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Review

Telomeric nucleosomes: Forgotten players at chromosome ends

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Abstract. Telomeres are the special nucleoprotein structures that protect chromosome ends from both recombination and degradation. In most organisms, telomeric DNA consists of short sequences repeated in tandem ending in single-stranded G-rich overhangs. In higher eukaryotes, about 80 % of telomeric DNA is organized in tightly packed nucleosomes separated by 10–20 bp of linker DNA. Several specific proteins contribute to telomeric structure. At the moment, a satisfactory description of telomere organization is still lacking. Whereas the role played by telomeric proteins in telomere function and regulation has been widely investigated, little is known about the contribution of nucleosomes to the protection of chromosome ends. In this review we present an overview on the chromatin organization in lower and higher eukaryotes, and discuss the recent results on the peculiar features of telomeric nucleosomes and on the epigenetic status of mammalian telomeres.

Keywords. Nucleosome, telomere, telomeric chromatin, telomere epigenetics, telomere structure.

Introduction

As they switched to linear chromosomes, eukaryotes had to face two main problems. The first is the so-called "end-replication problem" [1, 2]. Short RNA primers are required by DNA polymerases to initiate DNA synthesis; the removal of the terminal RNA primer leaves a gap that cannot be filled by the DNA replication machinery. The second problem is that chromosome ends must be distinguished from DNA damage sites by DNA repair machineries [3, 4]. To solve these problems, eukaryotes evolved a specialized nucleoprotein structure, the telomere [3–6], and mechanisms to counteract replication-associated loss of terminal sequences, based on retrotransposition [7], on recombination [8], or, in most cases, on a telomere-specific reverse-transcriptase, the telomerase [9, 10].

Apart from a few exceptions like *Drosophila,* whose telomeres are based on arrays of retrotransposons [7, 11], telomeric DNAs consist of 5–8-bp sequences repeated in tandem, ending in G-rich single-stranded 3' extensions (for review, see [12, 13]). The length of telomeres varies among the species, from a few tens of base pairs in ciliates to thousands of base pairs in higher eukaryotes [14–16]. Proteins that specifically recognize double-stranded telomeric DNA have been isolated in several species, such as Rap1 in budding yeast [17], Taz1 in fission yeast [18], TRF1 and TRF2 in mammals [19, 20]. The G-rich single-stranded DNA 3' overhang is also bound by sequence-specific proteins, including TEBP in ciliates [21], Cdc13 in *Saccharomyces cerevisiae* [22], and POT1 in fission yeast and mammals [23]. Several other factors, which do not directly bind telomeric sequences, are recruited

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to telomeres, cooperating to form the telomeric cap. Despite the great efforts spent in elucidating telomere structure and function, we are still far from a clear understanding of how telomeres are organized, especially for what regards higher eukaryotes. Moreover, it should be noted that most research has not taken into account the presence at telomeres of another complex that directly interacts with telomeric sequences: the histone octamer.

In eukaryotes, about 80 % of genomic DNA associates with an octamer of histone proteins (two copies each of H2A, H2B, H3 and H4) to form nucleosomes, the basic units of chromatin [24]. Nucleosomes have mainly a structural role, promoting DNA packaging within the nucleus by wrapping DNA around the histone octamer. However, the current view of nucleosomes is that they are not only static structural units but that they also represent dynamic players in several biological processes (for review, see [25]). Cellular processes such as gene expression and DNA repair require changes in chromatin organization that alter DNA accessibility to specific binding factors. This is achieved through at least three main mechanisms that involve the nucleosome: ATP-dependent nucleosome remodeling, histone post-translational modifications, and replacement of canonical histones with specific histone variants.

How nucleosomes and their modifications intervene in telomere dynamics and regulation is an issue of great relevance. To analyze the role played by nucleosomes at telomeres, we have to distinguish between lower and higher eukaryotes. The short telomeres of lower eukaryotes are prevalently organized as non-nucleosomal complexes, such as in budding yeast [26]. Nucleosomes formed on subtelomeric sequences are fundamental for establishing a protective structure at the end of yeast chromosomes [27]. The long telomeres of higher eukaryotes are mostly organized in tightly spaced nucleosomes [15, 28, 29]. In this review we outline the actual knowledge about the involvement of nucleosomes in telomere structure. We describe the organization of telomeric chromatin in lower and higher eukaryotes, trying to highlight similarities and differences, and the peculiar physicochemical characteristics of telomeric nucleosomes. Finally, we discuss recent findings on the epigenetic status of mammalian telomeres.

