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Human Chromosome 1 in Mouse Immortal Cell Background*

Maja Matulić,[a] Irena Jevtov,[a] Marina Ferenac,[a] Nikolina Škrobot,[a] Kaoru Tominaga,[b] and Ivica Rubelj[a,]**

[a]*Department of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia*

[b]*Department of Cellular and Structural Biology, Sam and Ann Barshop Center for Longevity and Aging Studies, STCBM Building, 15355 Lambda Drive, San Antonio, TX 78245-3207, USA*

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Telomeres are specialized structures at the ends of linear chromosomes and are essential for normal cellular function. Telomeres prevent degradation and aberrant recombination of chromosome termini and facilitate appropriate replication of chromosome ends. In this work, the telomere dynamics was followed in the immortal mouse cell strain A9 in comparison with A9+1. The latter is derived from A9 cells by introduction of human chromosome 1. In spite of the telomerase presence, a great decrease in telomere lengths was noticed in A9+1 compared to A9 cells. Behavior of individual human and mouse telomeres was also followed under the conditions of the observed gross telomere shortening. Human chromosome 1 followed the overall telomere length in hybrid cells. It is suggested that telomere lengths are primarily determined by the cell protein background.

Keywords
human chromosome 1
A9
A9+1
telomere
senescence
PNA-FISH

INTRODUCTION

Telomeres are specialized structures at the ends of linear chromosomes, composed of short repeat sequences, essential for normal cellular function. They prevent degradation and aberrant recombination of chromosome termini and facilitate proper replication of chromosome ends. In order to stabilize their conformation, tandem repeats of short G-rich sequences $(TTAGGG)_n$ form stable telomere loop (t-loop) structures in complex with telomere proteins.[1,2]

Telomeres shorten in each cell division as a consequence of the property of conventional DNA polymerase that cannot replicate the very ends of linear DNA.[3] Cells unable to maintain constant telomere length stop dividing when at least one of their telomeres shortens to a critical length. This irreversible growth arrest state, called replicative senescence,[4] is believed to be the mechanism that prevents cell immortalization.[5] Cells undergoing permanent divisions, such as tumor and stem cells, circumvent replicative senescence and maintain constant telomere length by constitutive telomerase expression.[6,7,8] In general, significant telomerase activity is absent in most somatic cells,[9] although recent data indicate that its presence in low amounts could have a role in maintaining the stable telomere structure of some normal human cells.[10]

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** Author to whom correspondence should be addressed. (E-mail: rubelj@rudjer.irb.hr)

In order to identify factors involved in mechanisms of cell senescence and immortalization, normal diploid fibroblasts were fused with various immortal human cells. Surprisingly, all obtained hybrids had a limited life span, clearly indicating dominance of cellular senescence.[11,12] Similar experiments also enabled identification of individual chromosomes that carried genes involved in induction of senescence, one of which is human chromosome 1. Using microcell-mediated chromosome transfer (MMCT) of human chromosome 1 leads to induction of senescence in various human immortal cell lines assigned to complementation group C[13], as well as in various rodent cell lines.[14,15,16] Although the mechanisms are still mostly unknown, it has been found that, unlike chromosomes 3, 4, 7 and 10, senescence induced by chromosome 1 is telomerase independent.[16,17,18,19,20] In the present work, changes were followed in the gross telomere length as well as telomeres of individual mouse chromosomes and human chromosome 1 upon its introduction into the immortal mouse cell line A9. Although introduction of this chromosome induces senescence in some other mouse cells, such as melanoma B16-F10,[21] A9+1 cells maintained the immortal phenotype. Telomere repeats of immortal mouse A9 cells in the culture are around 9 kb long, but hybrid cells with introduced human chromosome 1 have significantly shorter telomeres, around 4 kb. This could be explained by the influence of certain human proteins expressed in these cells that influence telomere conformation and stability.

EXPERIMENTAL

Cell Culture

A9, A9+1 and HCA2 cell lines were a gift from O. M. Pereira-Smith, Department of Cellular and Structural Biology, Sam and Ann Barshop Center for Longevity and Aging Studies, San Antonio, USA. A9+1 was created by microcell fusion of a single copy of a human chromosome 1 tagged with the selectable marker Neo and A9 mouse fibroblasts.[13,22] A9, A9+1 and young human diploid HCA2 fibroblasts were grown at 37 °C in DMEM (Dulbecco's modified Eagle's medium, Sigma), supplemented with 10 % fetal bovine serum (FBS) in the presence of 5 % CO_2.

