeric transcriptional silencing by folding telomeric DNA into a heterochromatin-like state (Grunstein, 1997), are essential for NHEJ to take place efficiently (Tsukamoto et al., 1997); Boulton, 1998 #6. Taken together with the fact that Sir4p interacts with Yku70p in the yeast 2-hybrid assay (Tsukamoto et al., 1997), these data have suggested that the Sir proteins might play a direct role in. Recent work by Rine and colleagues, however, has indicated that their effects on NHEJ might be largely indirect (Astrom et al., 1999).

Given the linkages between NHEJ and the Sir proteins, we have investigated whether DNA damage causes the derepression of telomeric silencing. These studies have revealed that DNA damage, particularly DNA DSBs, are capable of triggering the reversible derepression of telomeric transcriptional silencing. Moreover, this derepression is associated with the relocation of the silent information regulator, Sir3p, from discrete peripheral foci to multiple diffuse foci throughout the nucleus. Furthermore, we have demonstrated that this relocation of Sir3p is dependent on DNA damage signalling components, including Mec1p, which is related to the human ataxia-telangiectasia gene-product. The relocation of Sir3p is therefore a novel downstream effector of the DNA damage checkpoint response.

We have also investigated the possibility that DNA repair/recombination factors function in telomere length control in mammals. We have initially focused our investigations on two predominant DNA-end binding factors: Ku and poly(ADP-ribose) polymerase (PARP) (for review, see (Critchlow and Jackson, 1998); (Jeggo, 1997). The disruption of the genes for these factors in mice has already demonstrated their crucial roles in the response to DNA damaging agents and in the control of recombination events (Nussenzweig et al., 1996; Wang et al., 1995; Wang et al., 1997). We have analysed telomere lengths in such transgenic mice. Notably, we find that telomere length is severely compromised in both cases. Furthermore, cytogenetic analysis of primary fibroblasts from these animals reveals many chromosomal fusions and other genetic abnormalities. Their cellular growth rate is also affected. Telomerase activity, as detected by the TRAP assay, however, does not seem to be altered in cells from these animals. Therefore, as in yeast, DNA repair factors appear to play key roles in controlling telomeric functions.

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Title: Telomerase -/- mice show a high susceptibility to γ-irradiation.
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It has been proposed that telomere length may act as a biological clock and that the gradual telomere shortening may trigger cellular senescence or apoptosis. It is thought that damaged or shortened telomeres may be recognised as damaged DNA and, perhaps through p53, trigger an apoptotic response. Recent evidence has shown that elimination of TRF2, a protein which protects telomeres from fusing, does in fact cause an apoptotic response through a pathway involving ATM and p53.

Recent evidence from our laboratory has shown that, in the mouse telomerase knock out model, very short telomeres specifically affect tissues with a high proliferation such as testis, intestine and the germinal centres of the lymph nodes, and also embryonic development. Hence it is of great interest to determine the physiological effect of causing damage to DNA in the same experimental model.

Evidence will be presented showing that the fifth generation of mTR -/-mice are extremely sensitive to sublethal doses of γ-irradiation. Irradiation with a dose of 4 grays caused a great degeneration of the intestinal tract which was not present in the wild type counter parts. Currently, experiments are underway to determine whether the degeneration of the intestine is caused by apoptosis. Furthermore, chromosomal instability in these mice is being investigated in bone marrow samples obtained from these mice. The final aspect which is currently under investigation is whether the radiation-induced G1 check point function is intact in mTR -/- embryonic mice cells obtained from the sixth generation.

Session 4: The telomerase complex

Chair: Victoria Lundblad

ANALYSIS OF THE HUMAN TELOMERASE REVERSE TRANSCRIPTASE (hTERT) PROMOTER

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Telomerase-positive but not telomerase-negative cells contain mRNA for hTERT, the catalytic subunit of telomerase. Thus, regulation of telomerase activity may occur at the level of hTERT transcription. To understand regulation of hTERT expression, we have isolated the 5' flanking region of the hTERT gene. Mapping of the transcription start site shows that hTERT mRNA contains a short 5' UTR. The promoter region is embedded in a putative CpG-island and contains two GC-boxes and two E-boxes. The E-box sequences suggest high affinity for c-Myc/Max.

Consistent with the presence of E-boxes, overexpression of c-Myc induces hTERT expression (1, 2) by direct action on the hTERT gene (2). To test hTERT promoter function, different segments of the hTERT gene, spanning several kb 5' flanking region, were fused to a GFP-reporter gene. Expression was assayed upon transient transfection in different telomerase-positive and -negative cells. 260 nt upstream of the transcription start site are sufficient for hTERT promoter activity. We have found that reporter expression driven by the hTERT 5' flanking region does not depend on intact E-boxes in several c-Myc containing immortal cell lines. Thus, although c-Myc can induce hTERT expression, the lower levels of c-Myc in primary cells are not sufficient to explain lack of hTERT expression in these cells. Thus, other events than c-Myc upregulation may lead to hTERT derepression in some tumors.

Reporter expression is very weak in telomerase-negative primary lung fibroblasts but clearly detectable in all immortal cells tested, including a cell line that is negative in the TRAP-assay. However, this telomerase-negative cell line contains an alternatively spliced hTERT mRNA which is predicted to encode for a non-functional TERT. Upstream of the hTERT minimal promoter we identified a segment that downregulates reporter expression. This segment or a repressor that may bind to it could be mutated in some hTERT positive tumor cells.

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Molecular characterization of the hTERT promoter and studies on regulation of telomerase activity in human cells.

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Telomerase activity is absent in most human somatic cells but present in the majority of immortalized and tumour cells (1). Expression of the mRNAs for the catalytic subunit of the enzyme, hTERT, parallels that of telomerase activity pointing to transcriptional mechanisms for enzyme regulation (2-5). Transfection of normal cells with hTERT reconstitutes telomerase activity and results in cell immortalization (6,7), indicating that hTERT is the limiting factor regulating enzyme activity and the only missing telomerase component in normal cells, and highlighting the key role of the enzyme in the regulation of cell lifespan. The sufficiency of telomerase for cell immortality, combined with the absence of spontaneous immortalization of human cells, indicate that hTERT expression must be rigorously suppressed in most somatic cells.