Telomeric chromatin in lower eukaryotes

Telomere length has a rough bimodal distribution among eukaryotic species. Telomeres in lower eukaryotes are short, typically less than 1000 bp. In contrast, in higher eukaryotes telomere are several kbp long, with high length variation even in the same individual. These differences in telomere length also seem to correspond to marked differences in the organization of telomeric chromatin. Digestion assays with micrococcal nuclease (MNase), an endonuclease that cleaves preferentially the linker DNA between nucleosomes and is diagnostic of the presence of nucleosomes, have shown that telomeres are organized in a non-nucleosomal chromatin structure in lower eukaryotes such as ciliates [30, 31], slime molds [32, 33], and budding yeast [26]. In contrast, most of telomeric DNA in higher eukaryotes is organized in tightly packaged nucleosomes [15, 28, 29, 34–38].

However, lower and higher eukaryotes have many features in common. A relevant contribution comes from an accurate analysis of telomeric chromatin in *Tetrahymena thermophila.* Despite previous conclusions indicating that the telomeric DNA of *Tetrahymena* macronuclei is packed in a non-nucleosomal DNA-protein complex [30], prolonged digestion with MNase has revealed that 3–10 % of *Tetrahymena* telomeric chromatin is organized in nucleosomes with the same tight packing of vertebrate telomeres [39]. According to the authors, these findings indicate that structural differences between the telomeric chromatin of lower and higher eukaryotes could be quantitative, rather than qualitative. Other correlations come from the study of budding yeast telomeres, which are undoubtedly the best characterized. Even though nucleosomes have not been found on telomeric repeats, they play an essential role in the establishment of the telomeric capping structure in *S. cerevisiae.* Yeast telomeres consist of 250–400 bp of an irregular repeated sequence, $C_{1-3}A/TG_{1-3}$, ending in a short G-rich 3' overhang (Fig. 1A). The protein Cdc13 binds the single-stranded extension [22, 40]. Together with Ten1 and Stn1, Cdc13 forms a complex involved in telomere capping and length regulation [41, 42]. The ends of double-stranded DNA are bound by the Ku complex (Ku70/Ku80) [43], whereas telomere double-stranded repeats are covered by an array of Rap1 proteins [17, 44]. The DNA binding domain of Rap1 interacts with telomeric repeats *via* two homeodomains [45]. Through its C-terminal tail, Rap1 recruits two sets of proteins: the Rif factors (Rif1 and Rif2), which act as negative regulators of telomere length [46], and the Sir complex (Sir2, Sir3, and Sir4), involved in gene silencing at telomeres [47]. Sir proteins connect Rap1 to subtelomeric nucleosomes inducing the formation of a heterochromatic complex. The formation of yeast telomeric heterochromatin is initiated by the binding of Sir4 to Rap1 [47, 48]. Sir4 recruits Sir2 and Sir3, giving rise to the spreading of telomere heterochromatin as a consequence of a cascade of events. Sir2 is a NAD-depend-

