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In search of vertebrate telomeric proteins

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Indirect evidence suggests that vertebrate chromosome ends carry a protective nucleoprotein cap containing specific telomeric proteins. Telomeric proteins could explain the stringent sequence requirements for de novo *telomere formation, aspects of telomere length regulation, the unusual chromatin structure detectable in some vertebrate telomeres, and the attachment of telomeric DNA to the nuclear matrix. Although telomeric proteins have been identified in unicellular organisms, vertebrate telomeric proteins are not well characterized. The search for these factors is discussed in this review.*

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VERTEBRATE CHROMOSOME ends carry long arrays of telomeric TTAGGG repeats.[1,2] This DNA is maintained by telomerase, a specialized DNA polymerase that uses an internal RNA template to copy TTAGGG repeats onto 3' ends [ref 3; see ref 4 for review]. Telomeres protect chromosomes from a number of forces that threaten DNA ends. Unlike the ends of broken chromosomes, telomeres are resistant to degradation and ligation, and they are not detected by factors that scan the genome for DNA damage. The challenge ahead is to understand how telomeres cap chromosome ends and how they regulate their maintenance by telomerase.

Two main models could be proposed to explain the biological activity of telomeric repeat arrays. In one proposition, features of the telomeric DNA itself (e.g. one of the G–G base paired folded structures that can be formed by single-stranded TTAGGG repeats; reviewed in ref 5) would protect telomere termini and modulate their interaction with telomerase. The biological relevance of these structures has been difficult to evaluate in absence of proof for their existence at chromosome ends *in vivo*.

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The premise of this review is rooted in the alternate (but not mutually exclusive) proposal that the telomeric DNA interacts with telomere specific proteins. In fungi and in ciliates such factors have been characterized in detail (reviewed in ref 6). Hypotrichous ciliates carry a protein complex at their telomere ends and telomeres in the yeast *Saccharomyces cerevisiae* are coated by the telomeric protein Rap1p. Since many aspects of telomere biology are conserved, it is expected that vertebrate chromosome ends also carry a protein cap. Here I review the evidence for a specialized nucleoprotein complex at vertebrate telomeres and summarize what is known about candidate telomeric proteins.

Stringent sequence requirements for telomere formation in human cells

One of the arguments in favor of the existence of telomeric proteins in vertebrate cells is based on the stringent sequence requirements for telomere formation. After transfection of telomeric DNA into mammalian tissue culture cells, most stable transfectants carry a truncated chromosome with the introduced telomeric DNA at its broken end.[7,8] The fragmented chromosomes are stable, apparently because the transfected T_2AG_3 repeats seed a new functional telomere. In this setting, telomere seeds require at least 0.5–0.8 kb of telomeric DNA[7-9] and telomere formation displays a critical dependence on the T_2AG_3 sequence.[9] While T_2AG_3 repeats readily seed new telomeres, arrays of closely related telomeric repeats from other eukaryotes (TAG_3, T_3AG_3, T_2G_4, T_2AG_5, T_2AG_2C) are virtually inactive in this assay.[9] The lack of telomere formation with the heterologous telomeric sequence cannot be explained from the substrate requirements of human telomerase, which elongates heterologous primers.[3,8-10] In addition, since each of these repeats contains strings of guanines and functions at telomeres in other eukaryotes, it is unlikely that the heterologous telomere seeds are inactive because they fail to form a specific DNA structure. Instead, the data are most readily explained if incoming telomeric sequences need to engage a

protein that requires precise TTAGGG repeat arrays for its binding. The best candidate for this activity is TRF (see later), a TTAGGG repeat binding factor whose sequence specificity matches the sequence requirements for telomere formation.

Regulation of telomere length

A second hint at the presence of a telomeric nucleoprotein complex comes from observations on telomere length regulation in mammalian cells. There are a number of immortal human cell lines in which telomeres are stably maintained at constant length.[11-15] Thus, in these cells telomere attrition (due to replication-mediated sequence loss) and telomere elongation (by telomerase) are balanced; a situation commonly encountered in unicellular eukaryotes. A particularly informative example is an HPV-transformed keratinocyte cell line.[13] HPV transformed keratinocyte cultures undergo telomeric decline as they approach crisis, a culture stage at which proliferation is balanced by cell death. A post-crisis immortalized keratinocyte clone was found to have growing telomeres, most likely due to activation of a telomere maintenance system. However, eventually the telomeres reached a plateau in these cells, as would be expected if telomere length is subject to homeostasis.

