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Nedime SERAKINCI
Jorn KOCH

Telomeric Repeats of Immortal Hamster Cells

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The Danish Cancer Society, Department of Cytogenetics Tage Hansens Gade 2 DK-8000 Aarhus C. Denmark

Abstract: We have analyzed telomeres in four hamster cell lines to investigate how telomeres look in cells grown past the Hayflick limit. In all four cell lines telomeric signals were seen after detection with two different sensitive in situ methods (FISH with PNA probes and PRINS). The telomeric signals were not very strong, suggesting that the repeats are very short, but were nevertheless clearly visible. With the PRINS technique, it was further possible to probe also for the presence of variant telomeric repeats at the chromosome ends. With some of the variant repeat probes, a difference was seen between probes for the C-rich, and probes for the G-rich strand of the repeats. This is similar to findings on normal human chromosomes, and it could therefore be suggested that hamster telomeres are similar to human telomeres in having a discrete domain of variant repeats centromeric to the $(AGGGTT)^n$ repeats. Some differences in staining patterns with the variant probes were observed between the various cell lines, but the significance of this finding is unclear. It seems that the telomeres are maintained by telomerase in the cell lines investigeted, as a telomerase assay was positive in all four cell lines.

Key Words: Telomeric sequences, Hamster, PRINS, PNA-FISH.

Introduction

Linear chromosomes shorten with every round of replication, possibly as a consequence of incomplete lagging strand DNA replication (1). The chromosomal termini circumvent this problem by having renewable DNA at their very end. This DNA is added to the chromosome ends by the specialized enzyme telomerase and has the form of repetitive sequence elements (2). In human cells, telomerase is generally only highly active in germ line cells, here adding telomeric repeats to chromosome ends. Somatic cells, by contrast, has low or no telomerase activity, and telomeres shorten with replicative age (3). After a certain number of cell divisions, this telomere shortening causes the cells to stop dividing (the Hayflick limit). Cellular ageing is thought to be influenced by this mechanism, but it is unknown if the effect is related to shortened or to lost telomeres (reviewed in 4). Cancer progression is similarly influenced by the Hayflick limit, with the malignant cells having to pass a "crisis", before becoming immortal and able to grow unlimitedly. To escape from the crisis, the malignant cells must somehow obtain a potential for telomere elongation (reviewed in 5). This telomere elongation is usually accomplished through aberrant activation of the telomerase. However, not all immortal cells appear with detectable telomerase activity, so telomere elongation must be obtainable also by alternative mechanisms (6). It is not known at what point telomere elongation is forced, or what forces it?

Reports on the telomeric status of immortal hamster cells have previously been published. An absence of telomeric signals in chromosomes from four immortal Chinese hamster cell lines were found by Slijepcevic and Bryant (7), using FISH with DNA probes. Later Slijepcevic et al. (8) found telomere signals in two hamster cell lines, now using the more sensitive PNA probes. From this they concluded that the absence of telomeric signals in the first four cell lines were due to insufficient sensitivity of the previous assay, but it is not absolutely clear from the literature if any of the cell lines were tested with both methods.

Extending the range of cell lines tested, we here report the analysis of an additional four cell lines. This is done both with the PNA assay, and with the PRINS assay,

which gives stronger telomeric signals than the PNA assay (9, 10). We also expand the amount of data on each cell line by detecting not only the canonical AGGGTT-repeats, but also a variety of variant telomeric repeats, as well as by performing telomerase assays on the cells. We report telomerase activity, as well as telomeric signals in all the cell lines investigated. The signals are not very strong, suggesting that the repeats are indeed very short, but nevertheless clearly visible.

Materials and Methods

The cell lines investigated (Wg3-h-A2 (11), ALJ2 (12), GM7297, GM3701) have all been passaged well beyond the Hayflick, limit for the corresponding normal cells. All telomere detection's *in situ* were done on methanol acetic acid fixed spreads of metaphase chromosomes, prepared by standard methods and used freshly.

The PNA-assay of Lansdorp and coworkers (13) was performed with a commercially available kit (DAKO), and according to the supplier's instructions. The DAKO protocol represents a modification of the Lansdorp protocol designed to make the assay faster and to reduce the use of formamide, without compromising the staining efficiency.