Metaphase Preparation

Cells were treated with colcemid (0.1 μg/ml) for 4 h and harvested with trypsin. Cells were incubated in hypotonic KCl buffer prior to fixation with methanol-acetic acid (3:1). Slides were prepared as previously reported.[23]

Fluorescence in situ Hybridization

Metaphases were prepared after colcemid treatment (0.1 μg/ml), fixed in methanol-acetic acid (3:1) and air-dried slides hybridized with telomeric PNA probe labeled with rhodamine. After hybridization, slides were stained with DAPI and analyzed under a UV light microscope. Telomere length was analyzed by fluorescence *in situ* hybridization (FISH) according to the manufacturer's instructions (Applied Biosystems Framingham, MA, USA). Typically, slides were treated with pepsin (0.1g / 100 ml 0.01 M HCl), dehydrated in ethanol and, after denaturation at 70 °C for 6–8 min, hybridized using rhodamine-conjugated $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe in hybridization solution (10 mmol dm^{-3} sodium phosphate buffer pH = 7.4, 10 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris pH = 7.5, 70 % formamide, 1x Denhart solution, 0.1 μg/ml tRNA) for 2 h at room temperature. Slides were rinsed in PBS + 0.1 % Tween 20 at 57 °C for 20 min and counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma).

Slides were analyzed under an Olympus fluorescence microscope BX51, 1000x magnification. Relative signal intensities were analyzed by densitometry on Image Master VSD Software (Pharmacia). Mean metaphase intensities were obtained by subtracting the mean pixel value of the background from the mean pixel value for all telomeres in the metaphase. Relative intensities of individual telomeres were obtained by dividing the mean pixel value associated with that telomere by the mean pixel value of all telomeres in the metaphase.[24]

DNA Preparation and Southern Blot Analysis

High molecular weight genomic DNA was prepared using the Qiagen DNeasy Tissue Kit according to the manufacturer's instructions. DNA was digested with restriction enzymes Rsa I and Hinf I (Roche). Telomere probe was digoxigenin labeled by PCR. Primers specific to telomere sequence (F: $(CCCTAA)_4$, R: $(TTAGGG)_4$) were amplified by non-template PCR (94 °C / 1.5 min, (94 °C / 45 s, 52 °C / 30 s, 72 °C / 1 min, 72 °C / 10 min; 30 cycles)). Southern blot was performed by alkaline transfer[25] and the membrane (Hybond N+, Amersham) was hybridized with digoxigenin labeled telomeric probe. Signal detection was performed according to manufacturers' instructions (Dig DNA labeling and detection kit, Roche). Mean molecular weight of DNA fragments was estimated by densitometric analysis using the Aida 2.0 software.

Telomerase Activity Assay

Telomerase repeat amplification protocol (TRAP) assay was performed using a TRAPEZE kit (Chemicon) according to the manufacturer's protocol with a few modifications.[26] 10^5 to 10^6 cells were resuspended in cell lysis buffer, incubated for 30 min on ice, supernatants were collected after centrifugation and stored at –80 °C. Protein extracts (0.6 μg) were incubated for 30 min at 30 °C in the PCR mixture and PCR was performed under the following conditions: 90 °C / 90 s, 94 °C / 30 s, 50 °C / 30 s, 72 °C / 45 s, 35 cycles. Products were separated on 12.5 % polyacrylamide gel and visualized with ethidium bromide under UV light. Telomerase activity was calculated as TPG (Total Product Generated) according to the formula:

$$\mathrm{TPG} = \frac{(x - x^{\circ}) / c}{(r - r^{\circ}) / cR} \times 100$$

where x is the activity of telomeric bands of the sample, x° is the background, c is the activity of the sample internal control, r is the activity of the telomeric bands of the quantitation control, r° is the activity in primer-dimer/PCR contamination control and cR is the activity of the internal control of quantitation control. Densitometric analysis was performed on Image Master VSD Software (Amersham).

RESULTS

Telomere Restriction Fragment (TRF) Analysis of A9 and A9+1 Cells

Introduction of a single normal chromosome 1 into several immortal human and rodent cell lines led to the growth arrest and appearance of a senescent phenotype.[13,14,15,27] In contrast, the mouse A9 cell line appeared as a »general recipient« and these cells retained the immortal phenotype after introduction of all human chromosomes by microcell-mediated transfer, with the exception of chromosome 9.[18,22] Indeed, A9+1 growth rate was similar to their parental A9 cells. Surprisingly, analyzing telomere restriction fragments obtained from A9 and A9+1 cells we found great differences in their telomere lengths: southern blot densitometric analysis showed that A9 cells had significantly longer telomeres (mean 9.3 kb) than A9+1 (mean 4 kb). In comparison, human young fibroblasts (HCA2) at PD ≈ 25 (PD, population doublings) showed a mean telomere length of 8.3 kb (Figure 1). These results suggest the possible involvement of proteins expressed from chromosome 1 influencing telomere length.