Additional arguments for regulation of telomere length in vertebrates comes from the wide variation in telomere length in different species. For instance, *Mus musculus* has long telomeres (~50 kb) whereas the telomeres of the closely related mouse *Mus spretus* are about 5 kb.[16] Crosses between these species should be informative on genetic aspects of telomere length setting. Interestingly, when new telomeres are formed by transfection of TTAGGG repeat DNA into *Mus musculus* cells the telomere seeds quickly grow to ~50 kb.[8] By contrast, in human cells such seeds grow to mean lengths ranging from 2–10 kb, consistent with the length of the endogenous telomeres.[9] Thus, in de-novo telomere formation, the length of the newly formed telomeres is dictated by the host cell.

These observations indicate that vertebrate cells have the ability to determine the length of their telomeres. The simplest models to explain this phenomenon invoke a telomeric protein that measures the length of each telomere by binding to the double-stranded telomeric repeat region. For example, the binding of multiple copies of a telomeric repeat binding factor to longer telomeres could result in telomeric complex that sequester this terminus from telomerase. In yeast, there is good evidence that Rap1p, which binds along the length of the telomere, contributes to the regulation of telomere length (ref 17; reviewed in ref 18). One of the main goals of dissecting the vertebrate telomeric complex will be to understand the mechanism of this type of regulation.

Alternate telomeric chromatin structures

Most chromosomal elements, including genes, replication origins and centromeres reveal their presence as altered local chromatin.[19] This paradigm clearly holds for telomeres as well, since in unicellular organisms the short telomeric tract is packaged in a specialized non-nucleosomal chromatin structure.[20-22] The situation in vertebrate telomeres appears more complex. Two different telomeric chromatin arrangements can be discerned.

Small, densely packed nucleosomes in long vertebrate telomeres

The long telomeric regions of many vertebrates show a predominantly nucleosomal organization.[23-25] This is true for vertebrate telomeres in the 20–100 kb length range, including those of representative rodents (rat and mouse), birds (chicken), reptiles (turtle), amphibians (mud puppy), and fish (trout). The telomeric nucleosomes are similar to bulk nucleosomes in a number of physical properties, such as sedimentation behavior, electrophoretic mobility, and DNaseI digestion pattern, suggesting that telomeric and bulk nucleosomes have more or less the same protein composition. In addition, telomeric chromatin displays the same overall sensitivity to nucleases (MNase and DNaseI) as bulk chromatin (refs 23-25; Tommerup and de Lange, unpubl. observations).

Vertebrate telomeric nucleosomes also have a number of unique aspects.[23-26] As a rule, they are closely spaced and appear highly uniform, resulting in crisp MNase partial digestion products that extend far into the high MW range. While their size varies from species to species, telomeric nucleosomes are always consistently smaller (by about 40 bp) than the bulk nucleosomes from the same cells. Other telomere specific attributes are the relative depletion of histone H1, and that the mononucleosomes are hypersensitive to MNase, thwarting attempts to inspect TTAGGG repeat containing core-particles.

Altered nucleosomal organization in short telomeres

MNase digestion of the relatively short (2–6 kb) telomeres found in many established human cell lines has revealed an altered chromatin structure.[24] This chromatin is characterized by diffuse MNase patterns and a lack of the distinct higher molecular weight bands that are normally indicative of regularly spaced nucleosomal arrays. This unusual nucleosomal organization is confined to the TTAGGG repeat region; the nucleosomal organization of flanking subtelomeric sequences resembles bulk chromatin.[24]

The altered, diffuse telomeric chromatin is not a peculiarity of human tumor cell lines. Heterogeneous telomeric MNase products are also detected with primary human neutrophils in which the telomeres are short;[25] conversely, when human telomeres are long (20–30 kb range), as is the case in some immortalized cell lines, their chromatin structure is dominated by closely-spaced nucleosomes and the diffuse MNase patterns are not detectable.[24] Thus, human cells can show either of the two alternate telomeric chromatin structures depending on the length of the telomeres. Similarly, mouse telomeres can yield either type of telomeric chromatin depending on their length (ref 24; TdL, unpublished observations). These data indicate that the detection of the altered telomeric chromatin is strongly correlated with telomere length and argue that this unusual chromatin domain is a general feature of short vertebrate telomeres.