Telomeric PRINS reactions were done as previously published (10, 14), using a revised version of the original PRINS protocol (15). This revised PRINS exploits that labeling is seen only if a DNA polymerase catalyzes the *in situ* synthesis of labeled copies of the DNA flanking the target sequence. Certain DNA sequences, including telomeric repeats, lack one or more of the four bases of DNA. In such cases the base(s) not needed for chain elongation at the proper site(s) can be added as dideoxynucleotide(s), efficiently suppressing chain elongation at non-relevant sites. In brief, staining of the AGGGTT-repeats was obtained as follows: A reaction mixture was put on the slide and spread with a 25x50mm coverslip, and the slide was immediately put in a suitable incubator (Omnigene or Omnislide from Hybaid or Twin Tower from MJ-Research) set to a simulated slide temperature of 93°C (16). The reaction mixture contained 1µg telomere primer (Telo2: (CCCTAA)7), 100µM each of dATP, dCTP and ddGTP, 10µM digoxigenin-dUTP, 1U Tth-polymerase, 1xTth buffer and 10% glycerol in a total volume of 50µl. All these reagents were from Boehringer Mannheim, except for the primer, which was from DNA Technology (Denmark). After 3 minutes the slide was cooled to 55°C and incubated at this temperature for approximately 1 hour. The coverslip was loosened in stop buffer (50mM EDTA, 50mM NaCl) at 55°C. The slide was then washed for 5 minutes in wash buffer (4xSSC, 0.05% Tween20) and the digoxigenin incorporated into telomeric DNA *in situ* was stained with fluorescinated anti-digoxigenin antibody (Boehringer Mannheim) according to the suppliers instructions.

PRINS with Telo1, the primer complementary to Telo2, was similarly done according to the above protocol, except that ddCTP replaced dCTP, and dGTP replaced ddGTP.

Both slides reacted with PNA probes and slides reacted with PRINS primers were mounted in 0.4µM DAPI in Vectashield (Vector Laboratories) and analyzed in a fluorescence microscope (Leica DMRB) equipped with "Pinkel filters", and results recorded with a SenSys CCD-camera operated via the IP-Lab software.

The PRINS primers used for detection of variant repeats (14, 17,18) were:

"Telo3" ($GGG(TTGGGG)_2TTG$) corresponds to the telomeric repeat from Tetrahymena. This primer binds to the C-rich strand and supports the synthesis of the G-rich strand in the absence of dATP, dCTP, or both.

"Telo4" ($CCC(AACCCC)_2AAC$) binds to the G-rich strand of the same repeat as Telo3 and initiates the synthesis of the C-rich strand in the absence of dGTP, dTTP, or both.

"Telo5" ($(AGGGTTT)_5$) corresponds to the telomeric sequence in the malaria parasite *Plasmodium Falciparum*. This primer binds to the C-rich strand, and supports the synthesis of the G-rich strand in the absence of dCTP.

"Telo6" ($(CCCTAAA)_5$) binds to the G-rich strand of the same repeat as Telo5 and supports the synthesis of the C-rich strand in the absence of dGTP.

"Telo7" ($(GGTGAG)_4GGTG$) corresponds to a repeat variant that commonly occurs as the AGGGTT- repeat diverges at interstitial sites in the human genome. This primer binds to the C-rich strand and supports the synthesis of the G-rich strand in the absence of dCTP.

"Telo8" ($TCAC(CCTCAC)_4$) binds to the G-rich strand of the same repeat variant as Telo7, supporting the synthesis of the C-rich strand in the absence of dGTP.

All PRINS reactions for variant repeats were done according to the same basic protocol as used for the AGGGTT-repeat, except for two differences. Firstly, the choice of dideoxynucleotide used was adjusted to suit the particular primer. Secondly, PRINS with Telo4 was detected through the incorporation of digoxigenin-dCTP