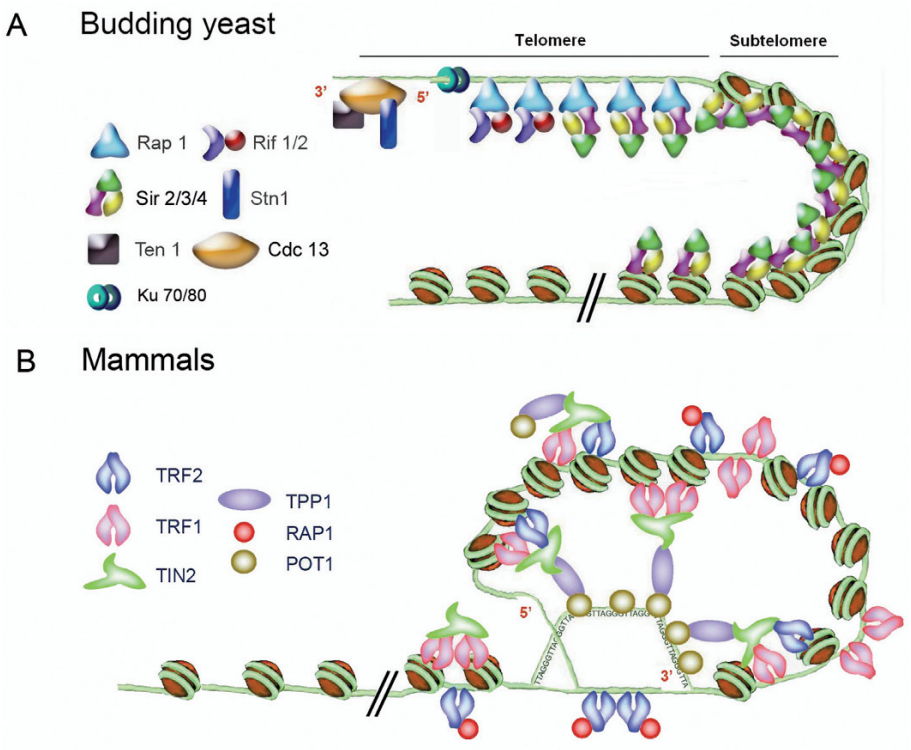

Figure 1. Models of the telomere structure in *S. cerevisiae* and in mammals. (*A*) Model of budding yeast telomere. Duplex telomeric DNA is bound by Rap1 (repressor/activator binding protein), which recruits either Rif1 and Rif2 (Rap1 interacting factors) that negatively regulate telomere length, or the Sir complex (silent information regulator protein) that interacts with subtelomeric nucleosomes and induce the spreading of heterochromatin. The very ends of double-stranded DNA are bound by the heterodimeric Ku complex (Ku70/Ku 80), whereas the single-stranded extension is capped by a complex formed by Cdc 13 (cell division control protein 13), Stn1 (suppressor of Cdc 13) and Ten1 (telomeric pathways in association with Stn1). (*B*) Model of mammalian telomere structure. The single-stranded G-rich overhang folds back and invades upstream double-stranded DNA, forming the so-called t-loop. TRF1 and TRF2 (telomeric repeat binding factor 1 and 2), which bind double-stranded telomeric DNA, and POT1 (protection of telomeres 1), which binds to single-stranded telomeric DNA, recruit TIN2 (TRF1-interacting protein 2), RAP1, and TPP1 (POT1/TIN2 organizing protein) to form the complex termed shelterin. In this model nucleosomes contribute to the establishment of the telomere structure.

ent histone deacetylase [49], and its activity favors the binding of Sir3 and Sir4 to hypoacetylated tails of H3 and H4 [50, 51]. Sir2's main target is lysine 16 of the histone H4 (H4K16), and, to a lesser extent, H3K9, H3K14, and H3K56 [49, 52]. As a consequence of telomeric heterochromatin spreading, expression of genes adjacent to telomeres is inhibited, a phenomenon named telomere position effect (TPE) [53], in which the Ku complex is also involved [54]. Interestingly, several lines of evidence indicate that yeast telomeres exist in a folded structure. Chromatin immunoprecipitation (ChIP) experiments localized Rap1 not only on telomeric repeats but also on subtelomeric chromatin [27, 55]. Moreover, an elegant experiment demonstrated that folding back of the telomere allows an upstream activating sequence (UAS), placed adjacent to a yeast telomere, to activate a gene located 1–2 kbp upstream [56]. Folding back of the telomeres and the association of telomeres with the nuclear periphery [57], both mediated by the Sir complex, represent two events that are thought to stabilize telomeric heterochromatin. However, yeast telomeres appear able to adopt different structures without apparent effects on cell viability. Conditions that disrupt telomere looping eliminate TPE but do not seem to have any effect on chromosome stability [55]. Moreover, an interesting example of the plasticity of yeast telomere structure comes from the realization of mutants in which the telomerase RNA template is modified so that vertebrate telomeric repeats (TTAGGG) are added to chromosome ends instead of the yeast telomeric sequence. Rap1, Rif proteins and the Sir complex do not associate with humanized yeast telomeres, which are bound by Cdc13, Ku80, and by the subtelomeric protein Tbf1, which has affinity for TTAGGG repeats [58, 59]. Despite the TPE being abolished, TTAGGG yeast telomeres are stable and the strains carrying these mutant telomeres have no growth defects [58, 59].