But why is this specialized chromatin not detected in long vertebrate telomeres? The simplest explanation is that the altered chromatin is confined to a short, terminal domain, whose presence is simply obscured by the nucleosomal organization in the remainder of the telomere. If the altered chromatin occupies a few kb of the TTAGGG repeat array, it would probably go unnoticed in telomeres of >20 kb.

Based on work in unicellular eukaryotes it is reasonable to expect that the altered chromatin structure is relevant to telomere function and reflects the presence of a non-nucleosomal complex.[20-22] However, it should be noted that heterogeneous MNase patterns can also originate from other circumstances, including variations in nucleosome spacing and increased nucleosome mobility. Future work will need to address these issues.

Subnuclear localization of telomeres in somatic and germline cells

With regard to searches for telomeric factors it is useful to consider the subnuclear localization of telomeres. This question has been approached by cytological and biochemical techniques.

Variable spatial distribution of somatic telomeres

In-situ detection of interphase telomeres using telomeric probes in combination with confocal microscopy has shown that mammalian chromosome ends can occupy a variety of subnuclear sites.[27-31] Some telomeric signals form a punctate pattern at central locations, others are more peripheral, some are adjacent to the nucleolus, while a small number of telomeres (perhaps 10%) directly adjoin the nuclear envelope.

Despite this variability, it is clear that individual telomeres can have non-random distributions and that their specific locale may depend on the cell type. For instance, the human Yq telomere dwells near the nucleolus in neurons but holds a peripheral site in astrocytes.[27] Furthermore, the spatial distribution of individual telomeres may change during progression from G1 to G2.[30] This was observed in mouse nuclei, where half of the telomeres directly abut the centromeres and are thus tagged with centromeric satellite sequences. These proximal telomeres move from their G1 position at the nuclear periphery to the interior of the nucleus in G2. At the same time, some of the distal telomeres move in the opposite direction.

These observations contrast the situation in the budding and fission yeast where telomeres are predominantly located at the nuclear periphery (reviewed in ref 32). A second remarkable difference is that while yeast telomeres are usually clustered or paired, there is no evidence for such associations in normal somatic mammalian cells. The only exception to this rule is the Barr body, the inactive heterochromatic X chromosome of female mammalian cells, which forms a loop with its telomeres in close apposition (<1 μm apart).[33]

Telomere distribution and movement in meiosis

In contrast to their variable locale in somatic nuclei, vertebrate chromosome ends occupy a peripheral position during the early steps of the first meiotic division (see for review ref 34). Often, the prophase

chromosomes are in the so-called RabI orientation in which centromeres and telomeres each occupy specific domains at opposite poles of the nucleus. Preceding synapsis, the telomeres move along the nuclear envelope, leading to a 'bouquet' in which all chromosomes loop out from a single site. Telomere congregation as well as the initiation of synapsis at chromosome ends are suggestive of a role for telomeres in this process. In human sperm, the telomeres are also at the nuclear envelope and display pairing (possibly p to q pairing within each chromosome) (ref 35; A.O. Zalensky, personal comm.). It will be of great interest to define the role of telomeric proteins in the association of telomeres with the nuclear envelope, the pairing of telomeres, and the movements of chromosome ends along the nuclear envelope. None of the involved factors have been identified.

Telomere–nuclear matrix interactions

Human, mouse and rat telomeres co-fractionate with the nuclear matrix (ref 36; and E. Luderus and TdL, unpublished observations). This is an operationally-defined nuclear remnant composed of specific DNA sequences, RNA, and a complex mixture of aggregated proteins, that are insoluble in the extraction buffers used to isolate nuclear matrices. Chromosome ends are attached to the nuclear matrix through their TTAGGG repeat sequences, while subtelomeric DNA can be dissociated from the nuclear matrix by digestion with appropriate restriction enzymes. Although the function of the matrix attachment of telomeres (or any other DNA sequence) is unclear, their tethering indicates that telomeric chromatin contains a component of the nuclear matrix.

