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Research article

THE HUMAN TELOMERASE CATALYTIC SUBUNIT AND VIRAL TELOMERASE RNA RECONSTITUTE A FUNCTIONAL TELOMERASE COMPLEX IN A CELL-FREE SYSTEM, BUT NOT IN HUMAN CELLS

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Abstract: The minimal vertebrate telomerase enzyme is composed of a protein component (telomerase reverse transcriptase, TERT) and an RNA component (telomerase RNA, TR). Expression of these two subunits is sufficient to reconstitute telomerase activity *in vitro*, while the formation of a holoenzyme comprising telomerase-associated proteins is necessary for proper telomere length maintenance. Previous reports demonstrated the high processivity of the human telomerase complex and the interspecies compatibility of human TERT (hTERT). In this study, we tested the function of the only known viral telomerase RNA subunit (vTR) in association with human telomerase, both in a cell-free system and in human cells. When vTR is assembled with hTERT in

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a cell-free environment, it is able to interact with hTERT and to reconstitute telomerase activity. However, in human cells, vTR does not reconstitute telomerase activity and could not be detected in the human telomerase complex, suggesting that vTR is not able to interact properly with the proteins constituting the human telomerase holoenzyme.

Key words: Telomerase, Holoenzyme, hTERT, vTR, Dyskerin, Marek's disease virus

INTRODUCTION

The major role of telomerase is to protect genome integrity by maintaining telomere length [reviewed in 1]. In vertebrates, this RNA-dependent DNA polymerase is involved in the catalysis of telomeric repeats (5'-GGTTAG-3') at the 3'-ends of chromosomes. This compensates for the telomere shortening that is induced by semi-conservative DNA replication [2]. Thus, telomerase plays a key role in highly proliferative cells in most eukaryotic organisms. Although many normal somatic cells maintain a low basal level of telomerase, its up-regulation is associated with immortalization processes and as a consequence, tumorigenicity [3]. Indeed, telomerase activity is detected in more than 85% of human cancers [4].

The telomerase holoenzyme is composed of a number of proteins involved in the assembly, processing and stability of the complex *in vivo* [reviewed in 5, 6]. The two main subunits of this ribonucleoprotein, the reverse transcriptase protein moiety (telomerase reverse transcriptase, TERT) and the RNA component (telomerase RNA, TR), are sufficient to reconstitute telomerase activity *in vitro* [7, 8]. The synthesis of telomeric motifs at the ends of chromosomes is performed by reverse transcription of a template region present in TR by the catalytic subunit, TERT. Regulation of the number of telomeric repeats added by telomerase is a complex process that is dictated by several factors [reviewed in 5, 9]. Studies have demonstrated that the human telomerase enzyme is one of the most processive telomerase complexes, especially compared to the murine one [10, 11]. Human telomerase processivity is mediated by template and non-template regions of human TR (hTR) and by regions in human TERT (hTERT), specifically the C-terminal and N-terminal RNA interaction domain 1 [12-17].

The ectopic expression of hTERT enables the immortalization not only of human primary cells, but also of other primary vertebrate cells such as leporine, bovine, porcine, simian and cervine cells [18-23]. More precisely, several studies demonstrated that hTERT can bind to heterologous vertebrate TRs, such as leporine TR and bovine TR, and those complexes respectively reconstitute telomerase activity in rabbit and bovine lens epithelial cells [22, 24]. Interestingly, hTERT expression in murine TERT^{-/-} embryonic stem cells in early passages is sufficient to prevent the signal-free ends and end-to-end fusions typically observed in mTERT^{-/-} ES cells in late passages [25]. Conversely,

mTERT cannot reconstitute or weakly reconstitutes telomerase activity in pig, chicken and human cells [25-27]. mTERT cannot promote the immortalization of primary human cells expressing the SV40 early region, nor can mTERT elongate the shortest telomeres in human cancer cells with alternative lengthening of telomeres (ALT) [25, 26].

In a previous study, we demonstrated that mTERT is functional with the only described viral telomerase RNA subunit, vTR [28]. vTR was identified in the genome of Marek's disease virus (MDV), a herpesvirus that induces T-lymphomas in its natural host, the chicken [28, 29]. The involvement of vTR in virally induced tumorigenesis has been clearly established [30, 31]. In another study, we showed that vTR is overexpressed during infection and associated with an increase in telomerase activity, especially at the time of lymphoma development [32]. Moreover, deletion of the two copies of the vTR gene induces a decrease of about 60% in tumor incidence in chickens [30]. *In vitro* studies established the function of vTR with its natural partner, chicken TERT (chTERT), and further demonstrated that vTR and chTERT reconstitute a more active complex than the native one (chTR/chTERT) [33]. Although the alignment of the primary sequences of vTR and hTR reveals only a weak sequence homology (30%; data not shown), the overall secondary structure predicted for vTR shows high similarity to the consensus functional structures described for hTR, including the conserved regions CR1 to CR8 [28, 34, 35].