Nucleosomal organization in higher eukaryotes

Telomeres in multicellular eukaryotes have additional levels of complexity due to the different proliferative activity of the diverse tissues. Telomeric DNAs are long, ranging from few kbp as in *Arabidopsis thaliana* [60] to more than 100 kbp in species such as mouse and tobacco [15, 16, 61]. Telomere length maintenance is in most cases linked to telomerase activity. In plants, telomerase activity is high in meristematic cells and low in differentiated tissues [62, 63]. In humans and other mammalian species telomerase is active in germ and stem cells [64]. In most somatic cells, telomeres shorten with every round of DNA replication, until they reach a critical length that leads to cellular senescence [65]. If growth arrest checkpoints are disabled, telomeres continue to shorten, leading to deprotection of chromosome ends and generalized genome instability, and resulting in massive cell death (for a review, see [66]). Occasionally, cells manage to escape crisis by acquiring a mechanism of telomere maintenance, and consequently the ability to proliferate indefinitely. The limitless replicative potential of cancer cells is linked to the establishment of mechanisms of telomere maintenance that, in most cases (85–90 %), consist in telomerase reactivation [67].

In addition to the complexity of the functions associated to the telomere, other elements make it difficult to obtain a satisfactory description of mammalian telomere organization. Telomeric DNA is homogeneous in composition, consisting of TTAGGG repeats, but heterogeneous in dimensions, with large variations among tissues and also among chromosomes of the same cell. Moreover, a plethora of proteins has been found associated with the telomere. An attractive model of the telomere capping structure is the telomeric loop, or t-loop (reviewed in [68]) (Fig. 1B). In this model, derived by electron microscopic analysis of purified telomeric DNA from human and mouse cells [69], the 3' overhang circles back and inserts into the upstream duplex telomeric DNA region displacing the G-rich strand. T-loops have been found also in other eukaryotes, such as in ciliates [70], trypanosome [71], and pea [72]. The t-loop model elegantly explains how chromosome ends can be protected, but it likely represents only one of the structures that telomeres can assume. Another proposed model is the G-quadruplex, a four-stranded structure derived by intramolecular folding of the G-rich single-stranded extension [73]. G-quadruplex structures have been identified *in vivo* in ciliates [74]. Although direct evidence of G-quadruplex structures in mammalian cells is still lacking, the ability to inhibit telomerase of a class of molecules that can induce the formation of G-quadruplex *in vitro* (for a review, see [75]) suggest that this structure may represent one of the possible conformations that telomeres can adopt.

Among the numerous proteins interacting with mammalian telomeres, only three bind specifically to telomeric sequences. The protein POT1 binds to single-stranded G-rich overhangs [23], whereas double-stranded TTAGGG repeats are specifically recognized by TRF1 and TRF2 [19, 20, 76]. TRF1, TRF2, and POT1 recruit RAP1, TIN2 and TPP1 to form the complex named telosome or shelterin [77, 78], essential for telomere protection. An increasing number of factors have been shown to associate with mammalian telomeres. An incomplete list comprises the poly(-ADP-ribose) polymerases Tankyrase 1 and 2, the TRF1-interacting protein Pinx1, the 5' exonuclease Apollo, the ReqQ helicases WRN and BLM, the nuclease XPF1/ERCC1, the DNA repair proteins ATM, ATR, Ku, MRE11/RAD50/NBS1 (for a review, see [79]). Last but not least, the bulk of telomeric DNA in higher eukaryotes is packaged in nucleosomes characterized by an unusual repeat length, about 20–40 bp shorter than bulk nucleosome spacing. This peculiar feature has been found in telomeres of all vertebrate classes [28, 29, 34–36], in sea urchin [35] and in several plant species [15, 37, 38].

