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The organization and function of chromosomes

Workshop on Chromosome Structural Elements: from DNA Sequence to Function

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The EMBO Workshop on Chromosome Structural Elements was held in the Villa Mondragone, south of Rome, Italy, between 29 September and 3 October 2005, and was organized by F. Ascenzioni, S. Bacchetti, G. Novelli and M. Savino.

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Introduction

At the end of the summer of 2005, around 60 scientists came together in Rome to discuss various aspects of eukaryotic chromosome biology, including telomeres, centromeres, origins of replication, genome evolution and nuclear organization. Each of these topics is a well-established field with its own specialist meetings and, for the workshop's participants, this was an unusual and welcome opportunity to hear talks outside their own speciality: the centromere and telomere fields rarely meet! In this report, we consider just a few of the broad range of topics discussed.

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The telomere

In its opening session, the meeting focused on the telomere. In most eukaryotes studied (the notable exception being *Drosophila* and other dipterans), telomeres are composed of tandem, simple, G-rich repeats. Telomeres have two key functions. First, the complex of proteins that nucleates on the telomeric DNA maintains its structural stability and ensures that the natural end is distinguished from a double-strand break. Second, they provide a mechanism, which operates in immortal cells, to compensate for the progressive loss of DNA from the chromosome end that occurs as a result of 'conventional' DNA replication processes. The most common 'top-up' mechanism involves the ribonucleoprotein complex, telomerase, which synthesizes telomere repeats *de novo* at the terminus. Data were presented on telomeric DNA structure, the regulation of telomerase activity, and on non-telomeric roles for some proteins that were thought, until recently, to be telomere-specific.

Telomere structure

It has been known since 1989 that G-rich telomeric DNA from ciliates can form G-quadruplex structures (G4) *in vitro*, a process facilitated by the telomere-end-binding protein TEBP-β. Evidence that such structures could form *in vivo*, and thus be biologically relevant, has been elusive. Recently, however, antibodies raised against G4 DNA have been shown to label ciliate nuclei (Schaffitzel *et al*, 2001). H. Lipps (Witten, Germany) showed that RNA-interference-mediated knockdown of either TEBP-β, or its functional partner TEBP-α, in the ciliate *Stylonychia lemnae* prevents the detection of antiparallel, but not parallel, G4 DNA (Paeschke *et al*, 2005). Because the TEBP-α–TEBP-β–DNA complex normally blocks telomerase activity, disruption of the TEBP heterodimer would be required for telomerase-mediated telomere lengthening. Lipps showed that TEBP-β is phosphorylated in S phase, which prevents its association with TEBP-α-bound telomeric DNA and allows unfolding of the G4 DNA (Fig 1). These results show that G4 DNA is involved in telomere biochemistry and, given that G4 DNA-interacting molecules inhibit telomerase and tumour growth, these findings could be relevant to human telomeres.

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Although several proteins that interact with telomeres have been characterized, the role of nucleosomes in human telomeric chromatin is less well understood. S. Cacchione (M. Savino's group, Rome, Italy) showed that telomeric nucleosomes are intrinsically mobile and are less stable than non-telomeric nucleosomes. This seems to be a feature of the hexameric telomere repeat sequence TTAGGG. Furthermore, the binding of telomeric-repeat binding factor 1 (TRF1) and, to a lesser extent, TRF2 is able to remodel telomeric nucleosomes. Cacchione suggested that TRF1-binding induces the movement of nucleosomes and nucleosome–nucleosome interactions.

The DNA sequence of telomeres is generally composed of small G-rich tandem repeats synthesized by telomerase. However, J. Fajkus (Brno, Czech Republic) showed that over long periods of evolutionary time, the sequence of plant telomeres in the order *Asparagales* has switched from the canonical TTTAGGG to TTAGGG in a large phylogenetic group inside *Asparagales*. These were lost in a second switch, during divergence of the *Alliaceae* family, in the Allium ancestor, and not replaced by any detectable telomeric sequences. This might be another example of a eukaryote in which an alternative telomere-lengthening mechanism has evolved (Fajkus *et al*, 2005).