Given the conserved structures between vTR and hTR, the compatibility of hTERT with heterologous TRs, and the high processivity of hTERT, the aim of this study was to determine if hTERT is able to form an efficient complex with vTR in a cell-free system and in human cells.

MATERIALS AND METHODS

Constructs

The plasmids phTR+1, pCR3.1-Flag-hTERT, pET-14b-chTERT and pCDNA3-vTR were previously described [33, 36, 37]. The vTR gene was cloned in the pUC-119 vector, similarly to the cloning of hTR [36]. A 480-bp fragment containing the T7 RNA polymerase promoter and the +1-442 of the gene encoding vTR was generated from pCDNA3-vTR by PCR, as previously described [28]. The vTR gene was amplified with the forward primer M277F (5'**CTGCAG**taatacgactcactatagggACACGTGGCGGG TGGAAGGCT-3') and the reverse primer M278R (5'*TCTAGA***TGCGCA**TGTGGGAGCGACGCCG TCC-3'). M277F contains the T7 promoter (shown in lower case), and both primers contain specific cloning sites at the 5'-end: a PstI site in M277F and an XbaI site in M278R (respectively shown in bold underlined and italic underlined characters). An FspI site was also engineered in the reverse M278R primer (shown in bold characters). The vTR-amplified fragment was then purified and inserted into the pGEMTeasy cloning vector (Promega). It was then subcloned into the pUC-119 vector, which had been previously digested with XbaI/PstI.

The chTR gene under the control of the T7 promoter was obtained from the XbaI/PstI digestion of pBS-T7chTR [33], and directly cloned in the pUC-119 vector. All intermediate and final constructs were sequenced with appropriate primers to check the integrity of the T7 promoter and genes of interest.

In vitro expression of the telomerase components

The human and chicken TERTs were *in vitro* translated using the TNT Rabbit Reticulocyte Lysate (RRL) system (Promega) in the presence of [^{35}S] methionine or unlabeled methionine according to the manufacturers' instructions, respectively from the pCR3.1-Flag-hTERT and the pET-14b-chTERT vectors. Radiolabeled hTERT and chTERT were analyzed by SDS-PAGE and the gel was exposed to Phosphorimager to assess the size and quality of the *in vitro* generated proteins.

The DNA templates used for *in vitro* T7 transcription of vTR, hTR and chTR consist of the pUC-119 constructs encompassing the genes of interest, linearized at the 3'-end of each gene either with an FspI digestion (vTR and hTR), or with an XbaI digestion (chTR). TRs were synthesized from 10 μg of template DNA, as previously described [33]. The resulting RNAs were treated with 10 units of RNase-free DNase I (Amersham Pharmacia Biotech) at 37ºC for 15 min. RNAs were then purified with the RNeasy Mini kit (QIAGEN). The quantity and purity of *in vitro* synthesized RNAs were estimated by spectrophotometry (Nanodrop Technologies) and the concentrations of each TR were adjusted to 1 μg/μl. The RNA quality was then confirmed by electrophoresis. Prior to electrophoresis, 1 μg of RNA samples diluted in 10 μl of formamide (Sigma Aldrich) was denatured at 95ºC for 2 min and then resolved on a 2% Tris-Acetate EDTA agarose gel and stained with ethidium bromide.

Telomerase reconstitution

Assemblies of hTERT or chTERT with the different TRs in a cell-free environment were performed as previously described [33]. Briefly, 0.75 μl of TERT proteins expressed in RRL was incubated with 1 μg of *in vitro* transcribed TR at 30ºC for 2 h in 1X buffer (50 mM NaCl, 20 mM HEPES (pH 8), 2 mM MgCl$_2$, 0.2 mM EGTA, 2 mM DTT and 10% glycerol).

To assess the reconstitution of telomerase activity in cells, vTR and hTR genes were transiently expressed in VA13-hTERT cells, a telomerase-negative cell line stably transfected with hTERT [38]. The phTR+1 and pCDNA3-vTR plasmids, with pCDNA3 as a control, were transfected into cells using the Lipofectin reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection for protein and RNA extractions [28]. Experiments were performed a minimum of three times.

TRAP assay

Telomerase activity was assessed using the semi-quantitative fluorescence-based telomere repeat amplification protocol (TRAP) assay, as previously described

[33]. Three microliters of a 1:10 dilution of the RNP assembly mix and 0.5 μg of protein extract were used for the assay.

Direct primer extension assay

This assay was performed as previously described [14]. The chicken and human TERTs were respectively synthesized in RRL from 300 ng of pET-14b-chTERT and pCR3.1-Flag-hTERT plasmids. The reactions were performed in the presence of 300 ng of *in vitro* transcribed vTR, hTR or chTR.