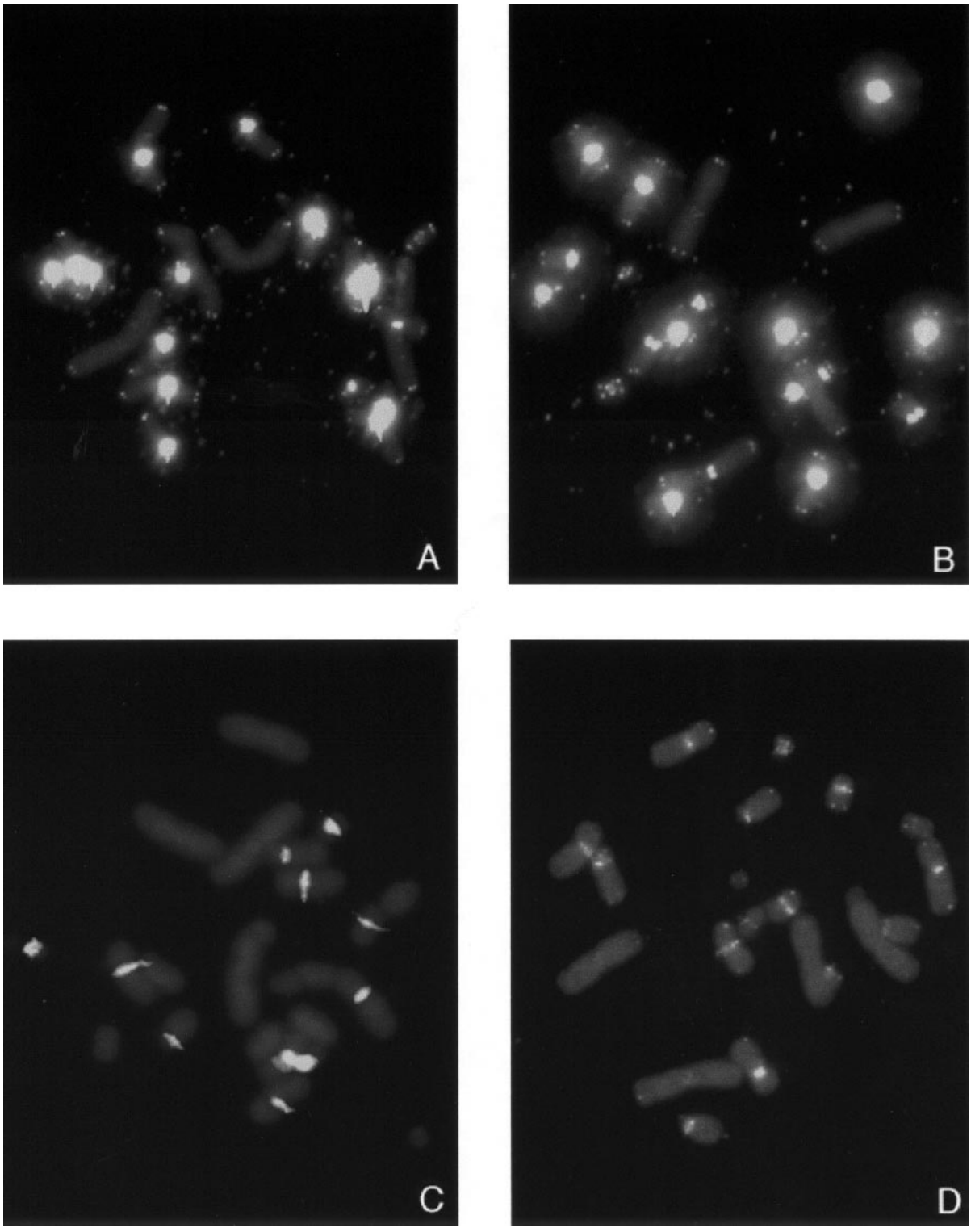

Figure 1. Top: PRINS with Telo2 on GM7297 (A) and GM3701 (B). Note the general staining of chromosome ends as well as the strong staining of some centromeres. Bottom: PRINS with Telo3 on GM7297 (C) and GM3701 (D). Telomeric signals are not seen in GM7297, but some signals are seen in GM3701, though these are both weaker and less abundant than the signals with Telo2. Not only telomeric, but also centromeric, staining is weaker with Telo3 than with Telo2.

(Boehringer Mannheim), as the Telo4 sequence contains no T-residues.

Telomerase activity was assayed with the Telomerase PCR ELISA (TRAP) assay from Boehringer Mannheim.

Results and Discussion

All our four hamster cell lines have been passaged extensively, and the telomeric repeats at chromosome ends in these cell lines must therefore represent telomere reconstitution, either through compensation for repeat loss, or through *de novo* generation of telomeres. In all four cell lines the TRAP assay was positive, and the cells thus expressed telomerase (data not shown).