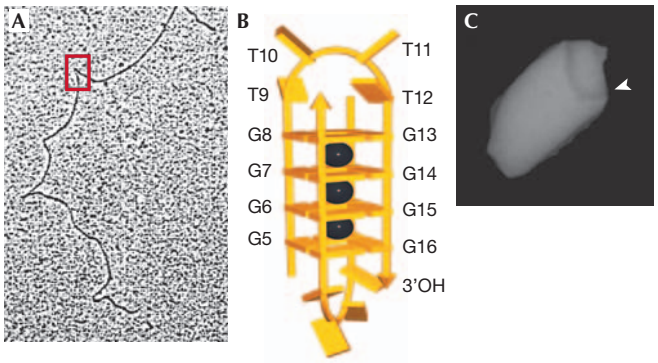

Fig 1 | Telomeric G-quadruplex DNA. (**A**) Telomere interactions (boxed area) as seen by electron microscopy (Lipps, 1980). (**B**) The formation of an antiparallel G-quadruplex (G4) structure from two G-overhangs (yellow). Stabilizing cations are shown as black spheres. (**C**) Staining of a nucleus with the anti-G4 antibody, showing that this structure is resolved during replication. The arrowhead points to the replication band (the zone of DNA replication in hypotrichous ciliate macronuclei). Images courtesy of H.J. Lipps, Witten, Germany.

Telomere-length control

The biochemistry underlying the regulation of telomere length by telomerase is an area of active research. V. Zakian (Princeton, NJ, USA) discussed the negative regulation of telomerase in *Saccharomyces cerevisiae* by Pif1. Her group has shown that Pif1 unwinds DNA:RNA hybrids *in vitro*, specifically when a 5' DNA overhang is present and that the protein binds single-stranded DNA, but not single-stranded RNA. The presence of Pif1 decreased processivity and released telomerase from the DNA. However, the activity of the released enzyme was maintained. Chromatin immunoprecipitation experiments showed that overexpression of Pif1 *in vivo* reduced the amount of bound telomerase, which is consistent with the observed reduction in telomere length. One possibility is that Pif1 regulates telomerase activity by unwinding the short telomerase RNA/telomeric DNA hybrid intermediate (Boule *et al*, 2005).

E. Gilson (Lyon, France) described mechanisms of chromosome-specific telomere-length regulation. The *S. cerevisiae* transcriptional activator Tbf1, which is related to TRF1 and TRF2, interacts with TTAGGG repeats in the subtelomeric region. Tbf1 acts as a *cis*-inhibitor of telomerase when it is located in subtelomeric regions, and when it is part of the telomere in 'humanized' yeast cells that synthesize vertebrate-type telomere repeats. Telomere-length regulation requires a specific transactivation domain of Tbf1. However, the effect does not seem to be mediated by changes in local chromatin structure, or by transcriptional activation. One possibility is that telomerase activity might be influenced by interaction between the telomere and the nuclear envelope. H. Schober (S. Gasser's group, Geneva, Switzerland) described the role of the DNA end-binding Ku70–Ku80 heterodimer in the characteristic tethering of telomeric silent chromatin at the nuclear periphery in yeast cells. The interaction of the RNA component of telomerase with Ku is required for tethering in S-phase, and this activity can be impaired by the mutation of Ku80. Whether telomerase-mediated anchoring at the nuclear periphery has a role in telomere-length maintenance is still to be resolved.