To gain an understanding of the regulation of hTERT expression, we have cloned genomic sequences encompassing the hTERT gene and characterized the organization of the gene and the regulatory function of its 5' flanking sequences (8). A genomic fragment extending from 4 kb upstream of the ATG to the beginning of the second exon of the gene, was linked to the luciferase reporter and transiently transfected into mortal and immortal cells. Luciferase activity was not detected in mortal cells, whether normal or transformed but pre-immortal, but was readily detected in immortal cells. 5'-deletion analysis of the 4kb upstream sequences identified sequences sufficient for promoter activity, at least in transient transfection assays, within a fragment encompassing 330 bp upstream of the ATG and the beginning of exon 2. No significant differences in luciferase activity were detected with constructs ranging in size from -4000 to -330 bp of upstream sequences. Luciferase activity was also produced upon transfection of promoter constructs into immortal telomerase-negative cells (ALT cells; 9). However, in these cells, levels of luciferase correlated inversely with promoter length, suggesting negative regulation by the most distal sequences.

The observed specificity of the plasmid borne hTERT promoter suggests it could be useful for the selective expression of genes in immortal cells. To explore this potential we have generated recombinant adenoviruses containing selected promoter constructs, and assessed promoter specificity upon infection of normal and immortal cells. Current data with first generation vectors indicate that promoter specificity is not retained in all normal cell types.

In additional studies we obtained evidence for estrogen-dependent transcriptional activation of hTERT in human cells derived from hormone responsive tissues. Ovary epithelial cells grown in the absence of estrogen are telomerase negative. Addition of the hormone resulted in induction of hTERT expression and telomerase activity within 3-6 hours of treatment.

Analysis of the hTERT promoter identified an imperfect estrogen response element (ERE) whose sequence binds recombinant human estrogen receptor (ER) in vitro. In vivo DNA footprinting in ER^+ breast cancer cells revealed alterations of the ERE region upon estrogen stimulation. Lastly, cotransfection of ER^- cells with an ER expression vector and ERE-containing promoter constructs resulted in production of luciferase activity only upon addition of estrogen. Hormone inducibility was dramatically decreased by mutation of the ERE or transfection of shorter promoter constructs lacking this element.

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Telomerase reverse transcriptase from *Tetrahymena thermophila*; identification of an amino acid involved in processivity

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Telomerase is a reverse transcriptase that utilizes an internal RNA moiety as a template for the extension of chromosome ends. Telomerase Reverse Transcriptase (TERT) has been identified as the catalytic subunit of telomerase in yeasts, mammals and the ciliated protozoan *Euplotes*. However, it was not reported among the protein components of purified telomerase from another ciliate, *Tetrahymena*, the first telomerase identified and the most thoroughly studied[1]. It therefore seemed possible that *Tetrahymena* used an alternative telomerase that lacked a TERT protein. This possibility was discounted by the cloning and sequencing of a *Tetrahymena thermophila* gene whose encoded protein has the properties expected for a TERT[2, 3]. Tetrahymena TERT (Tt_TERT) has reverse transcriptase sequence motifs with telomerase-specific features, as well as a new amino acid sequence motif (CP), conserved among the ciliated protozoan TERTs [2].

In this study we utilize *in vitro* –translated Tt_TERT to elucidate the functions of specific parts of the protein. We first constructed a synthetic version of the gene with codon usage adjusted for expression in non-ciliate systems, by synthesizing overlapping 100-mer oligonucleotides and joining them with a PCR-based technique. When the resulting gene was expressed in rabbit reticulocyte lysates together with *in vitro* –transcribed telomerase RNA, telomerase primer extension activity was observed. This activity was much less processive than that of native *Tetrahymena* telomerase. The processivity of the *in vitro* –translated TERT, unlike that of native TERT, increased with increasing dGTP concentration. Experiments titrating the primer substrate and preincubating it with *in vitro* –translated TERT suggested that the primer was being sequestered by a factor present in the reticulocyte lysates. This effect was abrogated by immunoprecipitation of the TERT-telomerase RNA complex from the lysates. Immunoprecipitated TERT retained telomerase activity, supporting the proposal that TERT and the telomerase RNA form the 'catalytic core' of telomerase and are sufficient for telomerase activity. Furthermore, immunoprecipitation partially restored processivity to *in vitro* –translated TERT. Processivity was not fully restored, however, suggesting that a factor not present in this *in vitro* system also contributes to telomerase processivity. Mutation of a single amino acid in reverse transcriptase motif C restored telomerase processivity to levels close to those of the native enzyme. The properties of this Tt TERT mutant, and others that affect the binding of telomerase RNA and primer, will be discussed.

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Assembly and Activities of Recombinant *Tetrahymena* Telomerase

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We have investigated the sequences and structures of recombinant *Tetrahymena thermophila* telomerase RNA necessary for physical association and activity with the catalytic protein subunit p133 TERT expressed in rabbit reticulocyte lysate. We find that phylogenetically conserved primary sequences and a phylogenetically non-conserved secondary structure are essential for telomerase RNA function. Telomerase RNA binding to p133 requires sequences 5' of the template and is highly sequence-specific. Other telomerase RNA sequences are required for enzyme activity and proper template use but not for protein interaction affinity. In addition, we demonstrate that the production of active recombinant telomerase requires a factor in rabbit reticulocyte lysate which promotes ribonucleoprotein assembly. These studies demonstrate multiple functions for the telomerase RNA and indicate that recombinant telomerase activity requires more than the catalytic protein and RNA components of the enzyme that have been identified to date.

We have also conducted a mutational screen to identify residues of p133 and telomerase RNA that are not required for activity in general but are essential for a specific property of activity. We are particularly interested in mutations that affect template positioning, substrate recognition, polymerase fidelity, repeat addition processivity, or nucleolytic cleavage. A specific change in any of these features can be detected by use of the appropriate reaction conditions.

Finally, we have investigated the repeat addition processivity of recombinant *Tetrahymena thermophila* telomerase. Although sub-micromolar concentrations of dGTP are sufficient for optimal nucleotide addition within a repeat, optimal addition of multiple repeats requires tens of micromolar dGTP. We addressed the mechanistic basis for the dGTP dependence of processivity using assays with altered template sequences and altered nucleotides. These and other experiments suggest possible models for the mechanism of telomerase's dGTP-stimulated repeat addition processivity.