***In vitro* RNA binding assay**

Competitive RNA binding assays were performed according to previously described protocols [25]. The viral and human TRs were *in vitro* transcribed in the presence or absence of [^{32}P] UTP. Three concentrations (3, 30 and 300 ng) of unlabeled RNA and 3 pmol of freshly synthesized ^{32}P-labelled hTR or vTR were added to RRL mixes containing [^{35}S] methionine and 500 ng of pCR3.1-Flag-hTERT plasmid. Immunoprecipitations were performed with 8.82 μg of α-Flag M2 antibody (Sigma) and the immunoprecipitated complexes were then separated in SDS-Polyacrylamide gels.

RNA isolation and RT-PCR

Total RNA was extracted from 10^6 transiently transfected cells with RNAble solution, (Eurobio) according to the manufacturer's instructions. Reverse transcription of RNA was performed with Oligo(dT) (Promega) or with the specific primers DS4 (5'-AGCCCGCTGAAAGTCAGCGAGT-3'; specific for vTR and chTR) and M441 (5'-CGCCCGCTGAAAGTCAGCGAGA-3'; specific for hTR). Expression of vTR and chTR was then detected by PCR with the forward DS1 primer (5'-CGTGGCGGGTGGAAGGCTCCG-3') and the reverse primer DS4, and expression of hTR was checked using the primer pair M440 (5'-CTGGGAGGGGTGGTGGCCATT-3') and M441. As an internal control, the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was detected by PCR with the primer pair GAPDH-F (5'-TGATGACATCAAGAGGGTGGTG AAG-3') and GAPDH-R (5'-TCCTTGGAGGCCATGTGGAGCCAT-3').

Immunoprecipitation of hTR and vTR

Immunoprecipitation of hTR/hTERT and vTR/hTERT complexes from VA13 cells was performed with an affinity-purified monoclonal hTERT antibody 2D8 (Abcam) or a rabbit polyclonal Dyskerin antibody (H-300, Santa Cruz, USA). RNA (hTR and vTR) bound to the immunoprecipitated hTERT was eluted by adding 100 μl of elution buffer (50 mM Tris-HCl (pH 8), 100 mM NaCl, 10 mM EDTA, 1% SDS) to IP beads (with 400 μg of whole cell extract) and incubated at 60ºC for 10 min. RNA was extracted by phenol/chloroform, followed by ethanol precipitation and resuspension in RNase-free water. Gene-specific RT for immunoprecipitated hTR and vTR was performed, respectively using the hTR-R3B primers (5'-TCTAACCCTAACTGAGAAGGGCGTAG-3') and vTR-R2 primers (5'- GACGCCGTCCGCGCGCCCCAC-3'). RT for non-immuno-precipitated hTR and vTR was performed using random primers (pd(N$_6$)). The

hTR was amplified by PCR using the primers hTR-F3B (5'-TCTAAC CCTAACTGAGAAGGGCGTAG-3') and hTR-R3C (5'-GTTTGCTCTAGAAT GAACGGTGGAAG-3'), as previously described [39] and vTR was amplified by the primers vTR-F2 (5'-CGTGGCGGGTGGAAGGCTCCG -3') and vTR-R2. Products were resolved on a 10% non-denaturing PAGE.

RESULTS AND DISCUSSION

hTERT and vTR reconstitute telomerase activity in a cell-free environment

In order to determine if hTERT could function with vTR, we first assembled the two subunits *in vitro* and determined the activity of the heterocomplex using a direct primer extension assay, which allows the detection of DNA synthesis and repeat addition processivity of telomerase enzymes. It was performed on complexes reconstituted with chTERT (as a control) or hTERT. The TERT subunits were expressed using RRL, in the presence of *in vitro* transcribed vTR, chTR or hTR. hTERT and hTR reconstituted complexes synthesized long products, as expected (Fig. 1A, lane 3). However, few repeats were generated by the other reconstituted complexes including vTR/hTERT and chTR/hTERT (Fig. 1A, lanes 1 and 2).

Low levels of DNA synthesis that can be difficult to detect using the direct primer extension assay can sometimes be more readily detected using the sensitive PCR-based telomerase assay (TRAP) [17, 40]. For instance, though we failed to detect substantial elongation by the chTR/chTERT (Fig. 1A, lane 5) and vTR/chTERT (Fig. 1A, lane 4) complexes, we previously demonstrated via TRAP assay that these complexes are able to reconstitute telomerase activity *in vitro* [33]. Therefore, we also tested the ability of hTERT and vTR to reconstitute telomerase activity in a cell-free system using the TRAP assay.