In these studies, nucleosomal organization at telomeres has been assayed by MNase digestion followed by southern blotting with a labeled telomeric probe. Due to the sequence uniformity of telomeric DNA and to the heterogeneity of length of the individual telomeres, it is impossible to say whether nucleosome organization is the same along the telomere, or whether the distal and the proximal part of the telomere are differently structured. Based on a more diffused MNase pattern found in short human telomeres, it has been proposed that the terminal part of the telomere is organized in a non-nucleosomal structure [29]. However, different interpretations are possible. Diffuse MNase ladders could derive by an irregular spacing due to the absence of positioning signals [80, 81] and to an uneven binding of histone H1 and/or non-histone proteins. Interestingly, an altered telomeric nucleosomal pattern has been found in Ataxia telangectasia (A-T) cells with respect to normal cells [36]. A-T is a human disease mediated by the protein ATM, which is involved in telomere protection (reviewed in [82]). In A-T cells, the nucleosomal ladder is more diffuse that in normal cells, indicating a less uniform nucleosome spacing [36].

Little is known about the higher order organization of telomeric chromatin. Two models have been proposed that take into account the short repeat length of telomeric nucleosomes. In the first model, telomeric

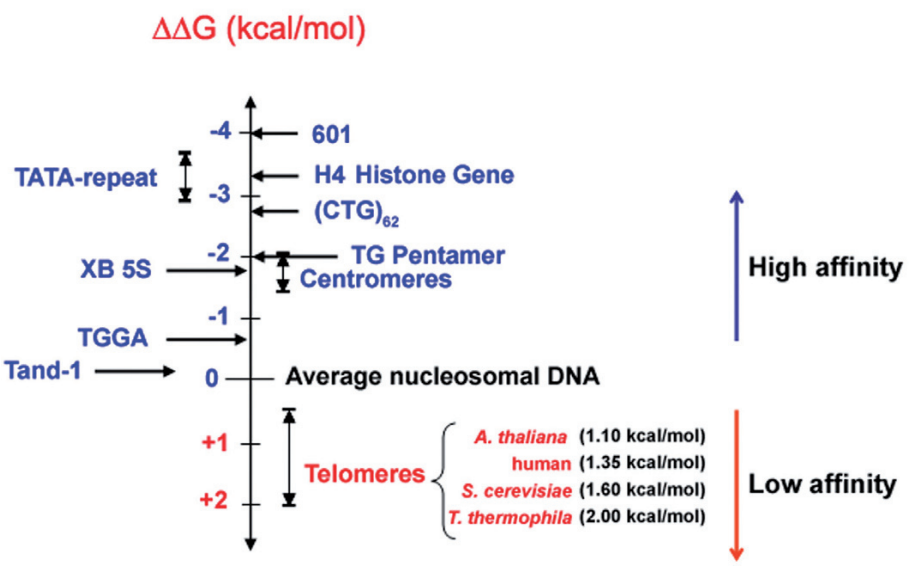

Figure 2. Comparative free energies in nucleosome formation expressed relative to average sequence nucleosomal DNA. Values are derived from the following works: 601 [127]; TATA-repeat [128]; $(CTG)_{62}$ and XB 5S [129]; TG pentamer [97]; centromeres [130]; TGGA [131]; Tand-1 [81]; telomeric sequences [80, 81].

nucleosomes are organized in a columnar chromatin structure while the short DNA linkers follow the same curved path as nucleosomal DNA [83]. The second model proposes a zig-zag structure with a fiber diameter of 30 nm [84]. The presence at telomeres of the histone H1, involved in the establishment of the 30-nm fiber, is controversial. Initially, H1 was not found on rat telomeric chromatin [28]. Subsequently, velocity sedimentation analysis and isolation of telomere nucleoproteins revealed the presence of H1 on rat telomeres [85] but at a lower H1/nucleosome ratio than bulk chromatin. A reduced H1 stoichiometry was found also in *A. thaliana* [86] and in human adult fibroblasts, where H1 at telomeres is nearly fourfold less than on inactive chromatin [87]. The reduced H1 content at telomeres is in agreement with their short nucleosome spacing, since there is a linear relationship between H1 stoichiometry and nucleosome repeat length [88]. It is worth noting that heterochromatic regions such as pericentromeric chromatin show a longer nucleosome repeat length [89] and higher H1 content [88] than telomeres. In contrast, telomere chromatin from chicken erythrocytes shows the same spacing as bulk chromatin and is associated with the linker histones [34].

A relevant contribution comes from the isolation in a native state of telomeric chromatin from mouse and chicken differentiated tissues. Electron microscopy analysis reveals that telomeric chromatin is organized in loops and fold in a 30-nm fiber, like bulk chromatin [90]. It remains to be established whether this feature is specific of highly differentiated tissues or is common to all telomeres.