Exploring the roles of TEP1 and TERT in mammalian telomerase activity

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Mammalian telomerase contains several components, including the telomerase RNA component TER, which provides the template for telomere synthesis, the telomerase reverse transcriptase TERT, and the telomerase-associated protein TEP1 (Blasco *et al.*, 1995, Feng *et al.*, 1995, Harrington *et al.*, 1997 a,b, Kilian *et al.*, 1997, Meyerson *et al.*, 1997, Nakamura *et al.*, 1997, Nakayama *et al.*, 1997,1998). TEP1 is a 240 kDa protein that is associated with the telomerase RNA and TERT, and contains an amino terminus similar to the *Tetrahymena* telomerase RNA binding protein, p80 (Harrington *et al.*, 1997a,b, Nakayama *et al.*, 1997). Using a reticulocyte lysate reconstitution assay, we and others have previously shown that only two exogenous components are required to reconstitute human telomerase activity *in vitro*: the telomerase RNA, and the hTERT cDNA (Weinrich *et al.*, 1997, Beattie *et al.*, 1998). Point mutations in the hTERT reverse transcriptase domain completely abolish telomerase activity in the reticulocyte lysate, suggesting that this region of hTERT contains the catalytic core of human telomerase (Weinrich *et al.*, 1997, Beattie *et al.*, 1998). Addition of hTEP1, however, is not required for telomerase activity in this assay (Beattie *et al.*, 1998).

In order to define the respective roles of mammalian TEP1 and TERT in telomerase activity and telomere length regulation, we have continued our biochemical and genetic analysis of these two telomerase-associated proteins. Using the reticulocyte lysate reconstitution assay, we analyzed systematic deletions of the amino- and carboxy-terminus of hTERT. In addition to the essential reverse transcriptase (RT) catalytic residues, regions outside the RT domain were also required for telomerase activity. Interestingly, there were regions of hTERT required for activity in reticulocyte lysates that were not required when transfected into immortalized human cells. The differences in telomerase activity between reticulocyte lysates and cells was not due to differences in hTER binding, since some inactive hTERT truncations in reticulocyte lysates could still bind hTER. Addition of human cell extracts to the inactive hTERT truncations did not restore telomerase activity to the reticulocyte lysates. These results suggest that residues outside the template domain of hTER and the conserved RT domain of hTERT are necessary for telomerase activity, and that the RNA binding activity and the catalytic activity of hTERT are separable. These findings also suggest that the *in vitro* reconstitution assay does not completely reconstitute native human telomerase, and that other non-diffusible factors may contribute to the formation of an active complex *in vivo*.

Recent studies of mice lacking the telomerase RNA have elegantly

demonstrated the importance of telomerase activity in telomere length maintenance and genomic stability *in vivo* (Blasco *et al.*, 1997, Lee *et al.*, 1998, Chin *et al.*, 1999, Greenberg *et al.*, 1999, Herrera *et al.*, 1999, Rudolph *et al.*, 1999). To determine the role of TEP1 and TERT *in vivo*, we generated murine embryonic stem (ES) cells and animals that were disrupted for mTEP1 or mTERT. For mTEP1, homozygous mTEP1 knockout cell lines and mouse tissues contained no detectable mTEP1 mRNA and protein, yet the mice were viable for up to six generations and showed no obvious differences in telomerase activity or telomere length. These results suggest that TEP1 is not essential for the catalytic activity of the telomerase ribonucleoprotein (RNP) complex *in vivo*. TEP1 may be functionally redundant with other telomerase components, or it may function in telomerase assembly, transport, or localization. In support of a more general role for TEP1 in RNP structure/function, our collaborators have determined that mTEP1 is a component of another RNP complex, the vault particle (Kickhoefer *et al.*, 1998). We have also begun to analyze mice disrupted for mTERT. Like the telomerase RNA component, mTERT is essential for telomerase activity *in vivo*, thus providing compelling genetic evidence that mTERT is the catalytic protein subunit of murine telomerase. It will be interesting to compare the phenotypic consequences of this telomerase-negative mouse strain to that of mice lacking the telomerase RNA.

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A GENETIC SCREEN FOR TELOMERE MAINTENANCE MUTANTS IN *S. cerevisiae* IDENTIFIES *MTR10*, A GENE INVOLVED IN NUCLEAR PROTEIN IMPORT OF hnRNPs.

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We have carried out a genetic screen in *Saccharomyces cerevisiae* for mutants unable to maintain a linear plasmid carrying short telomeric tracts at both ends. Six mutants with shorter telomeres have been identified from the screen. Two of them presented a senescence phenotype and are allelic to the gene *EST2*, the catalytic subunit of telomerase. Three other mutants are being actively characterized.

The remaining mutant has a growth defect, which is apparent in the dissection plate immediately after sporulation and germination. In addition, this mutant has a ts phenotype, being completely unable to grow at 37 °C. We took advantage of this phenotype to clone the gene by complementation with a yeast genomic DNA library. The gene responsible for the observed phenotypes is *MTR10*, a gene involved in nuclear protein import, in particular in the import of hnRNP-like proteins. At least 2 proteins are known to be transported by Mtr10p, Npl3p and Hrb1p. Both have several RRMs (RNA recognition motifs) and a glycine-arginine rich motif, which seems to be important for their transport to the nucleus. These motifs are also present in mammalian hnRNPs and in Gbp2p, a yeast protein homologous to Hrb1p. Since hnRNP A1 and Gbp2p have been recently involved in some aspects of telomere biology, it is tempting to speculate that the regulation defect in telomere length observed in a *mtr10* mutant could be mediated by hnRNP-like proteins. This possibility will be discussed in the light of upcoming results from experiments we are currently performing.

Session 5: Telomeres and telomerase in cancer and aging

Chair: Susan M. Gasser

Normal Differentiated Phenotype of hTERT-Immortalized Human Endothelial Cells.