As far as the telomeric repeats are concerned, both FISH with PNA probes and PRINS with Telo1 or Telo2 gave general staining of chromosome ends. The only obvious difference between the results with PNA-FISH or PRINS, was that the PRINS signals were clearly stronger than the PNA signals. The telomeric PRINS signals were thus visible directly in the microscope, also with a dual color filter that allowed the DAPI counterstain to be seen together with the PRINS signals. The PNA signals, by contrast, had to be viewed with a single color filter, not to be hidden by the counterstain. This difference in staining intensity means that, whereas we could determine that all chromosome ends held PRINS signals, we could not exclude that some chromosome ends escaped detection with the PNA FISH (data not shown).

In addition to the telomeric signals, much stronger centromeric signals were seen in all four cell lines (Figure 1A,B, Table 1) and with both techniques. The intense

Table 1. Summary of results on the four cell lines after PRINS with the eight primers used in this study. The results of the PNA-FISH corresponded to PRINS results with Telo2, except for a lower overall intensity of staining.
In general, the intensity of the telomeric signals varied with the cell line, increasing slightly from left to right in the table with most of the probes. It can therefore not be excluded that the absence of signals with some of the variant repeat probes in ALJ2 and GM 7297 reflects a lower overall stain ability of telomeric repeats in these cells, causing small target sequences to escape detection. However, after incubation with Telo3 or Telo7, centromeric signals are actually stronger in these two cell lines than in the other two cell lines, speaking against such a difference.
In general, signals were stronger with Telo3, 5 & 7 than with their even numbered counterparts. Telo1 and Telo2, by contrast, gave signals of equal intensity.
Telo7 and Telo8 gave a stronger staining of the chromosome arms than the other probes, though no specific sites could be identified. This is similar to our finding on human chromsomes (17).
Otherwise, scores are given for telomeric ("T") as well as centromeric ("C") staining for all probe/cell line combinations. The scores for the telomeres are given as "+" or "-", denoting respectively presence or absence of telomeric signals. After PRINS with Telo8 in ALJ2, signals could not be distinguished from the increased background fluorescence resulting from the use of the Telo8 primer, and the result is given as "not determined" (N.D.). The scores for stained centromeres are graduated according to intensity and given as "*", "**" or "***", the latter referring to the highest staining intensity.

	ALJ2		GM7297		Wg3-h-2A		GM3701	
	T	C	T	C	T	C	T	C
Telo1 $(TTAGGG)_7$	+	***	+	***	+	***	+	***
Telo2 $(CCCTAA)_7$	+	***	+	***	+	***	+	***
Telo3 $(GGG(TTGGGG)_2TTG)$	–	*	–	**	+	*	+	*
Telo4 $(CCC(AACCCC)_2AAC)$	–	–	–	–	–	–	–	–
Telo5 $(AGGGTTT)_5$	+	***	+	***	+	***	+	***
Telo6 $(CCCTAAA)_5$	–	*	–	*	–	**	+	**
Telo7 $((GGTGAG)_4GGTG)$	–	**	+	**	+	*	+	*
Telo8 $(TCAC(CCTCAC)_4)$	N.D.	**	+	***	+	***	+	***

centromeric staining saturated the CCD-images, and caused telomeric signals to be more easily seen directly in the microscope than on the computer screen. A strong staining of centromeres in hamster chromosomes was not surprising, as already Moyzis and coworkers noted this (19). However, comparing our images with the images from normal hamster cells presented in Moyzis et al. (19), the ratio between telomeric and centromeric staining seems lower in our cells. This difference is consistent with a loss of telomeric repeat DNA from the chromosome ends, but not from centromeres, and thus shorter telomeres in the cell lines. Slijepcevic et al. (8) similarly found short telomeres in the hamster cell lines they investigated, also reporting that telomeric signals in such cell lines are best seen directly in the microscope.

The staining intensity was for all four cell lines similar whether Telo1 or Telo2 was used as PRINS-primer. In normal cells from man and mouse, it has been found that PRINS with Telo1 leads to less telomeric signals than PRINS with Telo2 (20, 18). This has been explained by the existence of breaks in the C-rich strand of the AGGGTT repeat domain (20). In the present cell lines, we do not observe this difference between the two primers. A possible explanation for the lack of difference could be an absence of breaks from AGGGTT-domain in transformed cell lines, such as the ones analyzed here.

