Telomeric function of mammalian telomerases at short telomeres

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Summary

Telomerase synthesizes telomeric sequences and is minimally composed of a reverse transcriptase (RT) known as TERT and an RNA known as TR. We reconstituted heterologous mouse (m) and human (h) TERT-TR complexes and chimeric mTERT-hTERT-hTR complexes in vitro and in immortalized human alternative lengthening of telomere (ALT) cells. Our data suggest that species-specific determinants of activity, processivity and telomere function map not only to the TR but also to the TERT component. The presence of hTERT-hTR, but not heterologous TERT-TR complexes or chimeric mTERT-hTERT-hTR complexes, significantly reduced the percentage of chromosomes without telomeric signals in ALT cells. Moreover, heterologous and chimeric complexes were defective in recruitment to telomeres. Our results suggest a requirement for several hTERT domains and interaction with multiple proteins for proper recruitment of telomerase to the shortest telomeres in human ALT cells. Late-passage mTERT$^{-/-}$ mouse embryonic stem (ES) cells ectopically expressing hTERT or mTERT harboured fewer chromosome ends without telomeric signals and end-to-end fusions than typically observed in late-passage mTERT$^{-/-}$ ES cells. The ability of hTERT to function at mouse telomeres and the inability of mTERT to function at human telomeres suggest that mechanisms regulating the recruitment and activity of hTERT at mouse telomeres might be less stringent than the mechanisms regulating mTERT at human telomeres.

Key words: Telomere maintenance, Telomerase activity, Telomerase processivity, ALT

Introduction

Telomeres act to preserve the integrity of chromosome ends, and prevent the incidence of chromosomal fusions and rearrangements (Palm and de Lange, 2008). Progressive loss of DNA at telomeres during replication limits the proliferative potential of human cells. Most human somatic cells express little or no telomerase, whereas most human cancer cells express telomerase, which counteracts telomere erosion (Harley, 2008). Thus, telomerase is an attractive target for cancer therapy. Telomerase is an RNA-dependent ribonucleoprotein with two minimal moieties: the telomerase reverse transcriptase (TERT) and the telomerase RNA (TR) (Autexier and Lue, 2006). TERT catalyzes the addition of 5′ TTAGGG 3′ repeats to mammalian telomeres using TR as the RNA template. Dyskerin, a small nucleolar protein involved in telomerase assembly, associates with a highly purified and active telomerase complex (Cohen et al., 2007; Fu and Collins, 2003).

Extensive telomere shortening activates the p53 and retinoblastoma protein pathways, resulting in cellular proliferation arrest and senescence (Itahana et al., 2004). If the DNA-damage checkpoints are abrogated, cellular division continues, accompanied by telomere erosion. Eventually, short telomeres are recognized as DNA double-strand breaks. Cells are committed to apoptosis, unless activation of a telomere maintenance mechanism occurs. Ectopic human TERT (hTERT) expression in human primary cells can restore telomerase activity, maintain telomere length and prevent cellular senescence (Bodnar et al., 1998; Counter et al., 1998b; Vaziri and Benchimol, 1998).

TERT is unique among reverse transcriptases (RTs), because telomerase reiteratively adds stretches of hexanucleotide sequences (repeat addition processivity) and because the telomerase RNA is an integral part of the ribonucleoprotein complex. TERT has an RT domain composed of prototypical RT motifs that are conserved among RTs (Autexier and Lue, 2006). Most TERTs possess characteristic accessory domains that are essential for telomerase function. Flanking either side of the RT domain is a large (400 amino acids) conserved N-terminal extension (NTE) and a short (150 amino acids) less conserved C-terminal extension (CTE). The NTE region harbours low- and high-affinity RNA-interaction domains (RID1 and RID2, respectively). Telomerase processivity is modulated by temperature, both primer and dNTP concentration (Morin, 1989), telomere- and telomerase-interacting proteins such as TPP1 and POT1 (Wang et al., 2007), and regions of TR (Chen and Greider, 2003; Martin-Rivera and Blasco, 2001; Moriarty et al., 2004). However, various structural elements in the NTE and CTE of TERT also modulate processivity control and telomere function (Armbruster et al., 2001; Banik et al., 2002; Huard et al., 2003; Moriarty et al., 2004; Moriarty et al., 2005).

Although human and mouse telomerases and telomeres share common features, distinct characteristics include differences in TERT expression, enzyme repeat addition processivity and telomere

length. Mouse telomeres are long and telomere attrition is not a major cause of mouse cell senescence (Itahana et al., 2004; Kipling, 1997). Moreover, fewer events are required for the transformation of mouse cells compared with human cells (Boehm et al., 2005). Human TR (hTR) and mouse TR (mTR) are ubiquitously expressed (Feng et al., 1995; Greenberg et al., 1998). However, hTERT expression is limited to highly proliferative cells and is negatively regulated in most human somatic cells, whereas mouse TERT (mTERT) is widely expressed in most mouse tissues (Greenberg et al., 1998; Nakamura et al., 1997). In vitro, mouse telomerase is less processive than human telomerase (Morin, 1989; Prowse et al., 1993). Differences in processivity and the incompatibility of human and mouse telomerases have been mapped to the RNA component (Chen and Greider, 2003; Garforth et al., 2006). However, the contribution of the TERT component to species-specific differences in enzyme activity and processivity is not well understood (Middleman et al., 2006). The response to telomerase inhibition can vary between human and mouse cells (Marie-Egyptienne et al., 2008; Sachsinger et al., 2001). Species-specific differences in cellular immortalization are also evident because, in the context of full-length hTERT or mTERT, the hTERT CTE but not the mTERT CTE can promote the immortalization of primary human cells expressing the SV40 early region (Middleman et al., 2006).

Mouse models are commonly used to validate anti-telomerase and anti-cancer therapies, such as GRN163L, which is currently in clinical trials (Dikmen et al., 2005; Harley, 2008; Wang et al., 2004). To shift anti-telomerase therapies from a mouse model and apply them to a clinical setting, it is important to understand how the distinctive features of telomerase and telomere biology between human and mouse might result from species-specific differences in enzyme activity and regulation. We have characterized the distinct contribution of TERT to telomerase activity, enzyme processivity, TERT-TR and telomerase-DNA substrate interactions, as well as human and mouse telomere function.

Results

Telomerase activity and processivity of heterologous TERT-TR complexes reconstituted in vitro

Using in vitro reconstitution assays or mTR$^{-/-}$ mouse cells, hTR can functionally replace mTR to reconstitute mouse telomerase activity, demonstrating that murine TERT is amenable to heterologous TR activation (Greenberg et al., 1998; Martin-Rivera et al., 1998). However, hTERT is not efficiently amenable to activation by mTR, and mTR cannot reconstitute the telomerase activity of a hTR-depleted human 293 cell extract (Autexier et al., 1996; Beattie et al., 1998). The enzyme activity and processivity differences that mediate cross-species incompatibility between human and mouse telomerase complexes are dependent on the origin of the TR (Chen and Greider, 2003; Garforth et al., 2006). Because elements in hTERT also contribute to human telomerase activity and processivity (Armbruster et al., 2001; Banik et al., 2002; Huard et al., 2003; Moriarty et al., 2004), we investigated whether TERT elements are also responsible for modulating the telomerase activity and processivity differences that mediate cross-species incompatibility between human and mouse telomerase complexes. We analyzed the in vitro activity and processivity of rabbit reticulocyte lysate (RRL)-reconstituted hTERT or mTERT complexed with hTR or mTR using the telomeric repeat amplification protocol (TRAP) (Fig. 1A) and direct primer extension assays (Fig. 1B), respectively.

By TRAP, reconstituted hTR complexes were active, whereas reconstituted mTR complexes were inactive (Fig. 1A). Notably,