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Introduction of the catalytic protein component of telomerase, hTERT (Nakamura et al., 1997), into a variety of normal human cells allows the cells to divide indefinitely (Bodnar et al., 1998), avoiding all signs of senescence, and without neoplastic changes in growth control (Jiang et al., 1999; Morales et al., 1999). Some researchers have compared cellular senescence to terminal differentiation. If hTERT allowed cells to bypass normal terminal differentiation signals, this could limit the utility of hTERT-immortalized cells in research, drug discovery, and medicine. To address this question, we immortalized several strains of human endothelial cells and studied the expression of differentiation markers and the ability of the cells to form angiogenic webs *in vitro*. hTERT was introduced by transfection or retroviral infection into endothelial cells from four sources: umbilical vein, saphenous vein, aorta and coronary artery. Cell lifespan was extended in all cases, with hTERT expressing clones and mass cultures having currently achieved ≈2-3-fold increase in lifespan compared to parental cells. hTERT cells have appropriate pRb phosphorylation in response to serum, cell density and hydroxyurea, and do not form colonies in soft agar. Analysis of the expression of endothelial specific markers such as von Willebrand's factor, V-CAM, I-CAM and E-selectin, as well as LDL uptake and angiogenic web formation indicated that the hTERT expressing cells retain the differentiated phenotype and functional characteristics of parental endothelial cells. Specifically, we have observed that compared to young cells, senescent endothelial cells were defective in expression of certain differentiation markers and in web formation, while hTERT cells were not. These observations clearly distinguish the senescence pathway from normal terminal differentiation. Moreover, they illustrate the dominant nature of hTERT in rescuing cells from a senescent fate, and the permissive nature of hTERT in cells undergoing a differentiation program in response to normal stimuli. We propose a therapeutic advantage of using hTERT transduced endothelial cells in treating vascular diseases via a cell or gene therapy approach.

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Are Telomeres Targets for Cancer Therapy and the Treatment of Age-Related Diseases?

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Telomerase, the enzyme that elongates telomeres, is present in almost all primary human tumors but is not detectable in most somatic tissues except for proliferative cells of renewal tissues. Low levels or lack of telomerase activity results in somatic cell telomeres becoming progressively shorter with each division both *in vitro* and *in vivo*. Progressive telomere losses leads to the onset of cellular senescence while ectopic expression of telomerase in normal cells prevents cellular senescence.

While the forced expression of the catalytic component of telomerase prevents both M1 (senescence) and M2 (crisis), there are the following considerations: 1) The failure of telomerase to immortalize human keratinocytes may be due to lack of adequate growth conditions. This leads to growth arrest of keratinocytes in defined culture medium by mechanisms that are not related to telomere-based replicative senesence; 2) The presence of telomerase activity does not always imply telomere maintenance; 3) In some cell types low levels of expression of telomerase results in telomere maintenance at levels shorter than senescent cells; 4) Human muscle satellite cells with forced expression of telomerase can exit the cell cycle, undergo differentiation, and form multinucleated myotubes expressing mature muscle markers; 5) Experiments introducing viral oncogenes (v-ras, SV40 T-antigen or HPV16 E6/E7) into normal human cells with transfected telomerase does not always lead to cancer progression. Concerns about long term effects on cancer incidence by immortalizing all the cells of the body are legitimate. However, immortalization or reversible immortalization of specific cell types which can be thoroughly characterized prior to telomere modification may have manageable risks. Thus, extending cellular life span by elongating telomeres may have utility in cellular and tissue re-engineering.

Since telomerase is detected in almost all human cancers but not adjacent tissues, telomerase inhibition is also an attractive cancer therapeutic strategy. A central question is how will anti-telomerase treatments be used in future clinical trials? Do cancer cells stop growing or undergo apoptosis if telomerase is inhibited? Will preventing telomerase expression in high risk patients without cancer function as a chemopreventive agent? Recent results inhibiting telomerase activity in breast and prostate cancer cells will be presented. In addition, an experimental system to prevent cellular immortalization by telomerase inhibition will be described. In summary, while we are still early in our understanding of telomere biology in human cells, there is the very exciting possibility that telomerase will have utility as an important target for preventing/treating cancer and for the management of some age-related conditions.

Telomere dynamics and aging in $mTR^{-/-}$ mice

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Mice genetically deficient for telomerase activity provide a unique opportunity to understand the role of telomere maintenance in organism viability, and to study the existence of telomerase-independent telomere maintenance mechanisms in the organism. $mTR^{-/-}$ mice, that lack the mouse telomerase RNA (mTR), are viable for only four to six generations depending on the particular genetic background; however, as telomeres shorten and chromosome fusions accumulate with increasing generations, these mice show defects in the germ line, the gut and the hematopoietic system. We will also present that late generation $mTR^{-/-}$ mice display a variety of phenotypes associated with immunosenescence such as a decreased immune system reactivity manifested by a defective germinal center formation after antigen immunization. These observations support the notion that telomere shortening may contribute to the loss of cell viability in a variety of organs as well as to the decrease in immune system reactivity characteristic of the elderly. Importantly, we will present evidence for telomerase-independent telomere maintenance mechanisms operating in the $mTR^{-/-}$ mice.

Mapping human genes that activate telomere-dependent and telomere-independent replicative senescence pathways in human breast cancer cells

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Replicative senescence has been proposed as a key mechanism of tumour suppression in both human and rodent cells. In most normal human cells a primary level of control limiting proliferative capacity is telomere shortening, due to constitutive transcriptional repression of the catalytic sub-unit of telomerase (hTERT). In contrast, we have shown (*Oncogene* **17**, 3417, 1998) that telomerase is active in normal diploid rodent cells in culture and that cells maintain long telomeres throughout their replicative lifespan. Senescence in rodent cells appears therefore to be mediated solely by a telomere-independent mechanism.

We have been using somatic cell genetic approaches to identify and accurately map novel genes present in the normal human genome that induce senescence on transfer to human cancer cells. Techniques based on microcell-mediated monochromosome transfer (MMCT) have led to the identification and localization of sequences on the short-arm of chromosome-3 that completely repress telomerase activity and induce delayed senescence in the early passage breast ductal carcinoma cell line, 21NT (J. Natl. Cancer Inst.**91**, 37-45, 1999). More recently, we have been keen to establish whether telomere-independent senescence pathways function in human cells. We have demonstrated the presence of sequences on chromosome 1p that induce a delayed senescence response superficially identical to that induced by chromosome-3p transfer. However, senescence in this case was not associated with repression of telomerase. Transfer of a panel of extensively deleted chromosome-1 derivatives enabled us to map accurately the new breast cancer senescence gene to an interval of around 1Mb at 1p34-35, within the most common site of cytogenetic loss (and a frequent site of loss of heterozygosity) in ductal carcinoma of the human breast.