Telomere proteins

Several groups reported the involvement of telomere-associated proteins in telomere-independent functions. P. Kaminker (J. Campisi's group, Novato, CA, USA) has investigated the distribution of telomeres and telomeric proteins during differentiation in human mammary epithelial cells. The growth arrest that is associated with acinar differentiation was accompanied by the reorganization of TRF1-interacting nuclear protein 2 (TIN2) into large domains without telomeres, but with heterochromatin protein 1γ (HP1-γ). Expression of mutant TIN2 alleles prevented formation of these large TIN2 domains and reduced the onset of growth arrest (Kaminker *et al*, 2005). Furthermore, TIN2 has two isoforms: a short form found in both telomeres and in the large, non-telomeric, TIN2 domains, and a longer form that co-localizes only with telomeres and is tightly associated with the nuclear matrix. Other roles for telomere proteins were also discussed by B. Meier (S. Ahmed's group, Chapel Hill, NC, USA), whose data suggest that the *Caenorhabditis elegans* telomerase reverse transcriptase, trt-1, might have a role in modulating the response to DNA damage (Meier *et al*, 2006).

Various data implicate telomere biology in chromosomal synapses and segregation in both meiosis and mitosis. S. Smith (New York, NY, USA) showed that telomeres require a specialized mechanism for mitotic chromosome separation. Knockdown of the poly(ADP-ribose) polymerase Tankyrase 1 by small interfering RNA (siRNA) led to metaphase arrest as a result of a failure in sister telomere separation (Dynek & Smith, 2004). The phenotype could be rescued by a double knockdown of Tankyrase 1 and TIN2. To investigate how TIN2 is involved in sister telomere cohesion, a two-hybrid screen was used to identify interacting proteins. One of these, TPP1 (also known as TINT1, PTOP and PIP1), is another telomere-associated protein involved in length control through its association with TIN2 and protection of telomeres 1 (POT1). Intriguingly, TPP1 was found to localize not only to the telomeres, but also along the length of the mitotic chromosome arm. siRNA knockdown of TPP1 showed

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that structural maintenance of chromosomes 2 (SMC2) was mislocalized and chromosomes remained under-condensed and unable to segregate. S. Bailey (Fort Collins, CO, USA) reported on genes that influence DNA repair and telomere stability. Mouse cell lines deficient in both the Werner syndrome protein (WRN) and telomerase showed markedly elevated levels of telomeric sister chromatid exchange (T-SCE), but not genomic SCE, suggesting that WRN specifically suppresses recombination in telomeres. Unequal recombination in telomere, or telomere-adjacent, sequences provides a mechanism whereby telomere length can be maintained in the absence of telomerase. M. Cerone (S. Bacchetti's group, Quebec, Canada) reported on the creation of an immortal cell line that is able to maintain telomeres, but does not show any known marker of alternative lengthening of telomeres (ALT) (Cerone *et al*, 2005). These data suggest that in the absence of both telomerase and unequal telomere recombination, other mechanisms for telomere-length maintenance might develop.

Origins of DNA replication

To ensure the precise duplication of genetic material at each cell division, DNA replication starts at many sites distributed along eukaryotic chromosomes. In *S. cerevisiae*, replication initiates at specific sequence-dependent sites, but this simple relationship between replication initiation and primary DNA sequence does not seem to exist in other eukaryotes. In the most extreme cases of early embryos of *Drosophila* and *Xenopus*, replication initiation occurs at random sites along chromosomes, and becomes more restricted only around the midblastula transition (Debatisse *et al*, 2004). Even in differentiated somatic cells from mammals—in which DNA replication does not start at random locations—there is some variability in origin use, and the importance of primary DNA sequence in specifying regions as origins of replication is still unclear. S. Riva (Pavia, Italy) described a 1.2-kb DNA segment from the Lamin B2 gene, which shows origin activity when moved to ectopic locations in HeLa cells (Paixao *et al*, 2004). However, dissection of the 1.2-kb Lamin B 'replicator' did not reveal any 'magic' sequence that might be recognized by the origin-recognition complex. M. Debatisse (Paris, France) showed evidence for flexibility in the choice of initiation site for the adenosine monophosphate deaminase 2 (AMPD2) locus. Using molecular combing technology, six initiation sites could be identified in the ~270-kb region (Anglana *et al*, 2003). The availability of nucleotides was also found to influence use of the sites. When pools were high, most initiation events were at a single site—oriGNAI3—and the forks progressed quickly. Conversely, in nucleotide-starved cells, forks travelled more slowly and many initiation sites were frequently used in a single molecule. Once again, comparison of the DNA sequences failed to identify any notable features, suggesting an epigenetic involvement in the regulation of where and when replication is initiated.