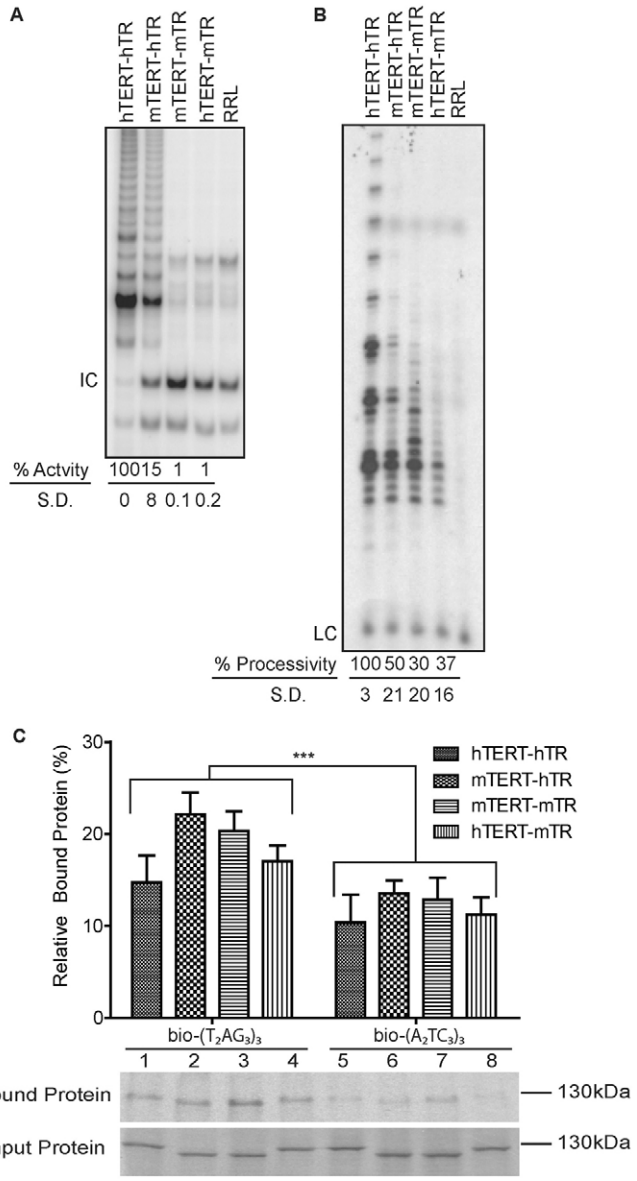

Fig. 1. Activity, processivity and DNA-binding affinity of in vitro reconstituted TERT-TR complexes. (**A**) TERT proteins were translated in RRL in the presence of previously transcribed hTR or mTR, and activity was assayed by TRAP. IC is the internal PCR control and RRL is the negative control. The decrease in activity of reconstituted mTERT-hTR, mTERT-mTR and hTERT-mTR complexes was statistically significant (compared with reconstituted hTERT-hTR complexes, $P<0.0001$). $n=3$. (**B**) TERT proteins were translated in RRL in the presence of previously transcribed hTR or mTR, and then incubated with biotinylated telomeric oligonucleotide $(TTAGGG)_3$ to measure processivity using the direct primer extension assay. LC is the loading control and RRL is the negative control. The decrease in processivity of reconstituted mTERT-hTR, mTERT-mTR and hTERT-mTR complexes was statistically significant (compared with reconstituted hTERT-hTR complexes, $P<0.05$). $n=3$. (**C**) L-[^{35}S]methionine-labelled hTERT and mTERT proteins were synthesized in RRL in the presence of in vitro transcribed hTR or mTR. TERT-TR complexes were then incubated with bio-$(T_2AG_3)_3$ (lanes 1-4) or bio-$(A_2TC_3)_3$ (lanes 5-8). Input protein represents the amount of protein before biotin binding and bound protein represents TERT bound to DNA primer. $n=5$. A two-way ANOVA test was performed showing statistically increased binding to bio-$(T_2AG_3)_3$ in the upper panel (***$P=0.0005$).

low levels of TRAP activity can sometimes be reconstituted by in vitro assembled mTERT-mTR complexes, possibly due to minor variations in reconstitution and TRAP-based assay conditions [our unpublished observations (Garforth et al., 2006)]. mTERT-hTR complexes retained 15% of wild-type activity (hTERT-hTR), suggesting that species-specific differences in telomerase activity also map to TERT (Fig. 1A). These results are supported by the observation that the activity reconstituted by the expression of mTERT in primary human BJ fibroblasts stably expressing the SV40 early region is lower than that of hTERT-transduced cells (Middleman et al., 2006).

To verify the processivity of heterologous TERT-TR complexes, we analyzed the ability of these in vitro reconstituted complexes to extend telomeric substrates using the direct primer extension assay (Fig. 1B). hTERT or mTERT complexed with hTR elongated the telomeric substrate $(TTAGGG)_3$. However, mTERT-hTR complexes retained only 50% of the processivity mediated by hTERT-hTR (Fig. 1B). Reconstituted mTR complexes elongated a telomeric substrate, but processivity was significantly reduced. Because of the design of the primers used in TRAP, the shortest products that can be amplified are 50 nucleotides in length (Gavory et al., 2002; Huard et al., 2003; Kim and Wu, 1997). Thus, the absence of long products of the mTERT-mTR and hTERT-mTR complexes (Fig. 1B) probably explains why no activity was observed by the TRAP assay (Fig. 1A).

Thus, in vitro reconstituted mTERT-hTR telomerase complexes are less active and processive than in vitro reconstituted hTERT-hTR complexes, suggesting that species-specific determinants of telomerase activity and processivity map to TERT. In vitro reconstituted mTR complexes form processivity-deficient telomerases, confirming previous reports that map species-specific differences in activity and processivity to determinants in the TR component (Chen and Greider, 2003; Garforth et al., 2006).

Heterologous telomerase-DNA interactions are not impaired

Wyatt et al. have shown that some hTERT mutants have reduced binding to their DNA substrates and reduced activity and processivity (Wyatt et al., 2007). To determine whether a defect in primer binding could explain the reduced activity of heterologous TERT-TR complexes, we used a primer-binding assay (Fig. 1C). We expressed L-[^{35}S]-methionine-radiolabelled hTERT and mTERT in vitro in RRL in the presence of in vitro transcribed hTR or mTR. We then incubated the reconstituted complexes with a biotinylated telomeric substrate, bio-$(T_2AG_3)_3$ (Fig. 1C, lanes 1-4), or a biotinylated non-telomeric substrate, bio-$(A_2TC_3)_3$ (Fig. 1C, lanes 5-8). All in vitro reconstituted TERT proteins displayed a statistically significant higher affinity for the telomeric bio-$(T_2AG_3)_3$ substrate than for non-telomeric oligonucleotide bio-$(A_2TC_3)_3$ (compare bound protein in Fig. 1C, lanes 1-4 and 5-8). We concluded that the reduced activities and processivities of in vitro reconstituted heterologous TERT-TR complexes are not due to decreased TERT-TR binding to its DNA substrate.

Relative telomerase activity and processivity of in vitro reconstituted heterologous telomerase complexes are not affected by dGTP concentration, permutation or length of the telomeric substrate

Elongation of DNA substrates by telomerase is regulated, in part, by alignment of the DNA with the template RNA, which differs in length between mTR (8 nucleotides) and hTR (11 nucleotides) (Autexier and Greider, 1995; Chen and Greider, 2003; Gavory et al., 2002; Greider and Blackburn, 1989). To determine whether the relative levels of activity and processivity of the heterologous complexes might vary with different substrates, we assessed the elongation of several additional telomeric oligonucleotides that have the potential to align differently and form distinct numbers of base pairs with the RNA templates. We tested primers containing diverse permutations of the telomeric sequence and different numbers of repeats, including $(GGGTTA)_4$ and TS-GTT; all are elongated efficiently by an in vitro reconstituted hTERT-hTR enzyme (Huard and Autexier, 2004; Moriarty et al., 2005). We also tested $(TG)_8TAG$, a primer predicted to align at the 3′ end of the hTR template (Huard and Autexier, 2004). Although we did not test an extensive number of different DNA substrates, we observed the same relative levels of activity and processivity seen with elongation of the telomeric $(TTAGGG)_3$ primer (Fig. 2). Telomerase activity and processivity are also affected by temperature, substrate concentration and dGTP concentration (Morin, 1989). As previously reported (Morin, 1989), we observed that longer elongation products were generated by hTERT-hTR in the presence of increased dGTP; however, the processivity of other TERT-TR complexes was not augmented (data not shown).

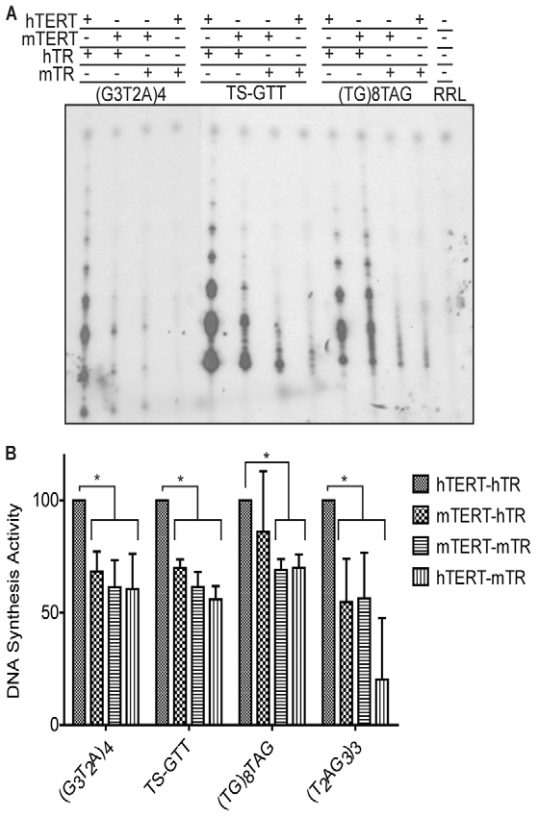

Fig. 2. Processivity of in vitro reconstituted TERT-TR complexes assayed using different telomeric substrates. (**A**) TERT proteins were translated in RRL in the presence of previously transcribed hTR or mTR, and then incubated with biotinylated telomeric [$(GGGTTA)_4$ and $(TG)_8TAG$] or non-telomeric (TS-GTT) oligonucleotides. (**B**) Quantification was performed from two independent experiments. The decrease in activity was statistically significant for reconstituted mTERT-hTR, mTERT-mTR and hTERT-mTR complexes (compared with reconstituted hTERT-hTR complex, $*P<0.05$).