TELOMERE- AND TELOMERASE REGULATION IN NORMAL SKIN KERATINOCYTES AND -FIBROBLASTS

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It is now well established that besides germ line cells also some other somatic human tissues are telomerase-positive and that most of them are permanently renewing tissues such as the epithelia of the gastrointestinal tract or the epidermis of the skin. In order to study telomerase regulation in normal cells, the skin is particularly suitable because it is composed of the continuously proliferating epithelium, the epidermis, and the non-proliferative dermis. While the dermis and the dermal fibroblasts do not express telomerase activity, we demonstrated earlier that the proliverative basal cells of the epidermis *in situ* and the cultured epidermal keratinocytes, are telomerase-positive. The unrestricted proliferation potential of the keratinocytes is believed to be warrented by a population of stem cells which represents 2 to 5 % of the basal keratinocytes. These cells only proliferate rarely before they give rise to the more actively proliferating socalled transit amplifying cells (TACs) which are then destined to terminally differentiate. By enriching the epidermal stem cell population to about 60% we were able to show that telomerase activity was increased in the TACs as compared to the stem cells and downregulated with differentiation in a calcium-dependent manner. In the epidermis, calcium is an important regulator of differentiation and it also seems to regulate telomerase activity by interfering with telomerase at the enzyme activity level. In immortal skin keratinocytes telomerase is still sensitive to calcium, however, induction of differentiation and downregulation of telomerase activity is delayed. A possible mechanism for the difference in calcium regulation between the normal skin keratinocytes and the immortal HaCaT cells will be discussed. We also will discuss the rate of telomere shortening during aging of telomerase-negative skin fibroblasts. Q-FISH and Southern blot analysis suggest that telomere erosion is not necessarily linear but, dependent on donor age, can be minimal. Thus, besides telomerase also other yet unknown mechanisms seem to be involved in telomere length regulation and in telomerase-negative fibroblasts these seem only to be active at certain stages of development.

TELOMERE ANALYSIS USING FLOW-FISH AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF HTERT ANTIBODIES.

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Telomere organization, telomere length maintenance and telomere-protein interactions are crucial for processes involved in cellular senescence and immortalization. Using a flow cytometric approach for telomere length quantitation, based on *in situ* hybridization with fluorochrome labeled PNA-probes ("flow-FISH"), we have analyzed a large series of cell lines and patient samples.

Overall a good correlation existed between T_2AG_3 strand lengths calculated by flow-FISH and data obtained by Southern blotting. In tonsil cell-subpopulations a significant *in vivo* telomere lengthening was verified in germinal center cells. In acute myeloid leukemias relapse samples showed decreased, stable or increased telomere lengths when compared with the diagnostic samples. The mean subtelomeric DNA was calculated to be about 3 kbp for a series of patient samples (benign and malignant) and circa 2 kbp for permanent cell lines, which might indicate alterations within the subtelomeric region during prolonged culture *in vitro*. The flow-FISH method allows analysis of the replication timing of specific repeat sequences during the cell cycle showing that telomere sequences were replicated earlier than or concomitant with the bulk DNA, in contrast to centromere DNA which consistently replicated in late S phase.

Telomerase activity and hTERT RNA expression have been shown to be tightly associated. If immunohistochemical (IHC) visualization of hTERT protein expression also correlate with telomerase activity, IHC could be used for telomerase evaluation in formalin fixed and paraffin embedded tumor samples which would be an advantage in the diagnostic tumor characterization. We have tested a series of polyclonal antisera raised against various peptides of the hTERT protein using ELISA, western blotting (WB) and IHC.

All antisera reacted in ELISA specifically with the peptide used as immunogen and not with irrelevant peptides. The WB patterns varied as did IHC staining patterns. One antibody (K370) showed a band at about 130 kD but in IHC strong reactivity was found with many cell types including presumably telomerase negative cells, such as resting lymphocytes, granulocytes and epithelial cells in all layers of squamous epithelium. A second antibody ("Aimy") raised against a C-terminal peptide showed a very similar staining pattern and revealed several bands in WB including one at about 116 kD.

In contrast, another antiserum ("Britten") directed against a peptide in the α deletion region indicated a specific band in a seminative WB and showed IHC reactivity in likely telomerase positive cells, such as germinal center cells and lymphoblasts in lymphoid tissues, Reed-Sternberg cells in Hodgkin´s disease, adenocarcinoma cells and spermatogenic cells in the testis. A fourth antiserum ("Irina") recognizing a peptide in the □ deletion region gave similar IHC staining patttern but was WB negative. These latter antibodies are presently characterized in more detail and can hopefully be useful in future IHC studies regarding hTERT expression.

The IHC part was performed in collaboration with Maria Blasco.
In the Umeå group following people have contributed to this study: Magnus Hultdin, Elisabeth Grönlund, Karl-Fredrik Norrback, Pia Nilsson, Ellinor Eriksson-Lindström, Ulla-Britt Westman.

POSTERS

Interactions Between Yeast and Human Telomerase Components.

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Telomerase is a reverse transcriptase that copies a small region (the telomeric motif) of its endogenous RNA, using as a primer the 3' overhang of the natural chromosomal ends, leading to telomere elongation. The core subunits of the enzyme have been identified in several species and consist of the telomerase RNA and one protein. The protein moiety shows considerable structural and sequence similarity in all species studied, with conserved RT motifs and a typical telomerase (T) motif. The RNA molecules differ widely both in size and in sequence. The role of the template region has been analyzed intensely in yeast and ciliates but little work has been done to functionally characterize the entire telomerase RNA. However, studies in yeast and ciliates indicate that non template regions are important for telomerase activity but that not all regions of the molecule are necessary for function.

We asked whether different telomerase RNAs can interact with the same protein complex leading to telomerase activity. For this purpose we have studied the *in vivo* interactions between the human telomerase RNA (hTR) and the protein complex of *Saccharomyces cerevisiae* telomerase. The hTR gene, cloned in the yeast expression vector γ-EPC under the GAL-CYC promoter, was introduced into the diploid yeast strain ΔT5, deleted for one TLC1 allele by the LEU2 selectable marker (TLC1/tlc1::LEU2), to generate strain ΔT5-hTR.