Peculiar features of telomeric nucleosomes

The nucleosome is the archetype of a DNA-protein complex in which direct recognition of the DNA sequence is not involved [91]. Nucleosome assembly requires that 146 bp of DNA wrap around the histone octamer, forming 1.65 superhelical turns. Interactions between DNA bases and histone proteins are prevalently electrostatic; as a consequence, nucleosomes can form practically on any DNA sequence. Yet, not all the DNA sequences in a genome have the same affinity for the histone octamer. Nucleosome positioning and nucleosome thermodynamic stability are determined by sequence-dependent DNA properties, such as DNA curvature and flexibility [92–94]. Besides, DNA molecules are usually anisotropically flexible, *i.e.*, can bend towards a preferred direction [95]. This property derives from the periodic occurrence in helical phase of short DNA sequences that can accommodate only small conformational variations. In the nucleosome, dinucleotides such as AA/TT are energetically favored in occupying sites where minor groove faces the histone octamer, whereas GC dinucleotides occupy preferentially positions where the minor groove points out [96]. These features affect nucleosome positioning, which can be described by two parameters: translational and rotational positioning. Translational positioning defines the position of DNA with regard to the boundaries of the nucleosome. In particular, it determines which sequences are found in the more accessible linker DNA regions. Rotational positioning describes which bases of DNA are exposed on the nucleosome surface, and is mainly determined by the periodic occurrence of short

sequence motifs in phase with the DNA helical repeat [96, 97].

In most eukaryotes, telomeric repeats (5–8 bp) are out of phase with DNA double helical period. As a consequence, telomeric DNAs are straight and it is reasonable to suppose that also nucleosome assembly is affected [15]. Some structural properties of telomeric nucleosomes have been extensively studied *in vitro*. The free energies of nucleosome formation of several telomeric sequences have been measured by competitive reconstitution, a widely used assay to measure relative affinities of different DNA sequences for the histone octamer. In this assay, a radiolabeled tracer DNA competes for a limited amount of histone octamer with a large excess of heterogeneous DNA. From the amount of nucleosome that forms on the tracer DNA after gradually lowering the ionic strength, it is possible to calculate the free-energy difference of nucleosome formation with respect to a reference DNA sequence [97]. Using this technique, it has been demonstrated that telomeric sequences have the lowest affinities for the histone octamer among the sequences so far tested [80, 81, 98] (Fig. 2). Free-energy values of nucleosome formation for the various telomeric sequences span from 0.80 kcal/mole to 1.80 kcal/mole of nucleosome, relative to average nucleosomal DNA (Fig. 2). The peculiarities of telomeric sequences have relevant consequences also on nucleosome positioning. DNase I and lambda exonuclease footprintings have shown that nucleosomes occupy multiple isoenergetic positions on telomeric sequences, spaced every telomere repeat [80, 81, 98]. These positions are isoenergetic, due to the recurrence along the overall telomeric sequence of the same repetition. These findings have been confirmed by theoretical methods that predict nucleosome positioning from DNA sequence [81, 94]. However, the telomeric DNA folding around the histone octamer is not different from that of bulk nucleosomes, showing the canonical periodicity of about 10.2 bp [80]. Interestingly, free-energy measurements of nucleosome formation after removal of histone terminal domains suggest that telomeric sequences have only minor interactions with histone tails [99].

The lack of positioning signals affects also nucleosome spacing, as shown by *in vitro* measurements of internucleosomal distances [100, 101]. Nucleosomal arrays formed on 800–1500 bp long human telomeric DNA and on multimers of the nucleosome positioning sequences 5S and 601 have been imaged by atomic force microscopy (AFM). Whereas nucleosomes are regularly spaced on 5S and 601 multimeric arrays, nucleosome positioning on human telomeric DNA is random at all degrees of nucleosome saturation. Interestingly, at increasing saturation, the distance between neighboring nucleosomes shortens, reproducing *in vitro* the 157 bp spacing found in cells [100, 101].

The dynamic properties of telomeric nucleosomes are also peculiar and sequence-dependent. Using a restriction enzyme assay and by AFM imaging, it has been demonstrated that human telomeric nucleosomes are highly intrinsically mobile under physiological conditions, whereas nucleosomes assembled on average sequence DNA mostly remain in the original position [102]. A likely explanation resides in the low energy barriers between the possible nucleosome positions along telomeric DNA that could allow thermal-induced movements of the histone octamer [102]. These findings suggest that nucleosomes could slide along telomeric DNA without the aid of nucleosome remodeling complexes.