The attachment to the nuclear matrix occurs through interactions along the TTAGGG repeat region. When human telomeres with ~20 kb of TTAGGG repeats are cleaved into fragments of ~2 kb with DNaseI, the majority of the telomeric DNA co-fractionates with the nuclear matrix (E. Luderus and TdL, unpublished observations). If the matrix attachment was confined to the telomere terminus, most of the 2 kb telomeric fragments should have been released by this treatment. Thus, the nuclear matrix interaction of telomeres extends through a large domain and the factor(s) reponsible for this tethering are expected to interact with double-stranded TTAGGG repeat arrays.

Which nuclear matrix protein binds the telomeric repeat region is not known. One of the best defined nuclear matrix components are the lamin/pore complexes of the nuclear envelope. However, only a small fraction (~10%) of human telomeres can be isolated with nuclear shells (E. Luderus and TdL, unpublished observations), a subfraction of the nuclear matrix largely composed of nuclear envelope remnants and associated DNA. This estimate is in agreement with the fact that only a minority of the telomeres are located at the nuclear periphery. One possibility is that these telomeres interact directly with the lamin polymers which are known to bind DNA (see below).

Telomeric DNA binding proteins

Work on unicellular organisms has revealed two classes of telomeric proteins (reviewed in ref 6). One class, so far uniquely represented by Rap1p in yeast, binds to the double-stranded part of the telomeric repeat array. A second class of telomeric proteins, first identified in Oxytricha, binds to telomere termini. These telomere terminus factors are single-stranded DNA binding proteins with a strong preference for G-strand telomeric repeats in a 3' overhang. Since preliminary surveys failed to reveal vertebrate homologs of the genes for Rap1p and the ciliate telomere terminus factors, the focus has been on biochemical approaches to isolate the protein components of vertebrate telomeres.

Double-stranded telomeric DNA binding factors

Searches for those proteins that bind along the length of the telomeric repeat array have so far yielded a single candidate, TRF (Telomeric Repeat binding Factor)[37] (Table 1). TRF is a DNA binding activity of moderate abundance (nuclear extracts yield in the order of 10 binding units per telomere) that is ubiquitously expressed in mammal tissues (ref 37; Chong, L., and de Lange, T., unpublished observations). TRF resembles the yeast telomeric protein Rap1p in that it binds along the length of the telomeric repeat array. Like Rap1p, TRF does not bind well to single-stranded telomeric DNA and neither factor requires a DNA end. Competition-titration experiments revealed that TRF has a strong preference for TTAGGG repeat arrays over a number of closely related sequences. For instance, arrays of T_3AG_3, T_4AG_3, T_2AG_4, T_2AG_5, T_2AG_2, TAG_3, T_2G_4, T_2AG_2C, and $TG_{1\text{-}3}$ repeats do not compete for TRF binding to vertebrate telomeric DNA (ref 37; J. Feng and TdL, unpublished observations).

Table 1. Vertebrate proteins that bind to telomeric sequences

	Species	MW [kD]	Abundance per somatic cell	Preferred substrate	Kd [M]	Reference
Double-stranded DNA binding factors						
TRF	mammals and birds	60	~10^3	ds$(TTAGGG)_{n(n\geq 3)}$	n.d.	37; L. Chong and TdL, unpubl.
Single-stranded DNA/RNA binding factors						
Not terminus specific						
hnRNPsA1, A2/B1, D, E	mammals	35–40	>10^6	(U2AG3)n RNA	~10^{-9}	50–52
Nucleolin	mammals	100	>10^6	(T2AG3)n DNA ss T-rich DNA	~10^{-9}	52,53
lamin A+C (soluble)	mammals	65–75	>10^6	telomeric oligos	(low)	44
lamin A and B aggregates	mammals	n.a.	n.a.	ss telomeric DNA (non-specific)	n.a.	E. Luderus and TdL, unpubl.
vimentin	mammals	53	>10^6	telomeric oligos	(low)	43,44
MF3	birds	n.d.	~10^6	G-G base-paired G-strings	~10^{-9}	47
Terminus specific						
XTEF	Xenopus	n.d.	~10^2	$(T_2AG_3)_2$ 3'	n.d.	48

n.d., not determined; n.a., not applicable.