PRINS with primers for variant telomeric repeats gave results that varied with the cell line and with the primer, as illustrated for Telo3 in Figure 1C,D and summarized for all the primers in Table 1. In general, the primers priming on the C-rich strand of the repeats (Telo3, 5 & 7) gave more and stronger telomeric signals than the primers priming on the G-rich strand (Telo4, 6 & 8). This asymmetry in staining between strands is similar to what we find in normal human telomeres. In human telomeres, the target sequences for the variant repeat probes are clustered in a discrete domain centromeric to the AGGGTT-repeat (21), and the difference in staining between our odd- and even-numbered Telo-primers is a consequence of this organization (17). It is not known whether hamster telomeres hold a similar telomeric repeat organization, but from our finding of the same strand polarity in staining as in the human telomeres, it may be suggested that they actually do. To the extent that the hamster cell lines do have a discrete variant repeat domain centromeric to the AGGGTT repeats, as suggested above, it thus seems that telomere shortening in these cells was stopped before it had proceeded to remove all telomeric repeats, and that the telomeres thus are "original" telomeres rather than new telomeres added to chromosome ends without telomeres.

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References

1. Watson JD. Origin of concatameric T7 DNA. Nat. New Biol. 239: 197-201, 1972.
2. Greider CW, Blackburn EH. A telomeric sequence in the RNA of *tetrahymena* telomerase required for telomere repeat synthesis. Nature 337: 331-337, 1989.
3. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RG. Telomere reduction in human colorectal carcinoma and with ageing. Nature 346: 866-868, 1990.
4. Harley CB. Telomeres and aging. In Blackburn EH, Greider CW (eds) Telomeres. Cold Spring Harbor Laboratory Press, pp. 247-264, 1995.
5. de Lange T. Tumor telomeres. In Blackburn EH, Greider CW (eds) Telomeres. Cold Spring Harbor Laboratory Press, pp. 264-293,1995.
6. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nature Medicine 3: 1271-1274, 1997.
7. Slijepcevic P, Bryant PE. Absence of telomeric FISH signals in chromosomes from immortal Chinese hamster cells. Cytogenet. Cell Genet. 69:87-89, 1995.
8. Slijepcevic P, Hande MP, Bouffler SD, Landsdorp P, Bryant PE. Telomere length, chromatin structure and chromosome fusigenic potential. Chromosoma 106: 413-421, 1997.
9. Serakıncı N, Koch J. Detection and sizing of telomeric repeat DNA in situ. Nat. Biotech. 17: 200-201, 1999.
10. Krejci K, Koch J. Improved detection and comparative sizing of human chromosomal telomeres in situ. Chromosoma 107: 198-203, 1998.
11. Goss SJ, Harris H. Gene transfer by cell fusion. Statistical mapping of human X-chromosomes by analysis of radiation induced gene segregation. J. Cell Sci. 25: 17, 1977.

12. Waldren C, Martin J, Sutherland J, Cram S. Use of somatic cell hybrids for quantitation of mutagenesis: reduction in background mutants by fluorescence-activated cell sorting (FACS). Cytometry 5: 584-588, 1984.

13. Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ. Heterogeneity in telomere length of human chromosomes. Hum. Mol. Genet. 5: 685-691, 1996.

14. Koch J. PRINS: PRimed IN Situ labeling and hybridization in one step. In Kessler C (ed) Nonradioactive labelling and detection of biomolecules, 2nd edition. Springer Verlag, in press.

15. Koch J, Kølvraa S, Gregersen N, Bolund L. Oligonucleotide-priming methods for the chromosome-specific labeling of alpha satellite DNA *in situ*. Chromosoma 98: 259-265, 1989.

16. Koch J, Hindkjær J, Kølvraa S, Bolund L. Construction of a panel of chromosome-specific oligonucleotide probes (PRINS-primets) useful for the identification of individual human chromosomes *in situ*. Cytogenet. Cell Genet. 71:142-147, 1995.

17. Krejci K, Koch J. An *in situ* study of variant telomeric repeats in human chromosomes. Genomics. 58; 202-206, 1999.

18. Serakıncı N, Krejci K, Koch J. Telomeric repeat organization- a comparative in situ study between man and rodent. Cytogenet. Cell Genet, in press.

19. Moyzis RK, Jones MD, Meyne J, Ratliff RL, Wu JR. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. USA 85: 6622-6626, 1988.

20. Therkelsen AJ, Nielsen A, Koch J, Hindkjær J, Kølvraa S. Staining of human telomeres with primed *in situ* labeling (PRINS). Cytogenet. Cell Genet. 68: 115-118, 1995.

21. Allshire RC, Dempster M, Hastie ND. Human telomeres contain at least three types of G-rich repeats distributed non-randomly. Nucleic Acids Res. 17: 4611-4627, 1989.