Centromeric DNA and artificial chromosomes

The centromere is an essential structural component of the eukaryotic chromosome and is required for faithful segregation in mitosis and meiosis. The term centromere refers to the chromatin on which the kinetochore assembles. The kinetochore, a complex and dynamic structure, provides the interface between the underlying chromosome and the mitotic spindle. In contrast to the telomere, in eukaryotes, the primary DNA sequence at the centromere is not evolutionarily conserved. In eukaryotes with a monocentric chromosome architecture, the primary constriction often encompasses thousands—if not millions—of DNA base pairs, with many types of repetitive DNA sequences present. This makes the identification of functional centromeric DNA difficult. In humans, α-satellite DNA is the only DNA sequence present at all normal endogenous centromeres. Experiments to test the competence of α-satellite DNA in *de novo* centromere formation have shown that large blocks of this DNA, with a higher-order repeat structure and centromere protein B (CENP-B)-binding sites, are able to form minichromosomes carrying *de novo* centromeres in cultured human cells. This confirms the long-held assumption that α-satellite DNA is the human centromeric DNA. H. Masumoto (Bethesda, MD, USA) presented evidence that α-satellite DNA, which is restricted to primate centromeres, can mediate *de novo* centromere formation in cultured mouse cells—although with lower efficiency. Furthermore, Masumoto and W. Earnshaw (Edinburgh, UK) reported the generation of artificial chromosomes carrying a conditional centromere. A dimer of alphoid DNA, consisting of one α-satellite monomer with a CENP-B-binding site, and another monomer in which the CENP-B box had been replaced by a tetracycline operator (tetO) sequence, was multimerized and then expanded by rolling-circle amplification. The 5–10-kb fragments produced were then cloned by transformation-associated recombination in yeast cells, producing an array of 50 kb (work done by V. Noskov, in the V. Larionov group, Bethesda, MD, USA). When introduced into human HT1080 cells, this long synthetic alphoid array generated artificial chromosomes that bound normal kinetochore proteins and could be visualized by expressing a red fluorescent protein (RFP):tetR fusion protein in the host cells. Binding of a tetR:VP16 fusion protein to the *de novo* structure resulted in destabilization and chromosome loss. Potentially, this could provide an exciting tool for investigating human kinetochore assembly and disassembly, an approach that until now has been available only in budding yeast.

Centromeric chromatin

It is now clear that, along with information encoded by the primary DNA sequence, epigenetics also has a key role at the centromere, although this interaction is far from being clearly understood. A characteristic of all eukaryotic centromeres is the presence of the histone H3 variant CENP-A. The CENP-A chromatin, on which most other kinetochore proteins are dependent for their localization, is embedded in heterochromatin. Using three-dimensional microscopy of the interphase human nucleus, M. Cremer (Munich, Germany) reported an overlap between centromere domains and chromatin that has been trimethylated on lysine 9 of histone H3 (H3K9triMe) and H4K20triMe, both of which are marks of a repressive chromatin state (Zinner *et al*, 2006). Using immunofluorescence on chromatin fibres, B. Sullivan (Durham, NC, USA) has explored the organization of the different types of chromatin in the centromere domain. It has been known for some time that, at human centromeres, only a subset of the extensive arrays of α-satellite DNA is typically associated with CENP-A. Sullivan and colleagues have shown that, even in the CENP-A chromatin domain, nucleosomes containing CENP-A occur in blocks interspersed with H3 nucleosomes (Blower *et al*, 2002). Extending these analyses using antibodies raised against specific modifications of