TRF is fairly remarkable in the way it binds to DNA. Improved binding is observed with longer telomeric repeat arrays, such that probes with six or 12 repeats have a much stronger interaction with TRF than probes with only three repeats.[37] This effect is not due to cooperative binding of multiple TRF units on the longer probes. Instead, it appears to reflect an intrinsic ability of TRF to differentiate between site sizes.

The human TRF protein was recently purified (Chong, L. and de Lange, T., unpublished observations). The cloning of the gene for TRF should help in defining its role at chromosome ends. Indirect evidence that TRF binds telomeric DNA *in vivo* comes from the analysis of the sequence requirements for telomere formation in human cells.[9] As detailed above, only telomere seeds that bind to TRF *in vitro* efficiently form new telomeres *in vivo.* Interestingly, this feature is also shared between TRF and Rap1p, whose binding appears to promote telomere formation in transformed yeast cells.[17,38]

Single-stranded telomeric DNA binding factors

The DNA configuration of vertebrate telomere termini is not known. An overhang of 3' TTAGGG repeat units is anticipated because single-stranded 3' protrusions are a conserved feature of chromosome ends in unicellular organisms.[39-42] In addition, such overhangs are the predicted product of lagging-strand DNA synthesis and telomerase-mediated telomere elongation.

Extensive efforts have gone into the identification of proteins that could complex with single-stranded telomeric termini. These searches revealed a bewildering wealth of factors that bind TTAGGG repeats *in vitro* (Table 1). Some are involved in RNA metabolism (hnRNPs, nucleolin) and some actually prefer RNA substrates. Lamins and other intermediate filaments also bind to single-stranded telomeric DNA,[43,44] especially when the repeat arrays are long (Luderus, E. and de Lange, T., unpublished observations), but these interactions are not very specific. Attempts to crosslink human telomeric DNA to either hnRNP D, hnRNP A2/B1, or lamins in living cells have so far failed (Luderus, E. and de Lange, T., unpublished observations). Although these factors are clearly not specific for telomeres, their contribution to telomere function requires further analysis. In this regard, it is suggestive that Chlamydomonas and yeast express a related G-strand binding protein which carries sequence motifs found in RNA binding proteins.[45,46]

Noteworthy among the abundant nuclear proteins that bind G-rich repeats *in vitro* is MF3 (Table 1). This factor was first identified as a candidate transcriptional regulator of chicken muscle genes and was found to bind to single-stranded telomeric TTAGGG repeats. Interestingly, it recognizes G–G base paired structures rather than the specific sequence of telomeric DNA.[47]

The ciliate telomere terminus proteins require a 3'

end near their binding site. The only vertebrate factor with a similar requirement for the proximity of a 3' end is XTEF (Xenopus Telomere End Factor).[48] Other features that are shared between XTEF and the ciliate telomere terminus proteins are a recognition site of approximately two tandem repeats, lack of requirement for a G-quartet structure, and relative stability of the complex in high salt. XTEF was first identified in Xenopus egg extracts where it is fairly abundant, possibly forming part of the stockpile of proteins required for the rapid generation of new chromosomes during the early cleavage divisions. By contrast, extracts of somatic frog and chicken cells contain in the order of one binding unit of this factor per telomere. The purification and cloning of MF3 and XTEF will be required to further address their roles at telomeres.

Perspective

Continued efforts are expected to unravel the telomeric complex of vertebrate chromosome ends in the near future. With (cloned) telomeric proteins in hand a number of important questions can be addressed. First, one would like to understand exactly how the telomeric complex ensures that DNA damage checkpoints and other unwelcome activities by-pass natural chromosome ends. As more components of the DNA damage checkpoint signaling pathways are identified, there is reason to be optimistic about the possibility that the protective activity of telomeres can be studied by reconstitution *in vitro* and by genetic manipulation *in vivo*. A related question is how the protective function breaks down in cells with critically shortened telomeres. Since loss of telomeric protection is thought to contribute to chromosome instability in cancer (reviewed in ref 49), this issue could have important medical implications. Finally, one would like to know how cells measure and control telomere length. In this regard, the interactions between the telomeric proteins and telomerase should be of interest. Prominent questions are whether protein-capped telomere termini are accessible to telomerase, how telomerase-mediated telomere elongation is regulated, and whether (components of) telomerase are only transiently associated with the telomeric complex or have a more stable association with chromosome ends.

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