The human and chicken TERT proteins were expressed in RRL and then incubated with *in vitro* transcribed vTR or hTR (Fig. 1C and D). The catalytic activities of these telomerase complexes were tested by TRAP assay and analyzed by capillary electrophoresis (Fig. 1B). Telomerase activity was found to be mediated by the hTERT- and vTR-reconstituted complex since products equivalent in length to 16 telomeric hexamers were synthesized, indicating that in a cell-free environment, human TERT is able to form a functional complex with vTR. It is noteworthy that similar activities were obtained with the vTR/chTERT and hTR/chTERT complexes. The reconstituted hTR/hTERT enzyme is able to synthesize products of 30 hexamers in length. The quantification of relative levels of hTERT-reconstituted telomerase activity demonstrated that vTR is less efficient than hTR, since vTR reconstituted only 5% of the telomerase activity reconstituted by hTR (Fig. 1B). However, in the context of chTERT, vTR and hTR are equally efficient in the reconstitution of telomerase activity. Moreover, the weakest activities observed in these assays were mediated by reconstitutions with chTERT, suggesting that chTERT may be less active than hTERT, regardless of the associated TR (Fig. 1B).

We then checked whether the reduced level of *in vitro* telomerase activity of the reconstituted vTR/hTERT compared to the hTR/hTERT complexes could be
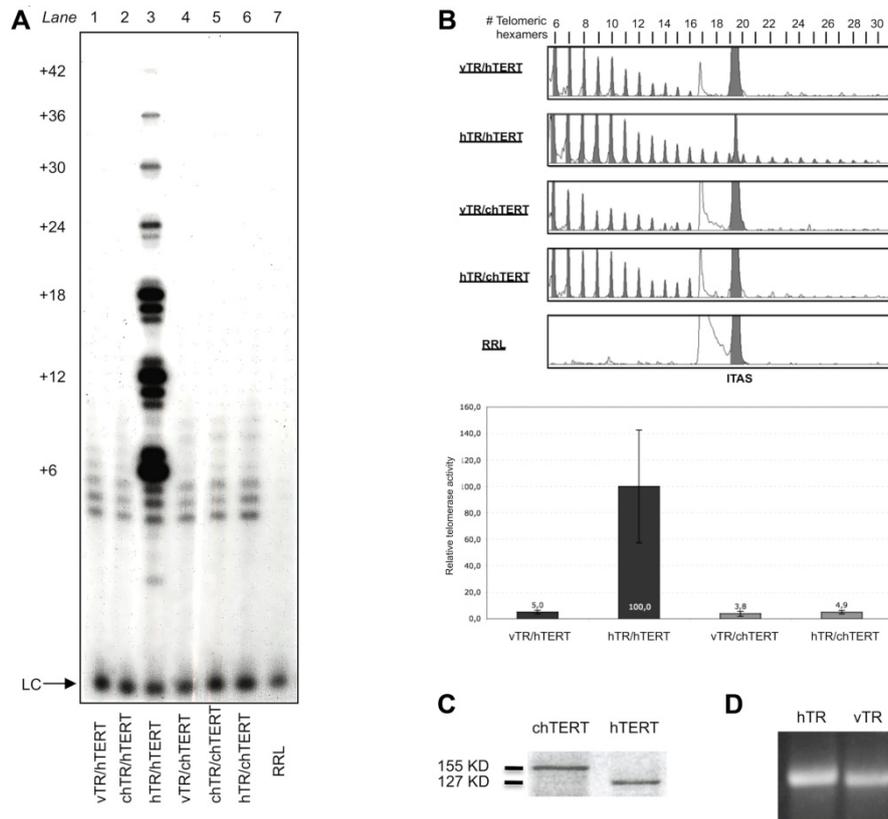

Fig. 1. Function of hTERT- and vTR-reconstituted telomerase complex *in vitro*. A – vTR/hTERT reconstitutes low levels of telomerase activity. RRL-reconstituted telomerase complexes composed of hTERT or chTERT assembled *in vitro* with vTR, chTR or hTR were tested using a $(T_2AG_3)_3$ 3' end-labeled biotinylated primer in the direct primer extension telomerase assay. The number of nucleotides added to the primer by telomerase is indicated on the left. LC: Loading control. B – hTERT and vTR reconstitute a functional telomerase activity *in vitro*. The hTERT and chTERT were synthesized in RRL and then assembled with *in vitro* transcribed vTR or hTR. Each assembly was performed three times and the resulting telomerase activity was detected by a modified TRAP assay, analyzed by capillary electrophoresis, and quantified, as previously described [28]. Upper panel: Electropherogram profiles illustrate the 6-bp TRAP products as filled-in areas. The number of telomeric repeats synthesized by the telomerase complexes is indicated above the electropherograms. ITAS represents the internal control used in quantification. Lower panel: Quantification of the observed telomerase activity. C – Expression profiles of hTERT and chTERT synthesized in RRL. Expression of both proteins was verified by SDS-PAGE from 1 μl of total translation RRL mixes containing [35S]-labeled hTERT or chTERT and the gel was exposed to Phosphorimager. The molecular weight of each protein is indicated. D – Expression profiles of *in vitro*