Heterologous TERT-TR interactions are not impaired

The impaired processivity of heterologous hTERT-mTR complexes can be partly attributed to nucleotides adjacent to the template and to the pseudoknot region of mTR (Chen and Greider, 2003). However, the interaction between hTERT and an mTR pseudoknot fragment (1-145) was not impaired, suggesting that the reduced processivity of the complex was not due to gross defects in TERT-TR interactions (Chen and Greider, 2003). Similarly, northern blot analysis of endogenous hTR coimmunoprecipitated with tagged hTERT or mTERT expressed in primary human cells did not reveal significant defects in TERT-hTR interactions (Middleman et al., 2006). To determine whether there might be any defects in TERT-TR interactions in the context of a full-length TR and in vitro reconstituted complexes, we quantitatively analyzed the binding of hTERT and mTERT to hTR and mTR (Fig. 3). Increasing concentrations of non-radiolabelled hTR reduced the binding of radiolabelled hTR* to hTERT from 100% to 70±6 and 41±11%, and reduced the binding of radiolabelled mTR* to mTERT to 98±2, 62±9 and 8±3%. Increasing concentrations of non-radiolabelled mTR reduced the binding of radiolabelled hTR* to hTERT to 85±7, 42±7 and 13±6%, and reduced the binding of radiolabelled mTR* to mTERT to 86±9, 33±5 and 2±2% (Fig. 3, lower panel). Human and mouse TRs did not show any preferential binding to their respective TERTs. For example, 30 ng of non-radiolabelled hTR displaced radiolabelled mTR* from mTERT with the same efficiency that it displaced radiolabelled hTR* from hTERT, and similar results were observed for mTR (Fig. 3, compare lanes 9 to 10 and lanes 11 to 12). Interestingly, we reproducibly observed that mTR has a higher binding affinity than hTR for TERT, regardless of the origin of the TERT (Fig. 3, compare lanes 9 to 11 and lanes 10 to 12). Perhaps the increased affinity of mTR for hTERT and mTERT alters the flexible structure of these TERT-TR complexes, modifying their activity and processivity.

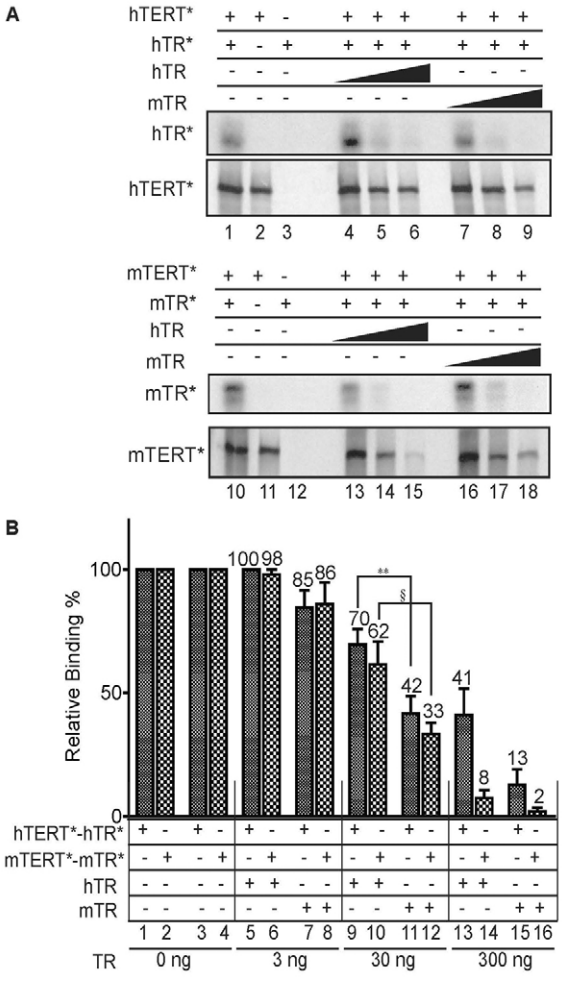

Fig. 3. In vitro reconstituted TERT-TR complex binding affinities. (**A**) FLAG-tagged hTERT and mTERT proteins were translated in RRL in the presence of L-[^{35}S]methionine and previously transcribed ^{32}P-labelled hTR or mTR. For competitive binding reactions, increasing concentrations of either unlabelled hTR or mTR (3, 30 or 300 ng) were added to labelled TERT-TR complexes. In vitro reconstituted TERT-TR complexes were immunoprecipitated with an anti-FLAG antibody, resolved by SDS-PAGE and detected by autoradiography. Radiolabelled species are denoted with an asterisk. (**B**) Numbers indicated above columns represent mean percentages of TERT binding to TR. n=5. The increased binding affinity of mTR for mTERT and hTERT, compared with that of hTR, was statistically significant (**,§P<0.05).

Nonetheless, the data confirm that the reduced processivity of in vitro reconstituted heterologous TERT-TR complexes is not due to significantly decreased binding of either hTERT or mTERT to full-length hTR or mTR. Our data show that the reduced activity and processivity of heterologous TERT-TR complexes cannot be attributed to reduced TERT-TR or TERT-TR-DNA interactions.

Non-functional heterologous TERT-TR complexes at the shortest telomeres in VA13 cells

To test whether heterologous TERT-TR complexes are functional at telomeres, we assessed the ability of these complexes to elongate the shortest telomeres. We used immortal human SV40-transformed WI38 lung-fibroblast-derived VA13 ALT cells that lack both hTERT and hTR expression, have no detectable telomerase activity, and maintain their telomeres through a recombination-based mechanism (Dunham et al., 2000; Wen et al., 1998). ALT cells have very long and heterogeneous telomeres (up to 50 kb) (Scheel et al., 2001). These cells also have an increased frequency of chromosomes with signal-free ends (SFEs), as measured by quantitative fluorescence in situ hybridization (Q-FISH) (Scheel et al., 2001). Telomerase preferentially elongates the shortest telomeres (Hemann et al., 2001; Teixeira et al., 2004). Restoration of human telomerase activity in VA13 cells elongates the shortest telomeres, which results in a marked decrease in the number of SFEs (Cerone et al., 2001; Grobelny et al., 2001; Perrem et al., 2001). Because VA13 cells are immortal, TERT function at the shortest telomeres can be evaluated independently of immortalization. Using this system, we tested whether heterologous TERT-TR complexes are able to reduce the number of SFEs in VA13 cells. We first generated stable VA13 clones expressing hTERT-hTR (h/h), mTERT-hTR (m/h), mTERT-mTR (m/m) and hTERT-mTR (h/m) complexes (Fig. 4A). Numbers after the designations represent clone numbers. The presence of hTERT, mTERT, hTR and mTR RNA was confirmed by reverse transcriptase (RT)-PCR (Fig. 4A), and the TRAP activity of in vivo reconstituted complexes was similar to the TRAP activity of in vitro reconstituted complexes (compare Fig. 4A and Fig. 1A). Similar to previously reported hTERT-hTR-expressing VA13 cells (Cerone et al., 2001; Grobelny et al., 2001; Perrem et al., 2001), the hTERT-hTR- expressing VA13 clones characterized in the current study, h/h5 and h/h9, had a significantly decreased number of SFEs (Fig. 4B). However, VA13 cells expressing TERT-TR