A9 and A9+1 Telomere Analysis by PNA FISH

Since A9+1 cells demonstrated drastic telomere shortening upon introduction of human chromosome 1, we examined individual telomere lengths of mouse chromosomes as well as human chromosome 1 and compared it to the original A9 cell strain. Telomeres of A9+1 and parental A9 cells were analyzed by PNA fluorescence *in situ* hybridization. Results are shown in Figure 2. Telomeres in A9+1 showed great variability in fluorescent signals, including sister chromatids, as compared to A9. Also, some chromosomes in both cell lines completely lacked telomeric signals. The fraction of unlabeled telomeres in A9+1 cells was as high as 44.2 %, and in A9 cells only 7.3 % (Figure 3a). Thus, the low percentage and intensity of labeled telomeres in A9+1 cells, in contrast to A9, match the dramatic telomere shortening observed by the TRF analysis.

Human Chromosome 1 Telomere Analysis

We further analyzed human chromosome 1 telomeres in contrast to mouse chromosome telomeres in the same

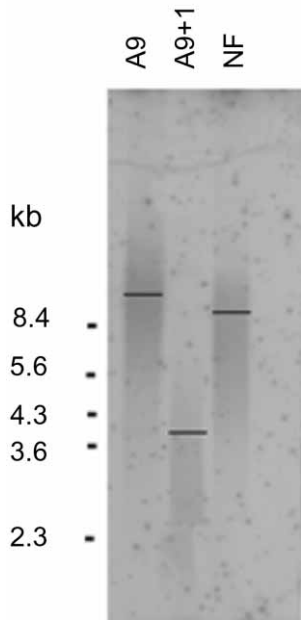

Figure 1. Telomere length analysis of A9, A9+1 and normal human fibroblasts. Genomic DNAs were isolated from A9, A9+1, and normal human fibroblasts HCA2 as a control, digested with RsaI/HinfI restriction enzymes in subtelomeric regions and hybridized with digoxigenin labeled telomere probe. TRF signals were analyzed with Aida 2.0 program. NF – young diploid human fibroblasts HCA2. Bars represent average telomere lengths. BstE2 marker on the left.

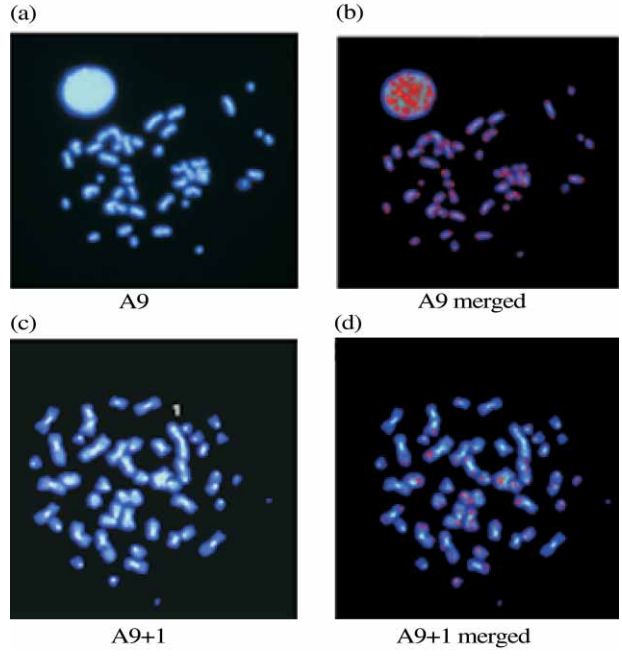

Figure 2. PNA FISH of A9 and A9+1 cell telomeres. Chromosomes were visualized with DAPI (blue) and telomere signals with Rho-labeled telomere PNA probes (red). a) A9 metaphase spread stained with DAPI, b) merged images of A9 metaphase spread and telomere signals, c) A9+1 metaphase spread stained with DAPI (1 labels human chromosome 1), d) merged images of A9+1 metaphase spread and telomere signals. Magnification 1000x.

cell. The percentage of unlabeled human chromosome 1 telomeres corresponded to mouse telomeres in A9+1 (46.3 % and 44.2 % respectively) (Figure 3a). Also, analysis of relative fluorescence intensities in A9+1 cells showed the same range of telomere lengths of human chromosome 1 as those of the surrounding mouse chromosomes (Figure 3b). These results indicate the crucial role of the cell protein background in telomere length regulation rather than specific chromosomal DNA properties.