transcribed hTR and vTR. One microgram of hTR and vTR transcribed with T7-RNA polymerase was resolved on a 2% TAE agarose gel and stained with ethidium bromide.
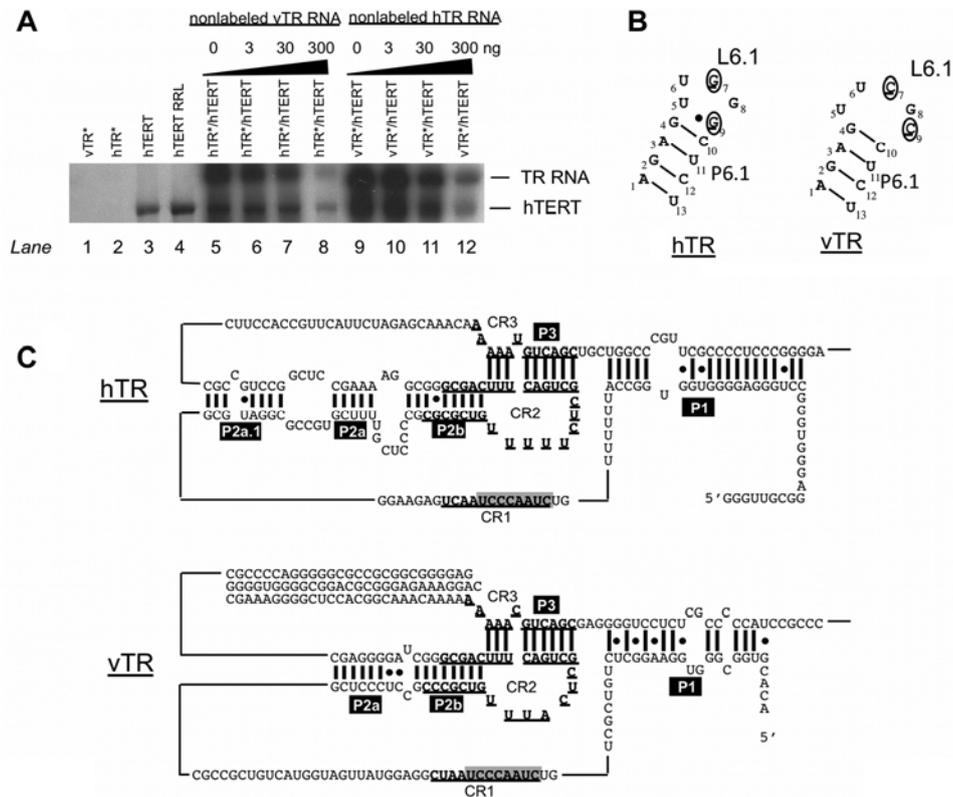

Fig. 2. vTR binds hTERT *in vitro*. A – ^{35}S-labeled Flag-hTERT was synthesized in RRL in the presence of equal amounts of ^{32}P-labeled hTR (annotated hTR*, lanes 5 to 8) or vTR (indicated as vTR*, lanes 9 to 12) and increasing amounts of unlabeled competitor RNA, vTR (lanes 5 to 8) or hTR (lanes 9 to 12). The amount of competitor RNA was respectively 3, 30 or 300 ng. Immunoprecipitation of the complexes was performed with the α-Flag M2 antibody and visualized by SDS-PAGE. Controls consisted of vTR* and hTR* incubated without hTERT (lane 1 and 2); hTERT synthesized in the absence of TR* and immunoprecipitated (lane 3); and the input hTERT synthesized in RRL without TR* (lane 4). The autoradiograph shown is representative of three experiments showing similar results. B and C – Schematic representation of the two regions of hTR required for hTERT binding: the P6.1 helix of the CR4-CR5 domain (B), and the pseudoknot/template domain and the equivalent vTR regions (C). The nucleotide divergences present in the L6.1 loop (B) are circled and the position of each nucleotide composing the structure is indicated.
In the pseudoknot/template domain (C), paired regions are numbered from 5' to 3' as P1 to P3 and indicated in black boxes. The template region is shaded in gray and the conserved regions CR1 to CR3 are identified in bold characters. Universal base pairing (according to

Watson-Crick) is represented by dashes, whereas G/U pairs and non-canonical A/C pairs are shown with dots.