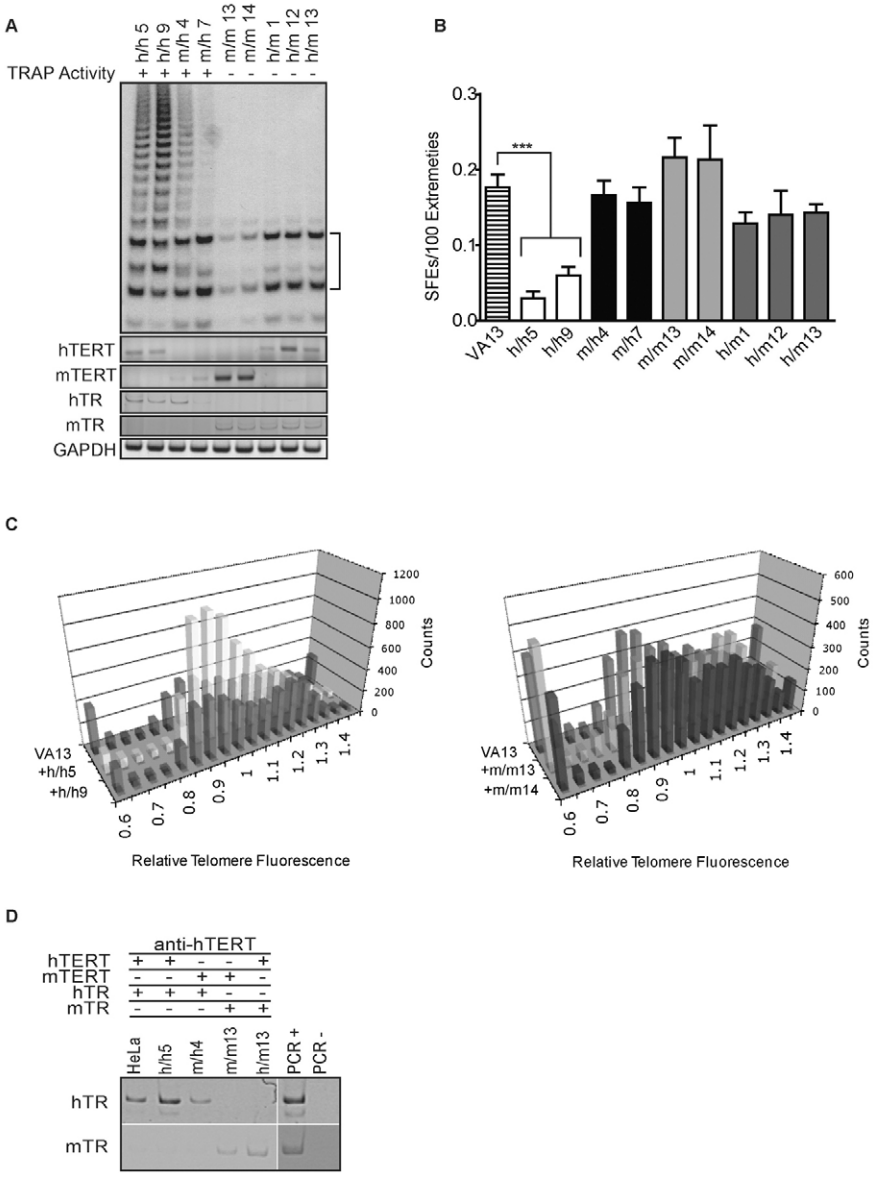

Fig. 4. Telomeric function of VA13 clones expressing TERT-TR. (**A**) Stable VA13 clones expressing hTERT-hTR (h/h), mTERT-hTR (m/h), mTERT-mTR (m/m) or hTERT-mTR (h/m) were established. The numbers after the designations represent clone numbers. Whole-cell extracts were used to test telomerase activity by TRAP. RNA was isolated to confirm the presence of hTERT, mTERT, hTR or mTR RNA by RT-PCR. GAPDH served as the RT-PCR control. The brackets indicate TRAP PCR primer dimers. (**B**) The number of SFEs was measured and quantified in chromosomal metaphase spreads from VA13 and VA13 TERT-TR clones. Each bar represents the number of SFEs per 100 chromosome ends in VA13 or VA13 TERT-TR cells (***P=0.0001). (**C**) The distribution of relative telomere length obtained by Q-FISH analysis. (**D**) Telomere-length profiles of h/h5 and h/h9 show decreased numbers of very short telomeres, and more homogeneous telomere lengths than VA13 cells; telomere length profiles of m/m13 and m/m14 are similar to those of VA13 cells. The presence of TR in anti-hTERT immunoprecipitated telomerase complexes was confirmed by RT-PCR from coimmunoprecipitated RNA. RNA extracted from HeLa whole-cell extracts was used as a positive control. PCR+ is a PCR control from VA13 cells stably expressing hTR or mTR. PCR– is a negative control from VA13 cells lacking TR expression.

complexes m/h4, m/h7, m/m13, m/m14, h/m1, h/m12 and h/m13 did not have decreased numbers of SFEs. Q-FISH analysis at the single-cell level revealed that the expression of functional telomerase complexes (particularly h/h5) (Fig. 4C, left panel) decreased the number of very short telomeres and rendered their lengths more homogenous. However, inactive telomerase complexes (m/m13 and m/m14) failed to significantly decrease either the number of very short telomeres or the telomere-length heterogeneity typical of VA13 cells (Fig. 4C, right panel).

Heterologous TERT and TR interactions in VA13 cells

Telomere-elongation defects of the heterologous telomerase complexes could be mediated by impaired TERT-TR interactions in cells. To rule out impaired TERT-TR interactions, we immunoprecipitated the complexes reconstituted in cells using an antibody raised against a peptide sequence present both in hTERT and mTERT (Moriarty et al., 2002). We analyzed the immunoprecipitated complexes for the presence of TR by RT-PCR. RT-PCR products were observed using RNA isolated from all four immunoprecipitated complexes (Fig. 4D). Thus, the inability of the mTERT-hTR complex to elongate the shortest telomeres was not due to a lack of activity (Fig. 4A) or the inability of mTERT and hTR to associate in cells (Fig. 4D). On the basis of the ability of hTERT-hTR, but not mTERT-hTR, to elongate the shortest telomeres, we concluded that the differences in telomere function of the heterologous complexes in human cells could result from inherent differences in the TERT components. Moreover, because hTERT-hTR, but not hTERT-mTR, elongated the shortest telomeres, the differences in telomere function of the heterologous complexes in human cells could also result from differences in the TR components.

Chimeric TERT complexes fail to rescue SFEs in VA13 cells

To further understand the lack of SFE rescue, and the reduced activity and processivity of the mTERT-hTR enzyme, we replaced conserved regions in mTERT with their hTERT counterpart. We hypothesized that replacing domains in mTERT with hTERT

domains would highlight hTERT and mTERT regions that differ in the regulation of activity, processivity and elongation of the shortest telomeres. We constructed chimeric TERT cDNAs and proteins by introducing unique restriction sites separating the main regions encoding the structural domains of hTERT and mTERT (Fig. 5A). The naming of the chimeric proteins reflects the identity, whether mouse or human, of the NTE, RT region and CTE. The proteins encoded by the original constructs containing the introduced unique restriction sites and used to assemble the chimeric TERT cDNAs and proteins were named HHH and MMM. Thus, we constructed HMM by exchanging the mouse NTE with the human NTE in MMM. Similarly, MHM and MMH were constructed by exchanging the mouse RT motif or CTE with the human RT motif or CTE in MMM, respectively. The NTE can further be subdivided into two regions: one containing the low-affinity RNA interaction domain RID1, which is implicated in regulating processivity, and a linker region; and the other containing the high-affinity RID2 domain, which is implicated in regulating activity (Moriarty et al., 2002). HRID1 consists of the human RID1-containing N-terminal region in an MMM backbone and HRID2 consists of the human RID2-containing N-terminal region in an MMM backbone. The in vitro expression of all constructs was verified in RRL (Fig. 5B). We first analyzed the TRAP activity of in vitro reconstituted chimeric TERT-TR complexes (Fig. 5C). We observed that most chimeric TERT-hTR complexes (MHM-hTR, MMH-hTR, HRID1-hTR and HRID2-hTR) reconstituted similar or reduced levels of activity compared with those reconstituted by mTERT-hTR (Fig. 5C). However, HMM-hTR, containing the N-terminal domain of hTERT, retained wild-type levels of activity, similar to levels reconstituted by hTERT-hTR (Fig. 5C). We tested the repeat addition processivity of in vitro reconstituted chimeric TERT-hTR complexes using the direct primer extension assay (Fig. 5D). We observed that the repeat addition processivity of MHM-hTR and MMH-hTR was comparable to that of hTERT-hTR, whereas that of HRID1-hTR and HRID2-hTR was similar to the repeat addition processivity of mTERT-hTR, despite lower levels of expression of HRID1-hTR and HRID2-hTR in vitro (Fig. 5B). Although HMM-hTR exhibited wild-type activity by TRAP (Fig. 5C), its repeat addition processivity was compromised (Fig. 5D).

