COMMENTSARY
Mammalian telomerase: catalytic subunit and knockout mice
David Kipling

Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK

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For the second time this year random cDNA sequencing, in combination with data from unicellular eukaryotes, has made a significant contribution to the analysis of human telomerase. Two groups have reported mammalian homologues of the Tetrahymena p80 telomerase-associated protein, in both cases the key breakthrough being mammalian cDNA clones with database matches to Tetrahymena p80. This has now been joined by the sequence of a candidate for the human telomerase catalytic subunit. The discovery that its message abundance closely follows telomerase activity could make a major impact on the utility of telomerase as a diagnostic marker for human malignancy. In addition, Blasco et al. report the phenotype of a transgenic mouse deleted for the mTR gene, which encodes the essential RNA component of telomerase. Interestingly tumour formation is unaffected in these mice, strengthening the argument that telomerase expression in mouse tumorigenesis is an innocent bystander rather than a necessary event. However, fundamental differences between the genomic organisation of mouse and human telomeres mean that the mouse is not a straightforward model to critically test the role of telomere loss and telomerase in human malignancy.

INTRODUCTION

Telomeres are the natural ends of linear chromosomes (reviewed in 1). At a very basic level, a telomere is whatever feature distinguishes a natural chromosome end from a simple double-strand DNA break, such as caused by ionising radiation. It has been known for many decades that telomeres do not behave like simple double-strand DNA breaks. The latter tend to be highly recombinogenic and fusigenic and, if created, can wreak havoc on karyotypic organisation and stability (1). For this reason many cells have DNA damage monitoring systems that can detect double-strand DNA breaks. In one of the clearest examples, a single double-strand DNA break in budding yeast caused by expression of the HO endonuclease will result in RAD9-dependent cell cycle arrest (2), thus providing time for the cell to attempt to repair the lesion. It is striking that the cell is able to detect this new lesion while at the same time ignoring a large number of other termini in the nucleus—the natural ends of the yeast chromosomes. That cells are able to distinguish double-strand breaks from telomeres reflects, at least in mammals, the presence of specific terminal DNA sequences (arrays of the hexamer TTAGGG) and associated proteins such as TRF1 (3) and TRF2 (4). Together these are believed to assemble a specialised nucleoprotein complex that sequesters the terminus of the chromosome away from genome damage monitoring and recombination systems.

Another problem faced by telomeres is the so-called end replication problem. As originally proposed by Olovnikov (5), conventional DNA replication faces a problem with the ends of linear molecules. This stems from the fact that conventional DNA polymerases cannot commence DNA synthesis de novo. Lagging strand replication starts with the synthesis of a short RNA molecule by RNA primase, which is then extended by DNA polymerase. Removal of the most terminal RNA primer leaves a small region that cannot be copied. In the absence of other activities a small but unavoidable loss of terminal sequence will therefore occur every S phase (1). Over many divisions essential chromosomal regions will be deleted, the first being the (TTAGGG)_n repeats responsible for telomere function.

Nature has devised a number of schemes either to overcome the end-replication problem (a circular genome being an obvious solution) or to compensate for end-replication losses by balancing them with de novo terminal sequence synthesis (1). In mammals the enzyme telomerase synthesises (TTAGGG)_n repeats de novo onto the ends of chromosomes (6), but crucially this activity is not present in all human cell types (7). It is not detected in most somatic cells and immortal cell cultures (with a few exceptions) but is readily detected in the germ line, most immortal cell lines and ~85% of tumour samples analysed to date (7). Telomerase reactivation is therefore the most common single biochemical change in human tumours and as such has raised enormous interest with respect to various areas of cancer biology (8,9).
A PATHOLOGICAL INTEREST IN TELOMERASE

There is scope for improvement in various areas of cancer diagnosis and management. For diagnosis and many aspects of prognosis, histopathology remains the ‘gold standard’. However, pathologists are now more frequently being asked to provide diagnostic and sometimes prognostic information on samples of vanishingly small size. Freehand and radiologically-guided fine needle aspirates, endoscopically obtained washings and brushings and other non-invasively obtained cytological specimens are all examples of clinical samples requiring a high level of skill and experience to interpret. Objective markers of the malignant phenotype applied to such material are required to improve the diagnostic yield. The extreme sensitivity of the TRAP telomerase assay (10) means that important prognostic information could be provided using specimens currently considered insufficient for full assessment. In other cases it is the nature of the tumour itself that presents the difficulty, especially when the aggressiveness of the tumour (and hence toxicity of the required therapy) is difficult or impossible to predict on histopathological grounds alone. Neuroblastoma has provided one example where telomerase activity can provide important prognostic data to guide choice of therapy (11); to be of clinical utility any test needs to provide information not currently available by other means.