The influence of hTR on telomere length was initially analyzed in the diploid clone ΔT5-hTR grown for about 200 generations under inducing conditions. hTR exhibited a dominant negative effect resulting in a reduction in telomere length over the first 60-80 generations that correlated partially with the amount of transcript present in the cells. Thereafter, telomeres remained short but stable for up to 200 generations. Upon plasmid curing, normal telomere length was restored. Haploid clones were isolated from ΔT5-hTR and the telomeres of one such clone, T2-3, were analyzed during prolonged growth in selective medium. Telomeric repeats were found to decrease from about 350 bp to 50-100 bp within the first 60 generations, but were then stably maintained for up to 200 generations. No activation of a RAD52-dependent telomere lengthening mechanism, typical of tlc1 strains, was observed. However, cells from which hTR had been removed by plasmid curing activated this alternative pathway of telomere maintenance.

To investigate the role of RAD52 in the telomere metabolism of hTR-containing cells, the RAD52 gene in ΔT5-hTR was knocked out with a kanMX cassette. After sporulation and dissection, haploid clones with the expected phenotype were selected. Three of them exhibited the desired genetic background (tlc1::LEU2, rad52::kanMX, γEPC-hTR) that was confirmed by molecular analysis. These clones grew for about 60 generations with a very long division time after which they ceased to divide. Telomere length analysis showed that, prior to cessation of growth, telomeres were only slightly shorter than those of the T2-3 clone.

Our results are compatible with a functional interaction between hTR and the yeast telomerase proteins. In a diploid background, this interaction appears to interfere with wild type yeast telomerase activity resulting in the initial telomere erosion. In tlc1/hTR haploid strains, telomeres shorten as well, but the presence of hTR ultimately arrests this process, allowing the cells to replicate indefinitely and preventing the activation of recombination. We show that this hTR-mediated telomere stabilization mechanism requires active RAD52 function.

Mutations affecting telomere behaviour in *Drosophila melanogaster*.

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We have undertaken a systematic study of the molecular basis for telomeric fusions, using *Drosophila melanogaster* as a model organism. We have found that mutants in the *Drosophila UbcD1* and *pendolino* genes display telomeric attachments, resulting in polycentric linear and ring chromosomes. These are indicative of interactions between both homologous and heterologous telomeres, and between the ends of sister chromatids. Both *UbcD1* (Cenci *et al*, Genes Dev 11: 863-875, 1997) and *pendolino* (unpublished data) encode ubiquitin-conjugating (E2) enzymes, suggesting that the gene targets are telomeric proteins that are removed from chromosome ends via ubiquitin-mediated proteolysis, in order to prevent telomere stickiness.

In order to identify other mutations affecting telomere behaviour in *Drosophila* somatic cells, we have cytologically screened more than 1000 EMS-induced lethals (generated in C Zuker's laboratory) that die at the larval/pupal boundary. This has led to the isolation of at least 5 additional non-allelic mutations that cause telomeric fusions. The genes identified by these mutations have been designated *michelangelo (mie), modigliani (moi), caravaggio (cav), tiziano (tiz),* and *beatoangelico (bao).* These are names of Italian trains, in that the multicentric chromosomes observed in larval neuroblasts resemble "trains" of chromosomes. We have focused on three parallel levels of research. We have begun mapping the above mutants over deficiency. We are undertaking the cytological characterisation of the mutant phenotypes in both somatic and meiotic cells. Finally, we are analysing whether the distribution of both the telomeric specific sequences Het-A and TART, and the Heterochromatic Protein 1 HP1, is disrupted in the mutants.

Results obtained to date along these three levels of observations will be presented.

REGULATION OF THE HUMAN *TERT* GENE UPON DIFFERENTIATION OF HEMATOPOIETIC CELLS

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The human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase, an enzyme responsible for the maintenance of chromosomal end structures. Recent evidence suggests that *hTERT* expression is rate-limiting for telomerase activity and ectopic expression of the *hTERT* gene is sufficient to prolong the lifespan of various cell lines [1]. The level of *hTERT* expression is correlated with the proliferative capacity of a given cell and almost all neoplastic tissues demonstrate significant *hTERT* expression. This observation and the finding that the *myc* oncogene activates *hTERT* expression indicate that *hTERT* may play a critical role in tumorigenesis. It is, however, not clear whether *hTERT* would have to be considered an *oncogene* or a *tumor suppressor gene* [2].

While telomerase becomes activated during neoplastic transformation, telomerase activity as well as *hTERT* expression decrease during differentiation processes which are accompanied by loss of proliferative potential. The mechanism by which *hTERT* expression is regulated during tumorigenesis or differentiation is largely unknown. To gain insights into the regulation of *hTERT* expression we have started to analyse the downregulation of *hTERT* mRNA upon differentiation of hematopoietic cells (HL-60 and U937) with phorbol ester like TPA or retinoic acid. Using RT-PCR we have demonstrated that the half-life of the *hTERT* mRNA is not altered by differentiating agents and nuclear run-on experiments revealed that the decline of the *hTERT* mRNA is caused by a decrease of transcriptional initiation of the gene. Interestingly, the decline of *hTERT* upon differentiation of U937 cells was not preceded by downregulation of the *c-myc* gene. This suggests that at least in these cells *c-myc* is not solely responsible for activation of *hTERT*. In contrast, our data are most compatible with the synthesis of a transcriptional repressor following initiation of differentiation by various agents. We are currently using transient transfections of reporter constructs as well as gel shift experiments to further analyse the *cis*-elements responsible for the downregulation of *hTERT* expression as well as to identify the *trans*-acting factors mediating this effect.

[1] Bodnar, A.G. *et al.* (1998) Science **279**, 349-352
[2] Rudolph, K.L. *et al.* (1999) Cell **96**, 701-712

Telomerase activity in human lung carcinomas. A prospective study.

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AIMS To evaluate in a prospective study the presence of telomerase activity in human non small cell lung carcinomas and to relate it with clinico-pathologic staging and tumour differentiation.