To determine whether wild-type activity or wild-type processivity is sufficient for elongation of the shortest telomeres, we stably expressed the chimeric TERT proteins HMM, MHM and MMH in VA13 cells expressing hTR. We analyzed the activity of stably expressed chimeric TERT-hTR complexes in VA13 by the TRAP assay (Fig. 5E). As observed with in vitro reconstituted enzymes, HMM11-hTR and HMM12-hTR reconstituted in VA13 cells retained wild-type levels of activity, whereas MHM12-hTR, MHM14-hTR, MMH1-hTR and MMH11-hTR did not exhibit any detectable activity when assessed in vitro by the TRAP assay. We then analyzed the ability of chimeric TERT-hTR complexes to rescue SFEs and elongate the shortest telomeres in VA13 cells. As previously described in Fig. 3, a functional hTERT-hTR complex (h/h5) was able to rescue SFEs in VA13 cells (Fig. 5F). However, similarly active complexes that were deficient in repeat addition

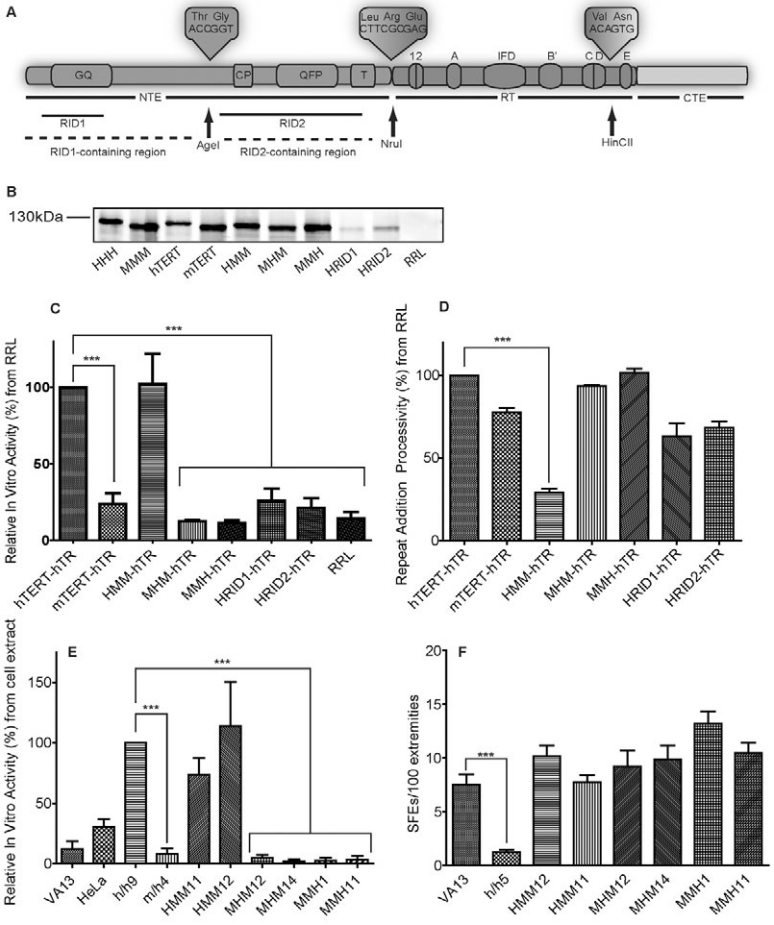

Fig. 5. In vitro analysis and telomeric function of chimeric TERT-TR complexes reconstituted in vitro and in VA13 cells. (**A**) Schematic representation of TERT with conserved regions in the NTE, RT region and CTE. Arrows indicate regions of unique restriction endonuclease sites engineered for chimeric cDNA construction. Call outs indicate the regions of identical sequences where restriction endonuclease sites were introduced. (**B**) TERT proteins were translated in RRL in the presence of L-[^{35}S]methionine and resolved on SDS-PAGE. HHH and MMM refer to the proteins encoded by the original constructs with mutated nucleotide sequences used to make the chimeric TERT constructs for chimeric TERT protein expression. (**C**) TERT proteins were translated in RRL in the presence of previously transcribed hTR. Telomerase activity was assayed by TRAP. RRL served as the negative control. n=3 (***P=0.0001). (**D**) TERT proteins were translated in RRL in the presence of previously transcribed hTR and then incubated with biotinylated telomeric oligonucleotide $(TTAGGG)_3$. Processivity was assayed using the direct primer extension assay. n=3 (***P=0.0001). (**E**) 1 μg of whole-cell extracts from stable VA13 clones expressing telomerase complexes was used to test telomerase activity by the TRAP assay. n=3 (***P=0.0001). (**F**) The number of SFEs was measured from chromosomal metaphase spreads from VA13 and VA13 TERT-TR clones and quantified. Each bar represents the number of SFEs per 100 chromosome ends in VA13 and VA13 TERT-TR cells (***P=0.0001).

processivity (HMM11 and HMM12) were unable to rescue SFEs in VA13 cells. Furthermore, chimeric enzymes that were activity deficient but repeat addition processivity proficient (MHM and MMH) also failed to rescue SFEs in VA13 cells. Our data suggest that minimum levels of both activity and processivity might be required for elongation of the shortest telomeres in VA13 cells.

Chimeric TERT-TR complexes fail to localize to the telomeres of VA13 cells

Recruitment of hTR from subnuclear sites to telomeres is co-dependent on the proper recruitment of hTERT during S phase (Tomlinson et al., 2008). We tested whether the lack of SFE rescue in VA13 cells expressing mTERT-hTR, HMM-hTR, MHM-hTR and MMH-hTR might partly be due to the lack of proper localization of these complexes to telomeres, in addition to defects in activity or processivity. To observe the localization of TERT-TR complexes to telomeres, we performed in situ hTR hybridization with three hTR-specific Cy3-conjugated (red) probes followed by telomeric-repeat-binding factor 1 (TRF1) immunofluorescence (green) (Tomlinson et al., 2008; Tomlinson et al., 2006; Zhu et al., 2004) (Fig. 6A). As reported previously, we did not observe any hTR-TRF1 foci in parental VA13 cells, because of the lack of hTERT expression and hTR transcription (Tomlinson et al., 2008) (Fig. 6). In HeLa cells expressing endogenous hTERT and hTR, we observed the formation of some hTR-TRF1 foci, as previously reported (Tomlinson et al., 2006), indicating proper recruitment of the telomerase complex to the telomeres. A previous report shows that hTERT expression is sufficient and important for the localization of hTR to specific nuclear foci (Tomlinson et al., 2008). In VA13 h/h5 cells, we observed the formation of both hTR and hTR-TRF1 foci, indicating proper recruitment of hTERT-hTR to telomeres, consistent with decreased levels of SFEs in these cells (Fig. 4B; Fig. 5F). However, in m/h7-, HMM12-, MHM14- and MMH11-expressing cells, we did not observe the formation of hTR-TRF1 foci, indicating impaired localization of m/h7 and chimeric TERT-TR complexes to telomeres (Fig. 6A). hTR localization with ALT-associated PML bodies (APBs) could also occur because APBs are formed by the coalescence of extremities with long and short telomeres (Draskovic et al., 2009). Localization of hTR to telomeres in h/h5 cells suggests that the telomerase complex in these cells can be recruited to telomeres.

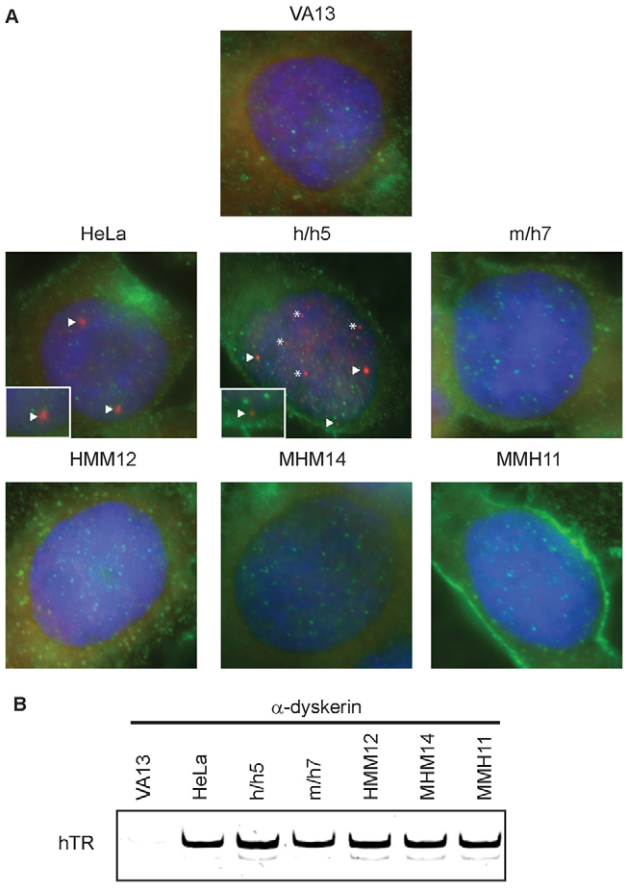

Fig. 6. Telomere recruitment and assembly of TERT-TR complexes. (**A**) Cells were prepared for in situ hTR hybridization and TRF1 immunofluorescence analysis. hTR probes are Cy-3 conjugated (red) and the TRF1 secondary antibody is Cy-2 conjugated (green). hTR and TRF1 colocalization is observed in HeLa and VA13 h/h5 cells, but not in VA13, VA13 m/h7, VA13 HMM12, VA13 MHM14 or VA13 MHM11 cells. Arrows indicate hTR foci that are colocalized with TRF1 foci; asterisks indicate hTR foci only. (**B**) The presence of TR in anti-dyskerin immunoprecipitated TERT-TR complexes was confirmed by RT-PCR using coimmunoprecipitated RNA. VA13 protein extract was used as a negative control and HeLa protein extract was used as a positive control for the absence and presence of a functional telomerase complex.