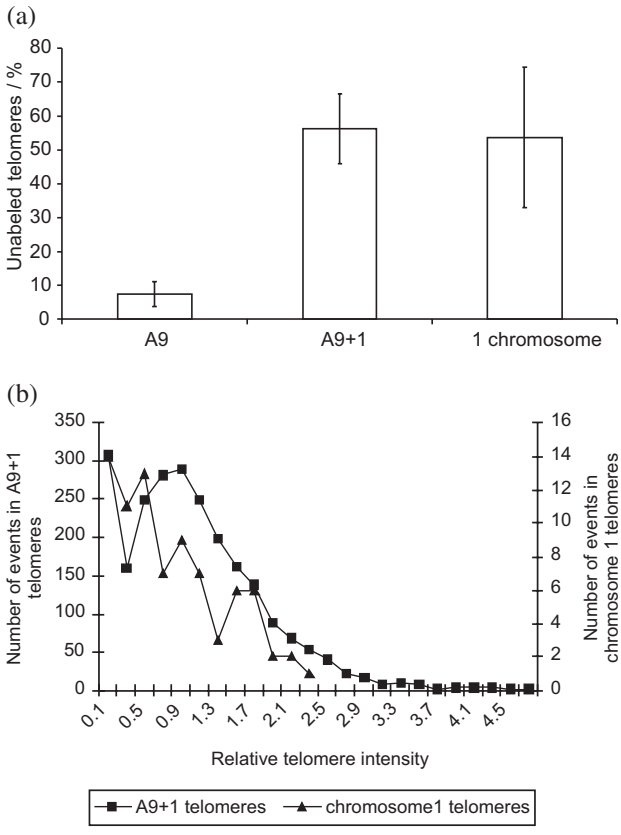

Figure 3. Telomere labeling statistics. a) Percentage of unlabeled telomeres in A9, A9+1 cells and human chromosome 1. b) Frequency of relative intensities of individual mouse telomeres in A9+1 cells compared to relative intensities of human chromosome 1 telomeres. Unmodified black and white PNA FISH images were used to estimate the mean pixel value for each telomeric signal. Images were analyzed using Image MasterVSD Software (Amersham).

Telomerase Activity Analysis

It is known that the level of telomerase activity could influence telomere length.[28,29] We therefore compared telomerase activity in both A9 and A9+1 cells, using the TRAP assay as described in experimental procedures. There was no significant difference in telomerase activity between these cell strains: A9 cells had the mean activity of 258.5 TPG (SD = 7.8) and A9+1 cells of 320 TPG units (SD = 46.7). These results demonstrate that telomerase activity did not contribute to the observed telomere length reduction upon introduction of human chromosome 1 into the mouse A9 cells.

DISCUSSION

Telomere length analysis in the mouse cell line A9 containing human chromosome 1, in comparison with their parental cell line, revealed that they have significantly shorter telomeres, as shown by PNA-FISH (relative signal intensity and labeling percentage) and TRF analyses. Human chromosome 1 also showed the same range of telomere lengths as mouse chromosomes in the cell. There are several possible explanations for the observed differences in telomere lengths between the hybrid and parental cell line. Although recombination between mouse and human chromosome 1 was not observed in A9+1 cells used in these studies (Ref. 13 and personal communication), it was demonstrated that they could lead to changes in the mouse cell protein background, including changes in the expression of some telomere-regulating proteins.[18] There is also the possibility that proteins expressed from human chromosome 1 affect the length of telomeres in these cells. This hypothesis is supported by experiments performed with some other A9 hybrid cell lines. It was found that total telomere lengths depended on the human chromosome introduced in these cells so that A9+3 cells showed a slight lengthening of telomeres, and chromosome 4 introduction showed a similar profile as A9+1 (data not shown). In comparison, experiments made with artificial »telomere seeds« introduced in cell lines containing telomerase showed an increase in their size with time in culture and followed telomere dynamics in cancer cell lines.[30,31,32] Differences in telomerase activity, as a cause of changes in the mean telomere length, could be excluded: A9 and A9+1 cells had nearly the same telomerase activity.[28,29]