On a practical level, telomerase assays on tumour material are hampered by the requirement for unfixed material and the loss of tissue morphology that is associated with what is an in vitro biochemical assay (10). This has prevented the application of telomerase assays to tissue sections, although some progress has been made in this area (12). The loss of morphology removes a whole level of information valuable in diagnosis. For this reason there has been great excitement over the possibility of using criteria for telomerase activity based on abundance of an RNA species or protein molecule, as this would enable techniques of RNA in situ hybridisation or immunocytochemistry to be utilised. The report of the sequence of the catalytic subunit of human telomerase (13–15) and its relative expression in mortal and immortal cells is a major step towards this goal.

TELOMERASE COMPONENTS AND MODEL SYSTEMS

Telomerase was first detected as a biochemical activity in the ciliate Tetrahymena (16). Like many ciliates Tetrahymena has an unusual life-cycle requiring de novo synthesis of many new telomeres, and as such, the macronucleus is a rich source of telomerase. In contrast, activity is much lower in human cells (17). Nevertheless, even with such an abundant source, the biochemical purification and cloning of Tetrahymena telomerase-associated proteins was a major tour-de-force (18). Curiously, the two telomere-associated proteins cloned from Tetrahymena (p80 and p95: ref. 18) have no obvious homologues in the Saccharomyces cerevisiae genome. Neither p95 nor p80 show significant homology to known polymerases or reverse transcriptases and thus neither would appear to be a good candidate for the catalytic subunit of telomerase. When faced with such a low abundance and delicate enzyme it is perhaps no surprise that similar attempts to clone mammalian telomerase-associated proteins have been unsuccessful, although the RNA template component has been cloned in both mouse (mTR; ref. 19) and human (hTR; ref. 20). For the protein components the breakthrough came with EST matches to Tetrahymena p80 which allowed the identification of mammalian homologues (TLP1) (21,22).

Table 1. Telomerase components

<table>
<thead>
<tr>
<th></th>
<th>Catalytic subunit</th>
<th>RNA template</th>
<th>p80</th>
<th>p95</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>hTRT</td>
<td>hTR</td>
<td>TLP1</td>
<td>?</td>
</tr>
<tr>
<td>Budding yeast</td>
<td>Est2p</td>
<td>TLC1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Fission yeast</td>
<td>Trt1p</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Euplotes</td>
<td>p123</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Tetrahymena</td>
<td>p123</td>
<td>Yes</td>
<td>p80</td>
<td>p95</td>
</tr>
</tbody>
</table>

The core catalytic domain of human telomerase in humans is hypothesised to consist of hTRT and the hTR template RNA. Homologues for both have been identified in most of the five species listed. In addition two telomerase-associated proteins have been identified (p80 and p95) in Tetrahymena. Neither have homologues in the complete S. cerevisiae genome sequence. A mammalian p80 homologue (TLP1) has been identified and is associated with telomerase activity. One explanation for the absence of yeast homologues is that p80/TLP1 and p95 might be accessory proteins, for example involved in polymerase fidelity, rather than absolutely required for catalysis.

In a separate study two telomerase-associated proteins from the ciliate Euplotes aediculatus were reported with molecular masses of 123 and ~43 kDa (23), and the sequence of p123 is now known (24). Euplotes p123 has all the indications of being the catalytic subunit, with the protein sequence containing a series of reverse transcriptase motifs. Euplotes p123 has homologues encoded by genes in both budding (EST2) and fission (trt1) yeast, with disruption leading to telomere shortening (13,24) and loss of telomerase activity (24). The apparent absence of a p123-like subunit of Tetrahymena telomerase has yet to be resolved (see Table 1).

A BLAST search of the sequence databases with the Euplotes p123 protein sequence identified a single human EST, consisting of 389 nt of 5’ sequence of an IMAGE Consortium (25) clone from a library made from human tonsillar cells enriched for germinal centre B cells by flow sorting. This partial sequence was sufficient to allow a full-length sequence to be obtained. It encodes a protein (hTRT) clearly homologous to Est2p, Trt1p and p123. hTRT is a 127 kDa protein which, like the homologous proteins in both yeasts and Euplotes, has a pl of >10. As has long been expected from its biochemical role in synthesising DNA using an RNA template, hTRT is a member of the reverse transcriptase family. It contains all seven conserved reverse transcriptase motifs, although with some characteristic variations. In addition, hTRT and its homologues share an additional unique motif (Fig. 1A), termed the telomerase or T motif (13,14,24). All four catalytic subunits are more closely related to each other than to any other reverse transcriptase family member, forming a discrete subgroup that is a deep branch in the evolution of reverse transcriptases, with the authors suggesting that this ancient group perhaps originated with the first eukaryote (13). Outside the central core containing the reverse transcriptase and T motifs, the four catalytic subunits show very weak sequence similarity, with the exception of a small region near to the amino terminus (Fig. 1B), and little by way of striking structural motifs.