Dyskerin is implicated in telomerase assembly and is part of a catalytically competent telomerase complex (Cohen et al., 2007; Fu and Collins, 2003). To understand whether impaired localization might be due to defects in complex assembly, we immunoprecipitated heterologous and chimeric telomerase complexes from VA13 cells using an anti-dyskerin antibody and then tested the presence of hTR in the immunoprecipitate by RT-PCR (Fig. 6B). Endogenous hTR from HeLa cells coimmunoprecipitated with dyskerin (Fig. 6B). Despite impaired localization to telomeres, heterologous and chimeric telomerase complexes also interacted with dyskerin. Thus, our results suggest a requirement for several hTERT domains and interaction with possibly multiple proteins for proper recruitment to short telomeres in human ALT cells. Moreover, the inability of mTERT to elongate short human telomeres suggests the existence of species-specific TERT interactions with telomerase-associated or telomere proteins.

hTERT expression in mTERT$^{-/-}$ ES cells prevents the accumulation of SFEs and end-to-end fusions

To assess the function of hTERT at murine telomeres, we used mTERT$^{-/-}$ and mTR$^{+/+}$ mouse embryonic stem (ES) cells (Liu et al., 2002; Liu et al., 2000). These cells exhibit genomic instability, aneuploidy and telomeric fusions at late passage. We infected early-passage (p35) mTERT$^{-/-}$ mouse ES cells with hTERT- and mTERT-expressing vectors, and followed their growth to passage 89 (p89). hTERT and mTERT expression was verified by RT-PCR (data not shown). mTERT, but not hTERT expression, reconstituted telomerase activity, as assessed in vitro by TRAP (Fig. 7A). We predict that levels of reconstituted activity might be low and/or that there are limitations of the in vitro elongation and PCR-based TRAP assays with regard to generating and detecting elongation products of the hTERT-mTR complex extracted from cells. These results are consistent with previous reports that hTERT is not efficiently amenable to activation by mTR (Autexier et al., 1996; Beattie et al., 1998). We performed cytogenetic analyses of early (p35) and late (p89) mTERT$^{-/-}$ (denoted –/–p35 or –/–p89, respectively), late-passage hTERT-expressing mTERT$^{-/-}$ (denoted

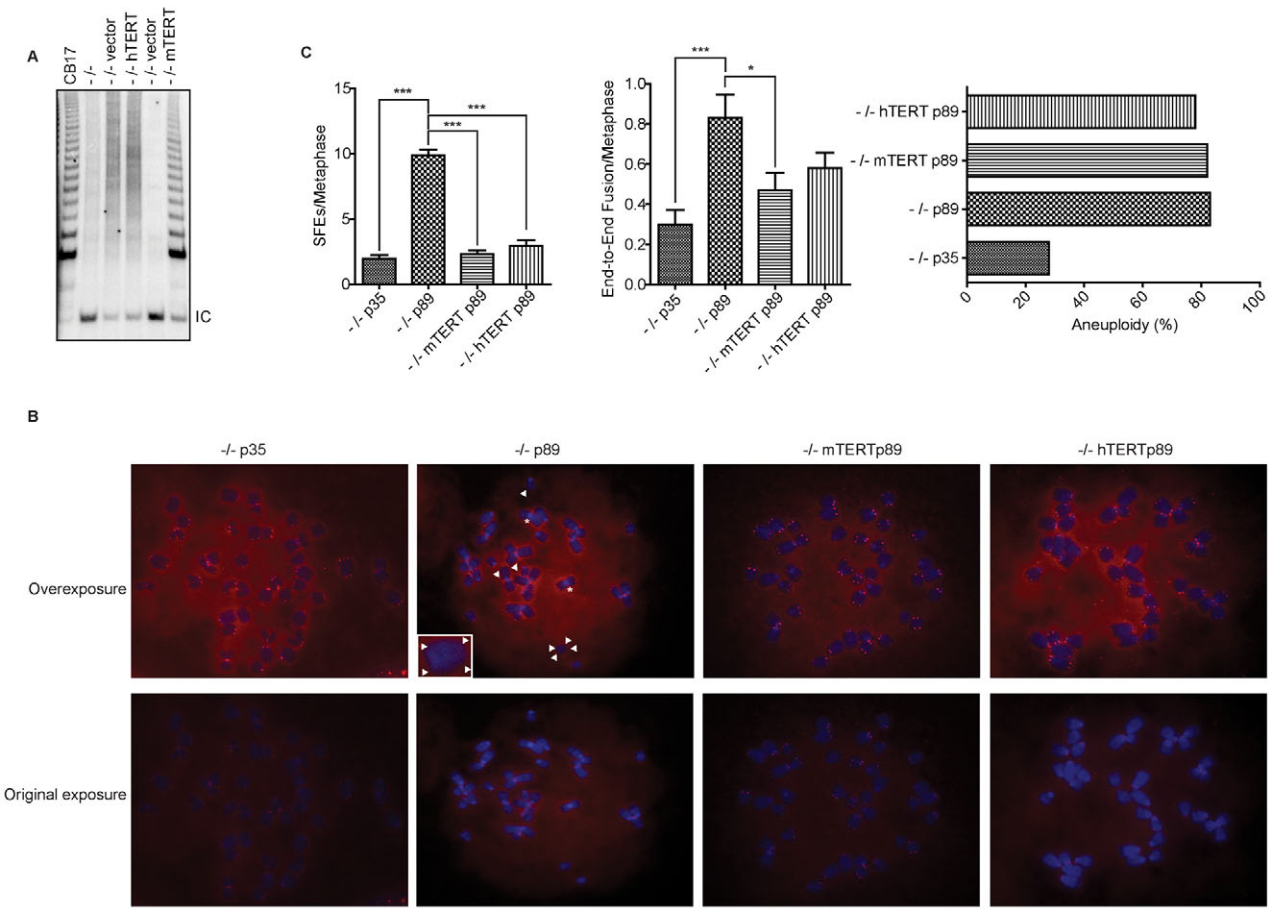

Fig. 7. Cytogenetic analyses of early (p35) and late (p89) mTERT$^{-/-}$ (denoted –/–p35 or –/–p89, respectively), late-passage hTERT-expressing mTERT$^{-/-}$ (denoted –/–hTERT p89) and late-passage mTERT-expressing mTERT$^{-/-}$ (denoted –/–mTERT p89) ES cells. (**A**) Telomerase activity of reconstituted hTERT and mTERT mTERT$^{-/-}$ ES cells was analyzed using the TRAP assay. CB17 are immortal mTERT- and mTR-positive mouse-derived cells. IC indicates internal control. (**B**) Representative metaphase spreads for each cell type hybridized with a Cy3-labelled PNA telomeric probe (red) and counterstained with DAPI (blue). Arrowheads indicate SFEs and asterisks indicate chromosomal end-to-end fusions. The lower part of the panel shows the original exposure. The upper part of the panel shows overexposed images to better visualize telomere signals. (**C**) Measurement of SFE, end-to-end fusion and aneuploidy levels. Cytogenetic analysis and quantification was performed on 50 metaphase spreads per cell type (***P<0.0001, *P=0.01).

–/–hTERT p89) and late-passage mTERT-expressing mTERT$^{-/-}$ (denoted –/–mTERT p89) ES cells (Fig. 7B,C). At early passage, mTERT$^{-/-}$ ES cells harboured minimal chromosomal aberrations and virtually no SFEs. However, late-passage mTERT$^{-/-}$ ES cells accumulated a substantial number of end-to-end fusions and SFEs (Fig. 7B,C). In mTERT$^{-/-}$ cells ectopically expressing wild-type mTERT, the numbers of SFEs and end-to-end fusions were significantly fewer at late passage compared with late-passage mTERT$^{-/-}$ cells. In late-passage mTERT$^{-/-}$ cells expressing wild-type hTERT, significantly fewer SFEs also occurred, similar to that observed in mTERT-expressing mTERT$^{-/-}$ late-passage cells (Fig. 7B,C). Additionally, hTERT expression prevented end-to-end fusions in late-passage cells. However, a significant increase in aneuploidy was observed in late-passage mTERT$^{-/-}$ ES cells and in mTERT- or hTERT-expressing mTERT$^{-/-}$ ES cells. We concluded that hTERT, like mTERT, is recruited to telomeres in mouse ES cells and prevents the accumulation of SFEs. Furthermore, the ability of hTERT to function at mouse telomeres and the inability of mTERT to function at human telomeres suggests that the mechanisms regulating recruitment and activity of hTERT at mouse telomeres might be less stringent than the mechanisms regulating mTERT at human telomeres.

