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Experimental Cell Research 306 (2005) 64–74

Experimental Cell Research

www.elsevier.com/locate/yexcr

Characterization of the telomere complex, *TERF1* and *TERF2* genes in muntjac species with fusion karyotypes

Nils Hartmann[a], Harry Scherthan[a,b,*]

[a]*Max Planck Institute for Molecular Genetics, Ihnestr. 73, D-14195 Berlin, Germany*
[b]*Institute for Radiation Biology Bundeswehr, Neuherbergstr. 11, D-80937 Munich, Germany*

Received 3 December 2004, revised version received 28 January 2005
Available online 13 March 2005

Abstract

The telomere binding proteins TRF1 and TRF2 maintain and protect chromosome ends and confer karyotypic stability. Chromosome evolution in the genus *Muntiacus* is characterized by numerous tandem (end-to-end) fusions. To study TRF1 and TRF2 telomere binding proteins in *Muntiacus* species, we isolated and characterized the *TERF1* and *-2* genes from Indian muntjac (*Muntiacus muntjak vaginalis*; 2n = 6 female) and from Chinese muntjac (*Muntiacus reveesi*; 2n = 46). Expression analysis revealed that both genes are ubiquitously expressed and sequence analysis identified several transcript variants of both *TERF* genes. Control experiments disclosed a novel testis-specific splice variant of *TERF1* in human testes. Amino acid sequence comparisons demonstrate that *Muntiacus* TRF1 and in particular TRF2 are highly conserved between muntjac and human. In vivo TRF2-GFP and immuno-staining studies in muntjac cell lines revealed telomeric TRF2 localization, while deletion of the DNA binding domain abrogated this localization, suggesting muntjac TRF2 represents a functional telomere protein. Finally, expression analysis of a set of telomere-related genes revealed their presence in muntjac fibroblasts and testis tissue, which suggests the presence of a conserved telomere complex in muntjacs. However, a deviation from the common theme was noted for the *TERT* gene, encoding the catalytic subunit of telomerase; *TERT* expression could not be detected in Indian or Chinese muntjac cDNA or genomic DNA using a series of conserved primers, while TRAP assay revealed functional telomerase in Chinese muntjac testis tissues. This suggests muntjacs may harbor a diverged telomerase sequence.

Keywords: *Muntiacus muntjak vaginalis*; *M. reveesi*; Telomerase; Telomere repeat binding factors; TRF1; TRF2

Introduction

Telomeres are specialized nucleoprotein structures that maintain and protect the ends of eukaryotic chromosomes [1]. In mammals, they consist of double-stranded, repetitive TTAGGG DNA-repeats complexed with associated proteins [2,3]. It has been found that invasion of the single-strand 3′-overhang of the telemere into the more internal telomeric duplex repeats leads to the formation of a so-called t-loop structure that stabilizes the chromosome ends [4]. Telomere repeat tracks are maintained by the enzyme telomerase which contains a template RNA (TR or TERC) and a catalytic subunit (TERT) [5,6]. Telomerase is capable of adding T_2AG_3 repeats to the 3′-overhang of telomeric DNA, which can compensate the loss of telomere sequences that occurs during DNA replication [7,8]. Furthermore, a growing number of proteins has been identified that associate with the chromosome ends. These involve components of the DNA damage response and repair pathways and are thought to protect the chromosome ends from degradation and fusions [9,10].

In mammals, the telomeric repeat binding factors TRF1 and TRF2 directly bind to double-stranded telomeric DNA. The two proteins are ubiquitously expressed, similar in size, and form homodimers [11–13]. TRF1 and TRF2 contain a myb-like domain at their carboxy terminus, which

* Corresponding author. Institute for Radiation Biology Bundeswehr, Neuherbergstr. 11, D-80937 Munich, Germany. Fax: +49 89 3168 2635.
E-mail address: scherth@web.de (H. Scherthan).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.02.001

is made up of a helix–turn–helix motif that is responsible for DNA binding [14]. TRF1 and -2 furthermore contain a central TRF-specific homology (TRFH) domain that mediates dimerization and interaction with other proteins. Despite the similarity of these domains, no dimerization was found between TRF1 and TRF2, which differ in their amino terminus that is acidic in the former and basic in the latter [13].

Over-expression of wild-type TRF1 results in reduction of telomere length, whereas inhibition of TRF1 increases telomere length. These data have implicated TRF1 in telomere length regulation, acting as a negative regulator of telomere length [15–17]. Over-expression of wild-type TRF2 also reduces telomere length; however, inhibition of TRF2 causes a variety of phenotypes [18]. In many human cell types, including primary lymphocytes, inhibition of TRF2 leads to immediate induction of ATM/p53-dependent apoptosis [19], while in other cell types, such as primary fibroblasts, loss of TRF2 results in p53/p21- and/or p16/Rb-mediated senescence [20,21]. Other consequences of TRF2 inhibition are the loss of the telomeric single-stranded 3′-overhang, the resolution of the t-loop, and an increase of chromosomal end-to-end fusions via the non-homologous end joining machinery [22]. In summary, loss of TRF2 leads to unprotected, fusigenic chromosome ends that are recognized as double-strand breaks [10].