Interplay between nucleosomes and telomeric proteins: Interaction or competition for telomeric sequences?

In higher eukaryotes, both specific proteins and the histone octamer bind to telomeric repeats. Whether histones and specific telomeric proteins compete for telomeric DNA binding (and hence occupy different telomere domains) or whether they cooperate in the formation of the telomeric complex is a relevant issue in order to understand telomeric structure and its dynamics.

In humans, TRF1 and TRF2 bind as preformed homodimers recognizing two telomeric TTAGGG repeats through their C-terminal Myb/homeodomain-like DNA-binding domain (DBD) [76]. In both proteins the homodimerization domain and the DBD are connected by an unstructured linker that is thought to confer binding flexibility [103]. An analysis of the association of several proteins to telomeres using a ChIP assay, showed that TRF1 binds to 20–30 % of telomeric tracts, whereas TRF2 binds to about 15 % [104]. Since most of telomeric DNA is packed in nucleosomes, a possible hypothesis is that TRF proteins, after saturating the short DNA linkers between nucleosomes, could interact with nucleosomal binding sites [106]. Indeed, yeast Rap1 protein and human TRF1 are able to specifically recognize their binding sites located on nucleosome surface, forming a specific ternary complex [105, 106]. yRap1 binds to the nucleosome with a threefold lower affinity with respect to naked DNA. Noticeably, the binding affinity is sharply higher for a site located near the edge of a nucleosome than for a site close to the nucleosome dyad axis [105]. The estimated affinity of hTRF1 for nucleosomal binding sites is about sixfold

lower than for naked DNA [106]. Strikingly, hTRF1 binding causes a marked alteration in nucleosome structure. Despite this, the hTRF1/nucleosome complexes are stable; no apparent dissociation of histone subunits is evident, even at saturating concentrations of TRF1 [106]. Moreover, binding of TRF1 to naked DNA is favored by the presence of an adjacent nucleosome (T. Ingegnere, A. Galati and S. Cacchione, unpublished results), maybe through non-specific interactions between the acidic N-terminal domain of TRF1 and the basic histone tails. Finally, TRF2 also seem able to recognize nucleosomal binding sites, even if with a lower affinity with respect to TRF1 (A. Galati, M. Savino and S. Cacchione, unpublished results).

These findings suggest that nucleosomes may play a relevant role in the architecture of higher eukaryotes telomeres. It is worth remembering that photobleaching studies have demonstrated that the association of the histone octamer with DNA is highly stable (residence half-time of >120 min for 40 % of H2B and essentially no exchange for H3/H4) [107, 108], whereas TRF1 and TRF2 exchange rapidly at telomeres (residence half-times of about 8 s for TRF1 and 73 % of TRF2, <44 s for the remaining fraction of TRF2) [108]. In this scenario, the contribution of nucleosomes to telomere dynamics could derive more from mobility along the DNA than from the association/dissociation of the histone octamer. Recent results indicate that TRF1 binding induces nucleosome sliding, enhancing the intrinsic mobility of telomeric nucleosomes (S. Pisano, M. Savino and S. Cacchione, unpublished results).