MATERIAL AND METHODS. We selected neoplastic and non neoplastic fresh lung tissue in 55 non small cell lung carcinomas received in our Department of Anatomical Pathology in 1998. Tissular samples were shock frozen in small pieces in liquid nitrogen and stored at -80°C. For cellular extract preparation, we made several 15µm frozen sections in a cryostat, and transferred them into a sterile 1,5 ml centrifuge tube. We performed a 5 µm Hematoxylin and Eosin stained control frozen section to assure the presence of normal or neoplastic tissue in each sample. Telomerase activity was assayed using TRAP-ELISA detection kit (Boehringer-Manheim), following the manufacturers instructions. We separate physically the preparative areas from the PCR amplification and analysis areas to reduce the potential for carry-over contamination. Occasionally no neoplastic cells were found in control frozen section. These cases were discharged from our study.

RESULTS. Telomerase activity mean was 1003,67 in neoplastic samples (95% confidence interval: 798,92 to 1208,43). In non neoplastic lung samples, telomerase activity was 182,44 (95% CI: 125 to 239,88). When comparing clinico-pathologic stage with telomerase activity by means of ANOVA test, differences were significative ($p<0,01$). Nodal status was also different according to telomerase activity ($p<0,05$). Telomerase activity was lower than 240 (superior limit for non neoplastic tissue) in 14 of 56 cases (25%). Two out of these 14 tumours were advanced stages (III or N2).

CONCLUSIONS.

* A high telomerase activity is present in 75% of non small cell lung carcinomas. Its activity is higher in advanced clinico-pathologic stages.
* A significative percentage of non small cell lung carcinomas (25%) have a telomerase activity similar to non neoplastic lung tissue. Some of them are advanced neoplasms (Stage III).
* Measurement of enzyme activity with a good morphological control could be necessary in Telomerase Activity assay.

Telomerase-independent telomere elongation during B cell expansion in the germinal centers.

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Germinal centers are characterized by an extensive clonal expansion and selection of B lymphocytes to generate B cell memory. It has been proposed that telomere maintenance by telomerase could be crucial to allow the extensive proliferation that B lymphocytes undergo in germinal centers.

Reduction of germinal center reactivity is thought to contribute to immunological dysfunction in the elderly and it is regarded as one of the landmarks of immunosenescence.

We describe that mice deficient for the telomerase RNA component ($mTR^{-/-}$), that lack telomerase activity and show telomere shortening, are greatly impared in formation of germinal centers upon immunization. In agreement with this, immunohystochemistry of the spleen shows that the catalytic component of telomerase is abundantly expressed in the follicles. These findings underscore the importance of telomerase and telomeres in the maintenance of the reactivity of the immune system.

TELOMERASE ACTIVITY IN HUMAN CARCINOMAS. CLINICAL CORRELATIONS IN NON-SMALL CELL LUNG CANCER.

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To contribute to the establishment of the role of telomerase in human carcinogenesis, we have measured the levels of telomerase activity in 65 human tumours and the corresponding adjacent normal tissues. For this purpose, we have used a Telomeric Repeat Amplification Protocol (TRAP)-based enzyme immunoassay (PCR-ELISA). We have also investigated both the RNA component of human telomerase (hTR) and its catalytic subunit (hTERT) expression in relationship to telomerase activity. Finally, we have determined clinical associations in a cohort of patients with resected non-small cell lung cancer (NSCLC). Thus, telomerase activity was detected in 83% of the tumours included in our population. A significant association was found between enzyme activity and both hTR and hTERT expression (P=0.004 and P=0.04, respectively). In NSCLC, we found an inverse association between patient age and telomerase activity (P=0.027), as well as a significant relationship between the levels of enzyme activity and histology of tumours (P=0.01). Thus, investigating telomerase activity by the PCR-ELISA protocol allows to a quick and reproducible analysis of large pools of samples. Moreover, telomerase activity may constitute a useful marker to predict aggressiveness of lung tumours.

Negative Regulation of Telomerase Activity by the Tumor Suppressor Protein p53

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Telomerase, a specialized RNA-directed telomere DNA polymerase, has been implicated as playing an important role in cellular replicative lifespan extension and cancer cell immortalization. The activity of telomerase is repressed in human somatic tissues and becomes activated during tumor progression in most human cancers. Since little is known of how telomerase is activated and maintained in cancer cells, the present study was undertaken to identify telomerase interacting proteins. By peptide affinity chromatography and immunoprecipitation, we demonstrate a direct interaction of telomerase with the tumor suppressor protein p53 in the nuclear lysates of human breast cancer cells, or with recombinant human p53. The interaction occurs between the carboxyl-terminal region of p53 and a region close to the amino-terminus of human telomerase-associated protein 1 (hTEP1). Incubation of recombinant p53 with nuclear telomerase extracts results in inhibition of telomerase activity, with the C-terminal region of p53 being essential for inhibition. This effect is not mediated by binding to telomerase substrate DNA, but requires the region near the N-terminus of hTEP1, in that a synthetic peptide derived from this region of hTEP1 similarly inhibits telomerase activity. Thus, the activity of telomerase may be regulated by p53, down-regulation of which in turn would favor telomerase activation in cancer cell development.

Telomeric DNA structure

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A DNA single-stranded short oligonucleotide, the sequence of which mimicks the cytosine-rich strand of human telomeres $(CCCTAA)_n$ may adopt an i-motif conformation *in vitro* [1,2], an unusual four-stranded structure that relies on the formation of hemiprotonated C.C+ base pairs. The complementary guanine-rich strand may also form a tetraplex involving the formation of G-quartets. The kinetics and thermodynamics of these structures may be followed *in vitro* by UV-absorbance [3-5] or fluorescence [6].

Association of the cytosine and guanosine-rich strands to form a DNA duplex requires the disruption of these tetraplexes. We have investigated the factors that may interfere with duplex formation, by trapping one of the strands into a stable quadruplex. Tetraplex-specific ligands are currently investigated [7], and some of these molecules may act as telomerase inhibitors.

Optimal i-motif stability occurs at acidic pH, but the formation of this structure is still possible under physiological conditions. This prompted us to search for nuclear proteins that may recognize this motif. Human cells indeed contain several nuclear proteins that bind specifically to cytosine-rich nucleic acids. Two of these factors have been characterized and identified (manuscript in preparation).