hTRT message was weak or undetectable in several different telomerase-negative primary human fibroblast cultures (foetal lung, foetal skin, adult stromal prostate, foetal knee synovial, and neonatal foreskin) (13). In contrast it could be detected in colon, testes and weakly in lymphocytes, three tissue types which express telomerase (15). A number of telomerase-positive immortal
human cell lines (293, breast tumour, lung carcinoma, melanoma, leukaemia, colon carcinoma and colon adenocarcinoma) were also positive, as were the majority of tumour samples analysed (13,15). Interpretation of these data is made more complex by the occurrence in some tissues of various alternate splice forms. The extent to which these alternatively spliced messages encode either non-functional or possibly even dominant-negative (see below) versions of hTRT remains to be assessed.

Mutation in three highly conserved aspartate residues in *S.cerevisiae* Est2p leads to a telomere shortening phenotype (24). Interestingly, this phenotype could also be seen when mutant protein was overexpressed in the presence of wild-type Est2p. Such a dominant-negative effect may result from mutant protein titrating away other telomerase components, such as the RNA template component. These three aspartate residues are conserved in hTRT, and it will be of interest to determine if a dominant-negative telomerase inhibition effect can be achieved with similarly mutant versions of hTRT.

How is telomerase regulated? RNA abundance of neither TLP1 nor hTR correlates well with telomerase levels, with significant amounts present in a number of telomerase-negative cells (13,20–22). This, together with the observation that protein phosphatase 2A is a potent inhibitor of telomerase activity *in vitro* (26), raised the possibility that telomerase might be predominantly regulated at a post-translational level, something that would have hampered the development of a simple and robust non-enzymatic assay. It is something of a relief therefore that hTRT transcript abundance appears to follow telomerase activity quite closely, with little if any transcript detected in immortal cells (13–15). This does not exclude additional layers of control in cells expressing hTRT, such as activating phosphorylation events as suggested by the PP2A data (26).
number of cell systems have been described where telomerase-positive human cells repress telomerase in response to exit from the cell cycle (27–30) and it will be of interest to determine if this also involves reduction in hTRT transcript levels. Regardless, it appears likely that the main reason mortal cells are telomerase negative is that they lack a key component. This opens the way to the development of non-enzymatic assays and thus more widespread clinical investigation of the usefulness of telomerase as a marker for malignancy.

mTR KNOCKOUT MOUSE

The production of viable mice deleted for the gene encoding the essential mTR RNA component of telomerase has recently been reported (31). Their viability for at least six generations in the homozygous null state gives some interesting insights into what is driving telomerase expression in tumours. These mTR−/− mice were created by standard gene disruption technology and then were backcrossed to C57BL/6 before being bred to homozygosity. The starting telomere length distribution is 10–80 kb as judged by PNA in situ hybridisation, in good agreement with the 20–60 kb range indicated by pulsed field gel electrophoresis of C57BL/6 (32,33) and a length considerably longer than in humans. mTR−/− mice have been bred for six generations in the homozygous null state, with no tissue containing any detectable telomerase activity. The number of generations, together with previous estimates for the number of cell divisions from fertilised zygote to spermatozoa in an adult mouse, enables the number of divisions mTR−/− cells have undergone in vivo to be approximated, >300 to date. By PNA in situ hybridisation telomere shortening in successive generations is observed. From a starting distribution of 10–80 kb there is a shortening of ~5 kb per generation until by the sixth generation the distribution is 0–50 kb. The entire distribution is being moved to a shorter telomere length, and as one cannot have a negative telomere length there is a noticeable ‘piling up’ at the lower end of the distribution in the later generations. Indeed, one of the striking observations is that by the sixth generation ~5% of chromosome ends have no detectable (TTAGGG)n. With this comes an increase in the frequency of aneuploid cells and apparent end-to-end fusions. The absence of telomerase in these mice is therefore leading to telomere shortening and in turn chromosome instability.

Mouse embryonic fibroblast (MEF) cultures from mTR−/− mice have apparently identical in vitro growth characteristics to wild-type MEFs, both undergoing senescence and immortalisation in the same fashion. MEFs from the first generation mTR−/− mice have now been passaged for >200 population doublings and remain telomerase negative; some evidence for telomere shortening is seen, even in the later passages. This appears to indicate that telomerase is not required for immortality in culture (but see below). mTR−/− MEFs transformed using E1A/rasV12 remain telomerase negative but produce tumours in nude mice in an identical fashion to wild-type MEFs. This indicates that telomerase is not required for tumour formation in this system.