attributed to a weaker interaction of vTR and hTERT (Fig. 2). The association of *in vitro* transcribed [^{32}P]-labeled hTR or vTR with RRL-synthesized Flag-tagged hTERT was examined following immunoprecipitation of hTR/hTERT or vTR/hTERT complexes using an anti-Flag antibody (Fig. 2A).We also used increasing concentrations of non-radiolabeled competitor RNA (Fig. 2A; lanes 10-12 show hTR and lanes 6-8 show vTR) to assess the affinity of vTR for hTERT. Increasing concentrations of non-radiolabeled vTR or hTR respectively efficiently inhibited the interaction of [^{32}P]-labeled hTR or vTR with hTERT, suggesting that in a cell-free system, vTR and hTR bind hTERT with similar affinities. The CR4-CR5 and pseudoknot/template domains are the two hTR regions required for hTERT binding [41, 42]. The sequence and structure of the P6.1 helix identified in the CR4-CR5 domain [34] consists of the high-affinity TR/TERT binding site [14, 41-43], and it is conserved between vTR and hTR. Also conserved are the uracil and guanine nucleotides (positions 6 and 8, respectively) of the L6.1 loop, described as essential for telomerase activity *in vitro* [34]. However, two point C→G transversions (positions 7 and 9) are present in the vTR L6.1 loop compared to hTR (Fig. 2B). These sequence divergences do not abolish the binding of vTR to hTERT *in vitro*, indicating that the wobble U.G base pair recently described for hTR would not be essential for TR/TERT binding [44]. However, despite the sequence variability at position 7, this nucleotide was demonstrated to be involved in a long-range interaction between the CR4/CR5 domain and the template-pseudoknot domain [45]. The function of such RNA-RNA interaction on telomerase activity or TR/TERT binding was not clearly established, but our results indicate that this long-range association, which would be compromised in vTR, is not essential for the binding of vTR and hTERT *in vitro*. Notably, the proposed secondary structure of the low-affinity TR/TERT binding site, the pseudoknot/template domain of vTR, differs significantly from that of hTR (Fig. 2C) [33]. Nevertheless, the nucleotide and structural divergences do not seem to prevent the interaction of vTR and hTERT *in vitro*.

In human cells, vTR is not able to reconstitute a functional human telomerase holoenzyme

The high tolerance of hTERT towards TR of different species was previously demonstrated using *in vitro* assays as well as in cellular systems [22, 24, 25, 27]. We further investigated if vTR would be able to reconstitute telomerase activity in a cellular context (Fig. 3). We transfected plasmids encoding vTR and chTR, as well as hTR as a control, into human VA13-hTERT cells. These hTR-negative, hTERT-negative ALT cells are stably transfected with hTERT and have been used for functional TR studies [25, 38, 40, 46-50]. The reconstitution

of telomerase activity in cells was analyzed by TRAP assay and the expression of TRs in the cells was verified by RT-PCR.

As reported previously, hTR reconstitutes telomerase activity in VA13-hTERT cells, with elongation products corresponding to 23 telomeric hexamers in length; no activity was detected in cells transfected with the empty pCDNA vector (Fig. 3A). However, neither vTR nor chTR was able to reconstitute telomerase activity with hTERT in a cellular context. It is noteworthy that the lack of telomerase activity in vTR- and chTR-reconstituted VA13-hTERT cells is not due to a lack of expression or accumulation of those TRs in cells (Fig. 3B).
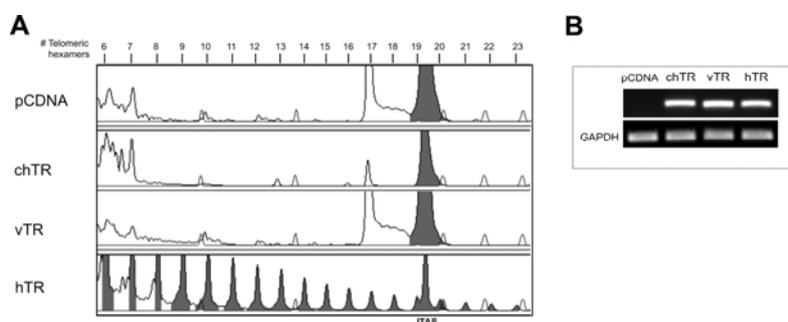

Fig. 3. vTR does not reconstitute telomerase activity in VA13 human cells expressing hTERT. The vTR, chTR and hTR genes as well as the empty vector pcDNA were transiently transfected into VA13-hTERT cells. A – 48 h post-transfection, protein extracts were prepared and 0.5 µg was assayed for telomerase activity using the TRAP assay and analyzed by capillary electrophoresis. B – hTR, vTR and chTR expression in VA13-hTERT cells was detected by RT-PCR with specific primers, as well as the internal control, GAPDH. As a negative control, RT-PCR was also performed on RNA extracted from cells transfected with the empty vector pcDNA with the specific primers used for vTR or chTR amplification.