Epigenetics of mammalian telomeres

Until recently little was known about the epigenetic status of mammalian telomeres. Chromosome ends in mammals were generally considered heterochromatic regions, as in yeast and *Drosophila*. This hypothesis was supported by the finding that telomeres in human and mouse cells are able to inhibit transcription of nearby genes, causing the telomere position effect [109–111]. In the last few years, a series of studies identified specific epigenetic marks in mouse telomeric chromatin ([112–117], reviewed in [118]). By ChIP analysis, it has been shown that mouse telomeres are enriched in dimethylated and trimethylated H3K9 [113] and in trimethylated H4K20 [114], two histone post-translational modifications that are typical of heterochromatin. Moreover, both subtelomeric and telomeric chromatin show hypoacetylation of H3 and H4 [116]. The inactivation of the histone methyltransferases (HMTases) SUV39H1 and SUV39H2, responsible for H3K9 trimethylation, cause a reduced binding of HP1 and abnormal elongation of telomeres [113]. RB1, RBL1 and RBL2, proteins of the retinoblastoma family of tumor suppressors, stabilize the trimethylation of H4K20 at telomeres [114]. Their inactivation causes hypomethylation of H4K20 and telomere lengthening [112]. Inactivation of the HMTase SUV4–20 h, responsible for trimethylation of H4K20, causes both telomere lengthening and increase of telomere recombination [117]. In addition, DNA methylation, one of the hallmarks of heterochromatin in mammals, also has a role in reinforcing TPE [111] and in telomere length control [115] in mouse cells. Since the known substrates for mammalian DNA methyltransferases (DNMTs) are CpG sequences, absent in telomeric sequences, DNA methylation likely occurs only on subtelomeric sequences, which are heavily methylated [111, 115]. Inactivation of DNMTs does not alter the levels of histone methylation at telomeres, but causes the increase of telomere length and of telomere recombination [115]. Heterochromatin marks have a strong influence on telomere length, but the reverse is true as well. In telomerase-deficient mice, progressive telomere shortening leads to a decrease of trimethylation of H4K20 and H3K9 at telomeric and subtelomeric regions, and a consequent reduced binding of HP1 [116]. In addition, there is an increase of histone acetylation, a decrease of subtelomeric DNA methylation, and an increase in telomere recombination [116]. All these modifications indicate that short telomeres may assume a more open chromatin state that could represent a signal for the preferential elongation of short telomeres, either by telomerase or by recombinational mechanisms. The relevance of epigenetic status in the regulation of telomere structure and function emerges from the recent characterization of the human histone deacetylase SIRT6 [119]. SIRT6 is a NAD-dependent deacetylase specific for H3K9, and is a member of the Sir2 family. Primary human fibroblasts, in which SIRT6 has been stably knocked down, show premature senescence and increased chromosome end-to-end fusions [119]. Moreover, the presence of SIRT6 at telomeres is required for the association of WRN, a DNA helicase defective in Werner syndrome, a disease characterized by premature aging [120].

Recently, two independent groups reported the transcription of telomeric DNA by RNA polymerase II in human, mouse and zebrafish cells [121, 122], showing that telomeres are not transcriptionally inactive as previously thought. Telomeric RNA is heterogeneous in length (100–9000 bp) and localize at telomeres. It is not clear yet what role telomeric RNAs play at telomeres but various data indicate that they may be

involved in telomere protection. Several effectors of the nonsense-mediated messenger RNA decay, involved in the inactivation of the X-chromosome mediated by Xist RNA [123], are enriched at telomeres and negatively regulate telomeric RNA synthesis [121]. In addition, telomeric RNA levels increase in cells depleted of HMTases [122].

Finally, two recent papers reported the involvement of siRNA in the formation of heterochromatin at telomeres [124, 125]. In the first study, the transient expression of GFP-siRNA in mouse fibroblasts produces a marked increase at telomeres of Argonaute-1, a component of the RNA interference (RNAi) machinery, of TRF1 and of HP1β, whereas levels of TRF2, POT1, HP1α and HP1γ do not change [124]. Moreover, also the levels of trimethylated H3K9 and of telomeric RNAs during GFP-siRNA transfection increase [124]. The second study shows that inhibition of Dicer1, a nuclease involved in RNAi, induces a decrease in DNMTs expression and hence telomere lengthening and increased telomere recombination [125]. A pathway emerges in which the microRNA290 cluster silences the retinoblastoma protein RBL2, which in turn regulates DNMTs expression [125]. These findings add another player to the crowd of molecules involved in telomere regulation.

Conclusions

Thirty years have passed since Elizabeth Blackburn discovered the repeated sequences at the ends of extrachromosomal rDNA in *Tetrahymena* macronuclei [126]. Nowadays, telomeres are an issue of outstanding relevance because of the role they play in chromosome stability and in the transmission of genetic information, and due to their involvement in aging and cancer. The role of nucleosomes in telomere structure and functions, in particular for higher eukaryotes, has been somewhat neglected since scientists focused their efforts mainly on the characterization of specific proteins that act at telomeres. Only recently has the relevance of nucleosomes in fundamental biological processes been acknowledged. It seems likely that also in telomere research the role played by nucleosomes will be much more important than previously believed.

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