Other proteins known to be associated with telomeres are recruited through their interaction with either TRF1 or TRF2. TRF1 has been found in association with tankyrase 1 and 2 [23], TIN2 [24], PINX1 [25], POT1 [26], and PTOP/PIP1 [27,28]. The tankyrase 1 and 2 enzymes can ADP-ribosylate TRF1 and thereby inhibit TRF1 binding to telomeric DNA [23,29,30]. TIN2 forms a ternary complex with TRF1 and tankyrase that may stabilize the interaction between both proteins. Recent findings suggest that TIN2 can bind TRF1 and TRF2 simultaneously, forming a link between these telomeric proteins [31]. POT1 plays an important role in telomere length control due to its ability to bind single-stranded telomeric DNA. It is recruited to the TRF1 complex through the recently identified protein PTOP/PIP [27,28].

TRF2 binds RAP1, a negative regulator of telomere length [16] and also recruits several recombination and repair proteins to the telomere, as is the case for the Mre11/RAD50/NBS1 complex [32], the WRN/BLM RECQ helicases [33], and the endonuclease ERCC1/XPF [34]. Furthermore, a heterotrimeric complex consisting of Ku70, Ku80, and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [35] is associated with both the TRF1 and TRF2 complex and contributes to the protection of telomeres from end-to-end fusions [36].

Chromosome fusions are the predominant rearrangement in the karyotype evolution of the Indian and Chinese muntjac deer [37], and have led to a dramatic reduction in chromosome number during a relatively short evolutionary time [38,39]. The present-day Indian muntjac karyotype (*Muntiacus muntjak vaginalis*, *Mmv*) comprises six chromosomes in the female and seven in the male [40], which is the lowest known chromosome number among mammals [41]. Numerous chromosome fusions, such as tandem and a few centric fusions, are thought to have shaped the exceptional muntjac fusion karyotype [38,42–45]. The closely related Chinese muntjac (*Muntiacus reveesi*, *Mre*) exhibits an intermediate chromosome number of 2n = 46 [46], whereas an ancestral chromosome number of 2n = 70 is considered for the cervid ancestor to the muntjacs and other Cervidae [47]. Molecular analysis revealed that repetitive telomeric and centromeric (satellite) DNA sequences were presumably involved in the tandem fusion process, but the underlying mechanisms remain ambiguous [48–50].

Due to the importance of TRF1 and in particular TRF2 for protection of telomeres [51], we were interested to learn about their structure as well as their expression in both muntjac species. Since many other telomere-associated proteins are also involved in karyotypic stability, we also analyzed the expression of a number of other genes involved in telomere metabolism in muntjac cells.

Materials and methods

DNA, chromosome, and RNA sources

DNA and metaphase chromosomes were prepared from Indian muntjac and Chinese muntjac fibroblast cell lines [52]. RNA was also isolated from muntjac fibroblasts as well as from testis, liver, and muscle of a 2-year old male Chinese muntjac, kindly provided by Dr. Ochs, Berlin Zoo, using the RNeasy Midi kit (Qiagen).

PCR amplification and cloning

The one-step RT-PCR kit (Qiagen) and gene-specific primers were used to amplify conserved regions of several genes from *M. muntjak vaginalis* (*Mmv*) and *M. reveesi* (*Mre*) (see supplementary Table 1 in Appendix A). After sequencing, further muntjac-specific *TERF* primers were designed to amplify the less conserved 5′- and 3′-cDNA ends of *TERF1* and *-2* using the Smart RACE cDNA amplification kit (Clontech), while the 5′-end of *TERF2* was amplified using an inverse PCR strategy. To this end, genomic muntjac DNA (5 μg) was digested by different restriction enzymes that cut on average every 1–4 kb in mammalian genomes according to the instructions of the supplier (NEB). For circularization, 500 ng of restricted DNA was ligated with the T4 DNA ligase (Promega) in a 100-μl volume overnight at 12°C. DNA was precipitated with ethanol and resuspended in H_2O. 100 ng of ligated DNA was used in an Expand Long Template PCR (Roche) using inverse primers that were designed to amplify the unknown 5′-region of *TERF2*. All PCR-products of interest were excised from a 1% agarose gel and cloned into the T/A overhang vector pGEM-T easy (Promega). Inserts were sequenced from both