The observation that hTERT is not able to function with the chicken telomerase RNA subunit was previously reported: the ectopic expression of hTERT was unable to stabilize avian telomere length and to reconstitute telomerase activity in chicken primary embryonic fibroblasts [27]. The 12% sequence divergence between vTR and chTR may account for the ability of vTR to confer a higher activity than chTR in telomerase complexes reconstituted with chTERT or mTERT. However, the sequence divergence does not seem to increase the function of vTR compared to chTR in the reconstitution of an active hTERT complex, both *in vitro* and in cells [28, 33]. To determine if the inefficiency of vTR and hTERT to reconstitute telomerase activity in the VA13 cells could be the consequence of a defective binding of those two subunits, we verified for the presence of vTR and hTR in the telomerase complex by hTERT immunoprecipitation-linked RT-PCR. At 48 h post-transfection, hTERT complexes were immunoprecipitated from cellular extracts of VA13-hTERT cells transiently expressing vTR, hTR or an empty vector, and RNAs in the

immunoprecipitated complexes were extracted. RT-PCR specific for vTR or hTR was then performed.

As expected, the expression of hTR in the cells leads to its detection 48 h post-transfection in the hTERT immunoprecipitated complex (Fig. 4, lane 2). Although vTR is expressed in the VA13-hTERT cells, no vTR was detectable in the hTERT immunoprecipitated complex (Fig. 4, lane 8), explaining the lack of activity of vTR/hTERT from human cells. Thus, while nucleotide and structural divergences between hTR and vTR do not seem to prevent the interaction of vTR and hTERT in a cell-free environment, such differences may contribute to the inability of vTR and hTERT to interact in a cellular setting.
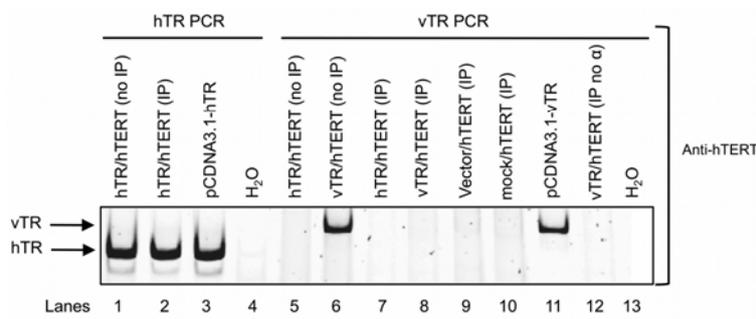

Fig. 4. vTR is not detected in hTERT-immunoprecipitated complexes from VA13-hTERT cells. Plasmids encoding vTR or hTR, or an empty vector were transiently transfected in VA13-hTERT cells. H_2O: cells were not transfected. At 48 h post-transfection, hTERT complexes were either subjected to immunoprecipitation with an anti-hTERT antibody (IP) or not (no IP). The RNAs bound to the immunocomplexes were extracted and subjected to RT-PCR using hTR-specific primers (lanes 1-4) or vTR-specific primers (lanes 5-13). Products were separated on a 10% non-denaturing PAGE. pCDNA3.1-hTR and pCDNA3-vTR are plasmids respectively used as size controls for the hTR-specific and vTR-specific PCR products.

Additionally, although the expression of TERT and TR is sufficient to reconstitute telomerase activity *in vitro*, in cells a large number of telomerase-associated proteins are required for a functional complex at the telomere [reviewed in 6, 51, 52]. Since vTR and hTERT are able to interact in a cell-free system but not in cells, one hypothesis would be that vTR does not allow the assembly of an active holoenzyme in human cells due to a defect in the interaction with proteins that bind the H/ACA domain and are involved in the maturation, stability and localization of hTR in cells (e.g. hGAR1, dyskerin or hNHP2) or proteins directly involved in assembly of the telomerase complex such as the 14-3-3 proteins, as well as in recruitment to telomeres [6, 53-57]. The sequence comparison of vTR and hTR shows only minor divergences in the H/ACA domains (Fig. 5A). Although the functional consensus sequence of the H box (AnAnnA) is conserved between vTR and hTR, a one-nucleotide difference occurs within the H box and several nucleotide differences are present

within the flanking regions of the CR6 domain. The ACA box is conserved for both TRs, but one adjacent base differs within the CR8 domain. In this context, it was of interest to test if vTR is able to interact with dyskerin, which binds the hTR H/ACA domain and which is also a component of the catalytically active telomerase complex [58]. Dyskerin is not required for an active assembly of the telomerase complex in a cell-free setting, but it is essential for the maturation and stability of TRs *in vivo* and thus for their ability to assemble into an active telomerase holoenzyme [8, 37, 56].