Discussion

Species-specific determinants of telomerase activity and processivity map to TERT

The lower activity and processivity of mTERT-hTR complexes could partly be due to an altered conformation that renders them less efficient than hTERT-hTR complexes at elongating substrates in vitro. However, the lower activity and processivity is not a result of either the inability of mTERT to bind hTR or decreased binding of mTERT-hTR to telomeric substrates (Figs 1, 2 and 3). hTERT mutants with decreased activity and processivity can be defective in binding telomeric substrates (Wyatt et al., 2007). However, mTERT-TR complexes reproducibly showed increased binding to the telomeric substrate bio-$(T_2AG_3)_3$ compared with hTERT-TR complexes (Fig. 1C). We speculate that the increased binding could disrupt enzyme translocation, consequently reducing the processivity of these complexes. Reconstituted mTR complexes did not rescue SFEs in VA13 cells (Fig. 4), indicating that species-

specific differences in telomere function also map to the TR component. The inability of reconstituted mTR enzymes to support minimal telomere elongation in human ALT cells might be due to their low telomerase activity (Fig. 1) (Garforth et al., 2006), although improper recruitment to human telomeres cannot be excluded.

Chimeric human-NTE-containing mTERT, HMM, exhibited wild-type TRAP activity consistent with previous reports (Middleman, 2006), but was defective in repeat addition processivity (Fig. 5). Human NTE is an important region mediating telomerase activity in vitro and in vivo (Armbruster et al., 2001; Beattie et al., 2000; Mitchell and Collins, 2000; Moriarty et al., 2005). Thus, humanizing the NTE in mTERT was perhaps sufficient to restore the activity of the reconstituted HMM enzyme. The NTE of hTERT in the chimeras contains additional amino acid sequences closer to the linker region compared with the NTE of mTERT present in the chimeras, which could potentially contribute to increased activity. Furthermore, the CTE in hTERT is important for processivity (Huard et al., 2003), which could explain the lack of wild-type repeat addition processivity of HMM-hTR. Replacing the mTERT CTE with the hTERT CTE can restore wild-type activity, maintain telomeres at a short length and induce immortalization of primary human cells (Middleman et al., 2006). We did not observe the same levels of telomerase activity in our reconstituted MMH enzyme. We postulate that this discrepancy might be due to differences in the exact hTERT CTE region that was introduced into mTERT. In an effort to maintain conserved amino acids, the hTERT C-terminal region we introduced was larger (amino acids 905-1133) than that introduced by Middleman and colleagues (amino acids 985-1133) (Middleman et al., 2006), and thus contained part of the human RT region. Additionally, the CTE contains a binding site for 14-3-3 protein (located between amino acids 1030 and 1040) flanked upstream by a nuclear-export-like sequence (between amino acids 974 and 980) (Seimiya et al., 2000). Disruption of 14-3-3 binding abrogates nuclear localization and enhances cytoplasmic export of hTERT by the nuclear export receptor CRM1. We speculate that additional hTERT amino acids present in our MMH TERT could alter 14-3-3 binding and enhance CRM1-mediated cytoplasmic export, which might explain the lack of telomere elongation by our reconstituted MMH enzyme.

Lack of mTERT recruitment to and elongation of the shortest human telomeres suggests divergent regulation of telomere maintenance in humans and mouse

Lack of elongation of the shortest telomeres in human ALT cells by mTERT-hTR and chimeric TERT-hTR molecules appears to be due to defects in telomere localization of these complexes (Fig. 4B; Fig. 5F; Fig. 6A). Species-specific determinants that are present in hTERT, but lacking in mTERT, or altered in the mTERT-hTR and chimeric TERT-hTR complexes, might be responsible for recruiting the telomerase complex to the shortest telomeres. Many cellular proteins affect the function of the telomerase holoenzyme (Cristofari et al., 2007). Proteins that modulate telomerase activity, processivity and/or recruitment to telomeres include TPP1, POT1, Pif1, EST1p and PinX1 (Snow et al., 2003; Wang et al., 2007; Xin et al., 2007; Zhang et al., 2006; Zhou and Lu, 2001). Specifically, TPP1 has been shown to mediate the recruitment of the telomerase complex through direct interaction between hTERT and POT1 (Xin et al., 2007). Thus, interactions with multiple recruitment proteins might be compromised in the chimeric complexes. Particularly, mTERT and the mTERT-like chimeric proteins might harbour species-specific elements that are not amenable to interactions with human telomerase-associated or telomere-binding proteins, thus causing improper telomere recruitment and lack of elongation of the shortest telomeres in immortal human cells. Multiple domains, including the N-DAT and C-DAT regions in the N and C termini of hTERT, have been implicated in recruitment, which is consistent with our results indicating the requirement for multiple hTERT domains for proper recruitment to, and elongation of, the shortest telomeres (Armbruster et al., 2003; Banik et al., 2002; Counter et al., 1998a).

hTERT can function at mouse telomeres

Telomere maintenance in mouse cells is mediated by mTERT and mTR (Blasco et al., 1997; Liu et al., 2000), despite the low levels of in vitro activity exhibited by mouse telomerase (Blasco et al., 1997; Prowse et al., 1993). Ectopic expression of hTERT or mTERT in mTERT$^{-/-}$ ES cells at early passage was sufficient to prevent the SFEs and end-to-end fusions typically observed in mTERT$^{-/-}$ ES cells at late passage (Fig. 7). Thus, hTERT was able to function at the shortest telomeres, despite the in vitro limitations of detecting reconstituted hTERT-mTR telomerase activity. It is probable that the hTERT-mTR complex reconstituted in murine cells elongates the telomeres sufficiently to prevent the generation of SFEs and end-to-end fusions. Although hTERT and mTERT were expressed at early passage in mTERT$^{-/-}$ ES cells, aneuploidy persisted, suggesting that aneuploidy was established prior to hTERT or mTERT expression. In the context of human ALT cells, TERT recruitment to the shortest telomeres appears to be stringently regulated, indicating that human proteins might have evolved to use only hTERT for telomere elongation. In mouse cells, recruitment of telomerase to telomeres appears more flexible, and both hTERT and mTERT are able to elongate short telomeres. Interestingly, mouse Pif1 has been reported to interact with hTERT, consistent with the notion that the interactions of mouse telomerase-associated proteins might be flexible (Snow et al., 2007). Additionally, species-specific elements in hTERT that control selectivity through protein-protein interactions could be missing in mTERT. Alternatively, because mTERT is less active, elongation of short mouse telomeres might require an increased stoichiometric presence of mTERT at telomeres. Hence, the selectivity of mouse telomerase-associated or telomere-binding proteins might be greatly reduced to maintain a high stoichiometric presence of mTERT at telomeres to ensure proper telomere elongation.

The ability of hTERT to function at mouse telomeres and the inability of mTERT to function at human telomeres suggests that mechanisms regulating the recruitment and activity of hTERT at mouse telomeres might be less stringent than the mechanisms regulating mTERT at human telomeres. Approaches such as those reported here and previously (Chen and Greider, 2003; Middleman et al., 2006) might lead to the identification of TERT sequences that confer similar functions in human and mouse cells. Such functions might be better therapeutic targets than domains with species-restricted functions, because one would predict that the effects of targeting conserved functions might lead to similar effects in rodent and human cells. Minimally, a better understanding of the differences and similarities between human and mouse telomerase and telomere regulation might allow the more accurate interpretation of the anti-telomerase effects observed in rodent models.

Materials and Methods

Plasmid construction

The plasmids pET28b-hTERT (Moriarty et al., 2002) and pET28a-mTERT were used as templates to generate the constructs pcDNA3-neomycin-FLAG-hTERT and pcDNA3-neomycin-FLAG-mTERT. pET28a-mTERT was constructed by subcloning a *Eco*RI-*Not*I fragment from pcDNA3.1-HA-mTP2 containing the mTERT cDNA (Liu et al., 2000) into pET28a. Digested *Eco*RI and *Not*I PCR products encoding FLAG-tagged hTERT or mTERT were cloned into *Eco*RI- and *Not*I-digested pcDNA3-neomycin (Invitrogen, donated by Anne Gatignol, Lady Davies Institute, Montreal, Canada). Construction of pcDNA3.1-hygromycin-hTR was previously described (Marie-Egyptienne et al., 2005). pcDNA3.1-hygromycin-mTR was generated using pcDNA3.1 (Invitrogen, gift of Antonis Koromilas, Lady Davies Institute, Montreal, Canada). *Hind*III- and *Bam*HI-digested mTR from pcDNA3-neomycin-mTR was cloned into pcDNA3.1-hygromycin digested with the same enzymes. pcDNA3-neomycin-mTR was constructed from pUC119-mTR (pmTR+1) digested with *Hind*III and *Bam*HI. pUC119-mTR (pmTR+1) was constructed by digesting mTR from pBKSmTR [obtained from Carol Greider, Johns Hopkins University School of Medicine, Baltimore, MD (Blasco et al., 1995)] with *Mse*I and *Not*I. The digested mTR fragment was used as a template for PCR amplification. The amplified fragment was digested with *Hind*III and *Bam*HI, and inserted into pUC119 vector digested with the same enzymes.