The telomere loss rate in vivo is estimated by the authors to reflect <100 bp of sequence loss per cell division, in excellent accord with telomere shortening rates in telomerase-negative human cells. This figure, together with the very different telomere structure in mice as compared with humans, gives some clue as to why these mice are alive. Because the starting telomere lengths are so long for most chromosomes, it is only by the sixth generation that significant numbers of ends without (TTAGGG)n are observed. That is, the length of mouse telomeres provide a substantial ‘buffer zone’ to allow cell division in the absence of telomerase. By the sixth generation, however, the presence of significant numbers of ends lacking (TTAGGG)n and the chromosome instability observed raises the possibility that cell division may soon be significantly compromised. It will therefore be of great interest to follow subsequent mTR−/− generations as their telomere lengths become even shorter and the frequency of karyotypic instability reaches such a level that cell viability may become compromised.

The data suggest that mTR−/− cells can divide for at least 300 divisions in vivo, and so it is not surprising that mTR−/− MEFs can be passaged for 200 divisions in vitro. We must therefore ask exactly what the criteria are for cell ‘immortality’. A culture able to divide for 200 divisions would normally be classified as ‘immortal’, but the new data suggest that for mTR−/− MEFs this might not be the case. Mouse cell lines established for several decades do exist and undoubtedly have undergone more divisions than a simple ‘long telomere buffer’ would allow. However, wild-type MEFs readily upregulate telomerase in the early stages of culture and thus telomere loss does not occur in such cell lines. The mTR−/− MEFs are a unique example where telomere loss continues to occur after apparent immortalisation. It would therefore be of great interest to passage these cells for many more generations to see if telomere shortening does eventually compromise cell viability and the ability of transformed mTR−/− MEFs to form tumours in nude mice.

TELOMERASE IN CANCER: INNOCENT BystANDER?

In light of the growth potential of the mTR−/− cells both in vivo and in vitro it is perhaps unsurprising that transformed mTR−/− MEFs are able to produce tumours in nude mice, as this requires only ~40–50 cell divisions. What is fascinating is comparing this behaviour with the normal process of tumourigenesis in mice where telomerase upregulation is a common feature (34). The growth of the mTR−/− cells indicates that this cannot simply be because telomere loss is rate-limiting for division. Indeed, the conclusion that telomerase upregulation does not reflect selection for telomere maintenance was made by Broccoli et al. in a model system of mouse tumourigenesis (35). Both normal and tumour samples had identical telomere lengths, with the identical hypervariable telomere fragment length fingerprints arguing that a transient shortening followed by regrowth had not occurred. Instead it seemed likely that long telomeres had been present throughout the history of tumour formation and no short telomeres were ever present to impose selective pressure to upregulate telomerase. If telomere shortening does not drive selection for telomerase expression, what does?

The answer to this may come from a somewhat surprising quarter—histology. In many cancers, progression to a more aggressive and invasive stage is associated with decreased cellular differentiation. Many early stage, indolent tumours are described as ‘well differentiated’, progressing to ‘poorly differentiated’ and finally ‘anaplastic’ as the tumour becomes more aggressive. The transition towards an immortal, undifferentiated cell could be considered as reflecting reversion to a more ancestral state with various layers of differentiation and
mortality control removed, consistent with the appearance of embryonic antigens in some tumours (36). Indeed, it is clear from cell fusion studies between mortal and immortal cells that mortality is dominant, that is to say an acquired characteristic (36). By analogy with other cellular signalling pathways it may be that the regulatory networks that produce mortality and differentiation are so highly interconnected that it is very difficult to alter one phenotype without affecting another. Thus selection for characteristics that enable a mouse cell to form a tumour in vivo (such as invasion and removal of normal growth control) may cause selection for cells that have reverted towards a more undifferentiated cell state, ‘hard-wired’ into this change being upregulation of telomerase (34). The mTR knockout mouse clearly indicates that tumours can occur without telomerase reactivation, arguing that telomerase is a biomarker of mouse tumourigenesis but not a necessary event.

**IMPLICATIONS FOR HUMAN TUMOURIGENESIS**

It has been argued elsewhere (34) that any phenotype of a mTR knockout mouse would have to be taken in the context of the very different telomere lengths in mice (32,33). As such, although the mTR−/− mouse does provide some useful insights, it does not provide a straightforward model to critically test the role of telomere loss and telomerase expression in human tumourigenesis. Human cells in culture have a very low spontaneous immortalisation rate compared with mice (<1 in 10^12). As originally shown by Counter et al. (38), telomere loss occurs in primary human fibroblasts until senescence and continues in the extended growth period after senescence is bypassed by use of viral oncoproteins. Not until rare foci come through the next phase of cell death (‘crisis’) do telomerase-positive cells appear. In Mus musculus a ‘crisis’ phase associated with very short (~2 kb) telomeres is not observed.

Telomeres are essential biological structures; no viable human chromosomes exist that are linear and do not terminate in (TTAGGG)n. The end replication problem is a fundamental aspect of chromosomes exist that are linear and do not terminate in (TTAGGG)n. The end replication problem is a fundamental aspect of chromosomes.

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