After overexpression of vTR in VA13-hTERT cells, we determined if vTR is part of the human holoenzyme complex by performing a dyskerin-linked RT-PCR (Fig. 5B). At 48 h post-transfection, hTR but not vTR could be detected by RT-PCR from the immunocomplexes (Fig. 5B, lane 2 and 8). This indicates that vTR is most likely not part of the immunoprecipitated complexes, and unlike hTR, vTR is unable to interact with the dyskerin protein.
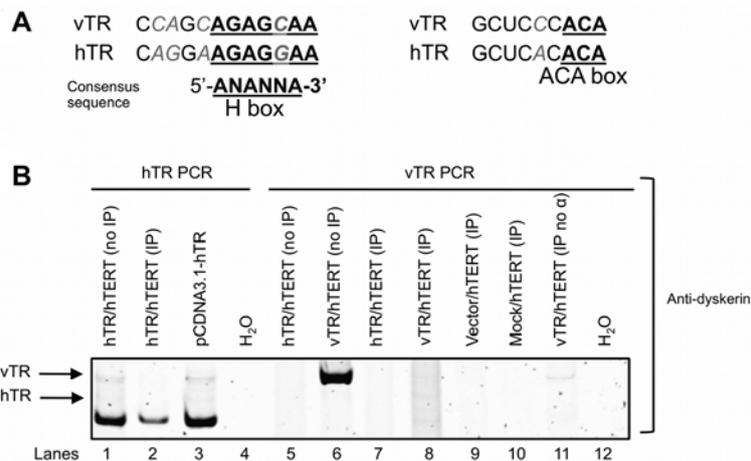

Fig. 5. vTR does not interact with dyskerin in VA13-hTERT cells. A – Sequence comparison of the H/ACA domains of vTR and hTR. The H and ACA domains are shown in bold underlined characters within the CR6 (left panel) and CR8 (right panel) regions of the telomerase RNA. The nucleotide sequence divergences between vTR and hTR are indicated in grey italic letters. B – vTR is not detected in dyskerin-immunoprecipitated complexes from VA13-hTERT cells. The plasmids encoding for vTR, hTR or an empty vector were transiently transfected in VA13-hTERT cells. H_2O: cells were not transfected. At 48 h post-transfection, an immunoprecipitation with an anti-dyskerin antibody was performed (IP) or not (no IP). The RNA bound to the immunocomplexes was extracted and subjected to RT-PCR using hTR-specific (lanes 1-4) or vTR-specific primers (lanes 5-12). Products were separated on a 10% non-denaturing PAGE. pCDNA3.1-hTR is a plasmid used as a size control for the hTR-specific PCR product.

Most studies on the dyskerin protein were performed in the context of the dyskeratosis congenita (DC) disorder, an inherited multi-system disease characterized by a variety of phenotypes [59]. Two main causes of this disease

are due to mutations in the dyskerin protein and/or in hTERT and hTR (especially in the H/ACA domain), leading to lower telomerase RNA accumulation, reduced telomerase activity and subsequent telomere attrition. Several reports have demonstrated the essential role of dyskerin in the maturation, stability and nucleolar localization of hTR, which are pre-requisites for a proper assembly of the telomerase complex in cells [7, 8, 60, 61]. Interestingly, vTR seems to be expressed at a similar level to hTR in VA13-hTERT cells, as shown by RT-PCR (Fig. 3B), indicating that the lack of binding with dyskerin may not affect vTR accumulation. Moreover, mutation of non-conserved H box consensus residues with the hTR H box (5'-AGAGGA-3' to 5-ATATTA-3') does not affect hTR accumulation [60], suggesting that the nucleotide difference in a non-conserved H box consensus residue in vTR compared to hTR (C instead of G) may also not affect vTR accumulation. The inability of vTR to form an active telomerase complex with hTERT in cells may therefore be due to a defect in maturation and/or a cellular mislocalization of vTR, likely caused by its lack of interaction with dyskerin.

In conclusion, complementary to our previous studies demonstrating the function of the homologous chicken TERT and the heterologous murine TERT with vTR, the current study demonstrates that hTERT can reconstitute a functional telomerase complex with vTR only in a cell-free environment. However, this heterologous complex is less efficient than the human complex. The lack of vTR/hTERT-mediated telomerase activity from cells is likely due to improper assembly of the telomerase complex. The inability of vTR to interact either with hTERT or with dyskerin in a cellular context suggests that the maturation of vTR in human cells does not occur in such a way to ensure the formation of a catalytically active telomerase holoenzyme. Consequently, our results suggest a species incompatibility between telomerase subunits that could result from a specific co-evolution of the viral telomerase subunit within the holoenzyme complex of its natural host species, the chicken. Although the distinct features acquired by vTR compared to chTR confer to vTR a better ability to interact functionally with hTERT, they are not sufficient to abolish the species incompatibility previously observed in an avian context between chTR and hTERT [62].

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