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Antisense affinity selection of the native human telomerase complex

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The yeast, ciliate and human telomerase complexes contain multiple protein subunits. We have undertaken a biochemical approach to identify additional human telomerase-associated proteins. Native telomerase is purified using an antisense affinity selection protocol. This technique has previously been used by Lingner and Cech (1996) for isolation of the *Euplotes aediculatus* telomerase complex. More recently, Schnapp *et al.* (1998) purified human telomerase from HeLa cell extracts with an antisense oligo complementary to the template region of hTR. We have used oligos targeted to regions in and around the human telomerase template to enrich for activity from Raji cell lysates. In order to avoid loss of telomerase-associated proteins, the protocol has been modified to eliminate exposure to detergent and high salt concentrations. Antisense affinity selection relies on accessibility of the target region. Our panel of oligos provides information regarding the availability of these regions within the ribonucleoprotein complex. The purified material is being analyzed using a variety of methods including UV cross-linking and EMSA. The results from our purification and characterization of human telomerase will be presented.

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DIFFERENTIAL AGING WITHIN THE CD4+ T CELL COMPARTMENT AT LONG TERM FOLLOW UP AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION.

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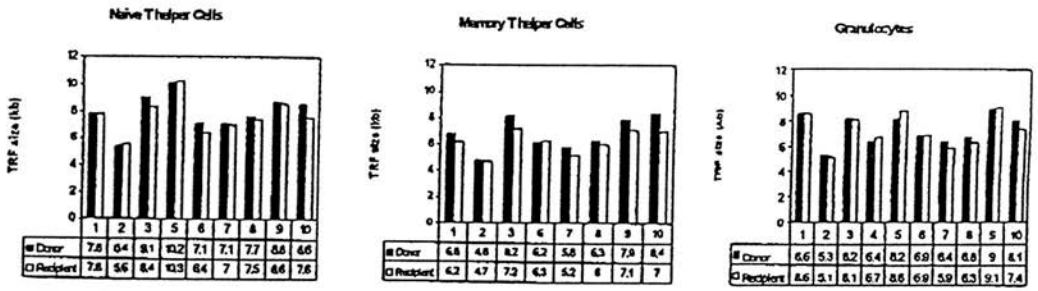
The loss of telomeric repeat sequences has been proposed as a possible mechanism for cell senescence, the length of telomeres decreasing with each cell division. Recent studies indicated telomere shortening in allogeneic bone marrow transplant (BMT) recipients with a relatively short follow up. With this in mind we undertook a study in allograft recipients who were transplanted between 11 and 25 years ago. Recipients received BMT as a treatment for aplastic anemia (n = 6), AML (n = 2) CML (n = 1) and ALL (n = 1) at an age between 7 – 34 years from an age-matched donor. Following informed consent 50 ml blood was collected from recipients and donors. Mononuclear cells were isolated, stained immuno-histochemically, and sorted into T cells (CD3), naïve ($CD45RA^+$) and memory ($CD45RA^-$) CD4 T cells, B cells (CD19) and monocytes (CD14). Sort purities were always more than 77% and typically 96%. Telomere restriction fragment size (TRF) was assessed by Southern blot analysis. Telomeres in naïve T cells were longer than those in memory T cells (0.1 – 1.8 kb) in each individual. No significant differences in telomere length between donor and recipient were detected in granulocytes or naïve CD4 T cells. However, a significant difference between donor and recipient was found in memory CD4 T cells.



TRF- size in different cell subsets of recipients and their corresponding donors

In summary long term follow up reveals no shortening of telomeres in the myeloid compartment and differential shortening in the lymphoid compartment of allogeneic bone marrow transplant recipients.

Disease states associated with telomerase deficiency appear earlier in mice with short telomeres.

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Mice deficient for the mouse telomerase RNA, $mTR^{-/-}$, and lacking telomerase activity, can be propagated for approximately six generations due to decrease male and female fertility and to an increased embryonic lethality consequence of a neural tube closure defect. Although late generation $mTR^{-/-}$ mice show defects in the hematopoietic system, they are viable to adulthood, only showing a decrease in viability in old age. To address whether telomerase deficiency would have a greater effect on the viability of other genetic backgrounds, we generated $mTR^{-/-}$ mutants on a C57BL6 genetic background, which showed shorter telomeres than the original mixed genetic background C57BL6/129S. Interestingly, these mice could be propagated for only four generations and the survival of late generation $mTR^{-/-}$ mice decreased dramatically with age as compared with wild-type counterparts. Fifty percent of the G4 mice die at only 5 months of age. This decreased viability with age in the late generation mice is coincident with telomere shortening, sterility, splenic atrophy, reduced proliferative capacity of B and T cells, abnormal hematology and atrophy of the small intestine. These results support the concept that telomere shortening in $mTR^{-/-}$ mice leads to progressive loss of cell viability in a variety of disease states, and that it could be an important factor in determining the life span of species with shorter telomeres, such as man.

Telomeres function to repress different kinds of natural genes.
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In *Saccharomyces cerevisiae*, the heterochromatin at telomeres spreads into subtelomeric regions and can silence the expression of reporter genes when they are transplanted near to their vicinity [1-4]. This transcriptional repression is conditioned by the strength of the promoters that are silenced and decreases rapidly with the distance to the telomeres [1, 2]. Thus, silencing at natural subtelomeric regions is expected to depend on the nature of the specific subtelomeric genes and on their location. Hence the importance of identifying natural systems undergoing telomeric silencing. Such systems should be suitable models for the characterization of this kind of silencing and should help to understand its biological significance. Only one natural transcriptional unit, the Ty5-1 retrotransposon, has been shown to undergo telomeric silencing in its natural context [5]. In fact, telomeric heterochromatin seems to be involved in the control of Ty5 elements [5-8]. Here we describe the identification of a second transcriptional unit from *S. cerevisiae* that undergoes natural telomeric silencing. This transcriptional unit is a unique gene which indicates that silencing at telomeres is not restricted to transposable elements. We have found that the spreading of telomeric silencing is confined within the limits of heterochromatin in a specific telomeric region. In addition, we show that natural telomeric silencing is not a generalized feature at the centromere-proximal regions of Telomere Associated Sequences (TAS).

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