There are several lines of data indicating the role of human chromosome 1 in cell senescence. Pereira-Smith[33] found four complementation groups of tumor cells indicating that there are four basic mechanisms responsible for cell immortalization. Introduction of a single normal chromosome 1 into immortal human cell lines assigned to complementation group C caused loss of proliferative potential and induction of the senescent phenotype.[13] Perhaps, human chromosome 1 did not induce senescence in A9 because these cells did not belong to an appropriate complementation group, but contributed to the changes of protein expression. Some of these changes could affect telomere maintenance and their reduction to a shorter length. It should also be considered that the entry into cell senescence is a complex mechanism, especially in tumor cells. These cells compromise control check points and certain signaling pathways cannot be activated by telomere length alone.[8] On the other hand, human chromosome 1 was able to induce senescence in an immortal Syrian hamster cell line,[14] a mouse melanoma hybrid cell line,[15] and a human uterine endometrial carcinoma cell line as well.[16] This suggests that human chromosome 1 is a carrier of some senescence-related

genes that are functionally conserved across evolutionary boundaries. Loss of the q arm of chromosome 1 by spontaneous deletion was unable to induce senescence in some experimental cell lines, indicating the presence of senescence associated genes in this region.[13,34] Structural changes and deletions of chromosome 1 have been documented in the cases of several clinical tumors.[35] Two putative senescence loci were localized on chromosome 1[36] and Yawata[21] identified region of ≈600 kb in 1q42-3 position involved in induction of cellular senescence. It acted in a telomerase independent pathway, since these cells retained telomerase activity upon restoration of the senescence phenotype.

Unlike chromosome 1, microcell-mediated chromosome transfer of human chromosomes 3 and 10 in the human tumor cell lines led to telomerase repression and progressive shortening of telomeres.[17,20,37,38] Introduction of chromosomes 2,[39] 4,[40] 6 and 7[20] in various immortal cell lines also induced senescence; most of them had suppressed telomerase activity.[40,41,42] Parallel experiments with several other chromosomes showed no changes in proliferation of various cell lines.[15]

A9+1 cells showed shortened telomeres, but remained immortal and telomerase positive. They are phenotypically identical to their parental cell line as well. We detected no significant increase in genome instability in hybrid cells or an increase in telomere associations observed under DAPI staining (data not shown). It is known that very short or missing telomeres could lead to chromosome rearrangements and fusions.[43] In spite of this, some cell lines manage to maintain stability and constant equilibrium of the mean telomere length.[8,32,44] Although human chromosome 1 influenced the mean telomere length, A9+1 cells maintained their telomeres at a constant length and avoided senescence. It is also likely that mouse chromosomes exert a defined influence on human chromosome 1 derived proteins and/or their expression and thus modulate the cell environment. In our future experiments we would like to investigate whether expression of some specific human chromosome 1 proteins could influence telomere lengths of the host chromosomes.

In conclusion, A9 mouse cell line showed telomere shortening after introduction of human chromosome 1 whose telomeres were also adapted to these changes. Telomere lengths are primarily determined by the cell protein background and the cause of telomere shortening in A9+1 cells could be explained by the expression of some human proteins.

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SAŽETAK

Humani kromosom 1 u mišjim imortalnim stanicama

Maja Matulić, Irena Jevtov, Marina Ferenac, Nikolina Škrobot, Kaoru Tominaga i Ivica Rubelj

Telomere su specijalizirane strukture na krajevima linearnih kromosoma i esencijalne su za normalnu staničnu funkciju. One sprečavaju degradaciju i pogrešnu rekombinaciju krajeva kromosoma i olakšavaju replikaciju kromosomskih krajeva. U ovom radu praćena je dinamika telomera u imortalnim mišjim staničnim linijama A9 i A9+1. A9+1 stanična linija dobivena je unošenjem ljudskoga kromosoma 1 u A9 stanice. Usprkos prisustvu telomeraze primijećeno je veliko skraćenje telomera kod A9+1 u usporedbi s A9 stanicama. Praćeno je i ponašanje ljudskoga kromosoma 1 u mišjim stanicama u uvjetima pod kojima je došlo do skraćivanja telomera. Raspon duljina telomera ljudskoga kromosoma 1 odgovarao je rasponu mišjih telomera stanica domaćina. Ovi rezultati ukazuju da na raspon duljina telomera najveći utjecaj ima ukupan sastav telomernih proteina stanice.