Construction of pcDNA3.0-neomycin-FLAG chimeric TERT plasmids was performed by introducing three unique restriction sites into *hTERT* and *mTERT* cDNAs between the regions encoding the major protein domains at the following nucleotide positions: hTERT 1110-1115, mTERT 1125-1131 (RID1-RID2), hTERT 1841-1846, mTERT 1811-1816 (NTE-RT), hTERT 2770-2775, mTERT 2749-2754 (RT-CTE). The proteins encoded by the original constructs containing the introduced unique restriction sites and used to assemble the chimeric TERT proteins were named HHH and MMM. The introduced restriction sites did not modify the amino acid sequence. Subsequently, the desired fragment in MMM was exchanged with the corresponding hTERT domain. The naming of the chimeric proteins reflects the identity, whether mouse or human, of the NTE, RT region and CTE. Thus, we constructed HMM by exchanging the mouse NTE with the human NTE in MMM. Similarly, MHM and MMH were constructed by exchanging the mouse RT motif or CTE with the human RT motif or CTE in MMM, respectively. HRID1 consists of the human RID1-containing N-terminal region (amino acids 30 to 159) in an MMM backbone and HRID2 consists of the human RID2-containing N-terminal region (amino acids 350 to 547) in an MMM backbone.

Details of the construct pMSCV-puromycin-FLAG-hTERT are to be published (Yasmin D'Souza and C.A., unpublished data). pLPC-puromycin-FLAG-mTERT was constructed by PCR amplification of pcDNA3.0-neomycin-FLAG-mTERT. Digested *Hind*III and *Eco*RI PCR products were cloned into *Hind*III- and *Eco*RI-digested pLPC (donated by Gerardo Ferbeyre, AAA, BBB, CCC). Sequencing validated the integrity of all constructs.

Cell culture

GM847, VA13 and VA13-hTERT stable cell lines (Guiducci et al., 2001) (gift of Silvia Bacchetti, Istituto Regina Elena, Rome, Italy) were grown in α-MEM (Invitrogen) supplemented with 10% FBS (Wisent, Canada). VA13-hTERT cells were transfected with pcDNA3.1-hygromycin-hTR and pcDNA3.1-hygromycin-mTR. Clones were selected with 105 μg/ml hygromycin for 3-4 weeks and split in a 1:4 ratio at confluency. VA13 cells were transfected with pcDNA3-neomycin-FLAG-mTERT. Clones were selected with 250 μg/ml G418 for 3-4 weeks and split in a 1:4 ratio at confluency. Subsequently, cells stably expressing FLAG-mTERT were transfected with pcDNA3.1-hygromycin-hTR and pcDNA3.1-hygromycin-mTR. Clones were selected with 105 μg/ml hygromycin for 3-4 weeks and split in a 1:4 ratio at confluency. A5 mTERT$^{-/-}$ mouse ES cells [kind gift from Lea Harrington, University of Edinburgh, UK (Liu et al., 2000)] were grown on plates coated with 0.1% gelatin in DMEM (Invitrogen) supplemented with 17% FBS, β-mercaptoethanol (0.001%) and ESGRO (0.0001%) (Chemicon). Cells were split in a 1:4 ratio at confluency. A5 mTERT$^{-/-}$ ES cells were retrovirally infected with pMSCV-puromycin-FLAG-hTERT or pLPC-puromycin-FLAG-mTERT.

RNA isolation and RT-PCR

Total RNA isolation was performed using TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was generated from total RNA and PCR was used to detect hTERT with primers HT1 and HT5 (Bodnar et al., 1998); mTERT with mTERT-WTF (5′-GACATGGAGAACAAGCTGTTTGCTGAG-3′) and mTERTshortR (5′-ATCTTGTATATATTGATGCAGACTGTCTGG-3′) primers; hTR with hTR-F3B and hTR-R3C primers (Nakamura et al., 1997); and mTR with mTR-F2-RT (5′-CCTAACCCTGATTTTCATTAGCTGTGGG-3′) and mTR-R2-RT (5′-GAGGCTCGGGAACGCGCGGTGGCCC-3′) primers. Human and mouse GAPDH was amplified with primers RT11 and RT12 (Cerone et al., 2006).

In vitro transcription and translation

In vitro transcription and translation were performed using the T7-coupled transcription/translation RRL system (Promega) (Moriarty et al., 2002). Full-length FLAG-hTERT and FLAG-mTERT were synthesized in RRL in the presence of purified hTR or mTR and L-[^{35}S]methionine. hTR was synthesized from *Fsp*I-linearized phTR+1 (Autexier et al., 1996). mTR was synthesized from *Nco*I-linearized pmTR+1.

Oligonucleotide synthesis

5′-biotinylated and high-performance liquid chromatography (HPLC)-purified oligonucleotides were prepared by Operon (USA). Non-tagged desalted oligonucleotides were prepared by AlphaDNA (Canada).

TRAP, direct extension assay, TRAP quantification and direct extension assay quantification

The activity of FLAG-TERT-TR complexes was analyzed by TRAP (Moriarty et al., 2002). However, in some cases, radiolabelled dGTP was omitted and TRAP gels were stained with SYBR Safe (Invitrogen) and scanned on a Storm 840 imager. 1 μl of RRL expressing FLAG-TERT in the presence of TR was diluted in 50 μl IP buffer [10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1% NP-40, 10% glycerol, 150 mM NaCl]. 1 μl of the diluted RRL was assayed for telomerase activity by TRAP or direct primer extension assay. The processivity of FLAG-TERT-TR complexes was analyzed by the direct extension assay under the same experimental conditions of dGTP concentration, temperature and substrate concentration (Moriarty et al., 2004). TS-GTT is the standard substrate typically used in TRAP assay; the sequence of this substrate is: 5′-AATCCGTCGAGCAGAGTT-3′. The enzymatic activity of heterologous FLAG-TERT-TR complexes detected by the TRAP assay was quantified (see Huard et al., 2003). Repeat addition processivity was quantified as described (Moriarty et al., 2004). The telomerase activity of 1 μg whole-cell extract aliquots of VA13 cells, mTERT$^{-/-}$ ES cells and TERT-expressing mTERT$^{-/-}$ ES cells was analyzed by the TRAP assay.

In vitro RNA-binding assay and quantification

RNA-binding assays were performed as described (Moriarty et al., 2002), except 8.82 μg/ml of M2 anti-FLAG antibody (Sigma) was used to immunoprecipitate FLAG-hTERT and FLAG-mTERT. Quantification of FLAG-TERT-TR interactions was performed as described previously (Moriarty et al., 2002).

Immunoprecipitation of hTR and mTR

Immunoprecipitation of TERT-TR complexes from VA13 cells expressing the telomerase heterocomplexes was performed with an affinity-purified polyclonal anti-hTERT antibody raised against a peptide sequence present in both hTERT and mTERT (Moriarty et al., 2002). Immunoprecipitation of dyskerin in VA13 cells expressing TERT-TR and chimeric TERT-TR complexes was performed with affinity-purified rabbit polyclonal anti-dyskerin antibody H-300 (Santa Cruz Biotechnologies). Elution of RNA (TR) from these complexes was performed by adding 100 μl of elution buffer (50 mM Tris-HCl pH8, 100 mM NaCl, 10m M EDTA, 1% SDS) to IP beads and incubating at 60°C for 10 minutes. RNA was then collected by performing a phenol/chloroform extraction, followed by ethanol precipitation and resuspension in 30 μl RNase-free water. RT-PCR was performed using the primers hTR-F3B or mTR-R2-RT. PCR detection of hTR was performed as described above. PCR detection of mTR was performed with the following primers: mTR-F3-RT (5′-CATTAGCTGTGGGTTCTGGTCTTTTG-3′) and mTR-R3-RT (5′-CGCGC-GGTGGCCCGCTGCAG-3′).

In vitro DNA-binding assay and quantification

Primer-binding assays were performed as described (Wyatt et al., 2007), except streptavidin magnesphere paramagnetic particles were used for oligonucleotide pull down (Promega, USA) and immobilization of biotinylated oligonucleotides. TERT proteins were synthesized in RRL using L-[^{35}S]methionine to visualize and quantify the oligonucleotide-bound TERT. Quantification of primer-binding experiments was done as described (Wyatt et al., 2007), except Imagequant (GE Healthcare) software was used for quantification.

TRF and telomeric Q-FISH

Metaphase spreads were prepared as described (see Liu et al., 2000). Telomere-length analysis and Q-FISH analyses were performed as described (Cerone et al., 2006).

hTR FISH and TRF1 immunofluorescence

hTR FISH was performed as described (Tomlinson et al., 2008) using unsynchronized cells. hTR probes were Cy-3 conjugated (red). TRF1 was detected using a rabbit polyclonal anti-TRF1 antibody (Abcam) (1:300 dilution) and a goat anti-rabbit Cy2-conjugated (green) secondary antibody (Jackson) (1:100 dilution). Images were taken on a Zeiss microscope and analyzed with Axiovision software (Zeiss, USA).

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