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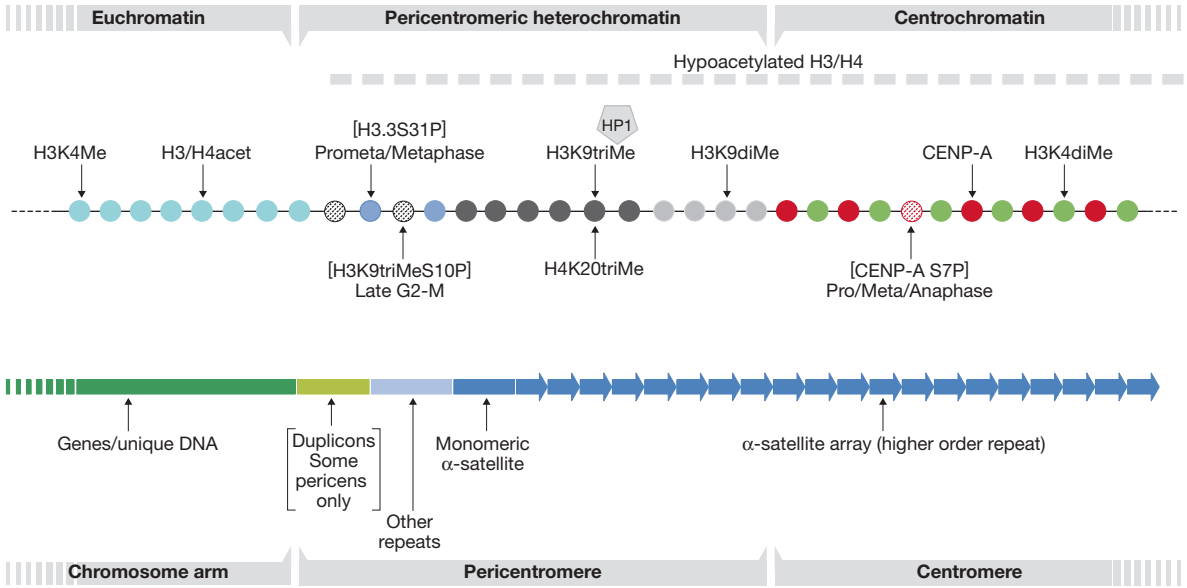

Fig 2 | The two-dimensional organization of human centromeric chromatin (one side of the centromere domain). The organization of the primary DNA sequence relative to the various chromatin domains is indicated. Some histone modifications are present at specific stages of the cell cycle. This schematic is a simplified representation of current findings (Zeitlin *et al*, 2000; Blower *et al*, 2002; Schotta *et al*, 2004; Sullivan & Karpen, 2004; Fischle *et al*, 2005; Hake *et al*, 2005; Hirota *et al*, 2005; Lam *et al*, 2005; Zinner *et al*, 2006). The relative distribution and importance of many of these variants and modifications at human centromeres remains to be established. CENP-A, centromere protein A; HP1, heterochromatin protein 1.

histone H3 or H4, a picture is now emerging of a much more complex chromatin domain organization than has previously been suspected (Sullivan & Karpen, 2004). In interphase, the blocks of histone H3 nucleosomes that lie between spots of CENP-A chromatin show histone hypoacetylation (typical of heterochromatin) and dimethylation of K4 (a modification previously identified in open chromatin), but lack H3K4triMe, H3K9diMe and H3K9triMe. In the regions immediately flanking the CENP-A domain the chromatin contains H3K9diMe, whereas H3K9triMe is located further out in the flanking pericentromeric chromatin. This indicates that, in addition to the presence of CENP-A, centromeric chromatin contains a distinct combination of histone modifications (Fig 2). Sullivan showed that slight overexpression of CENP-A in human cells causes the CENP-A chromatin to expand, replacing H3K9diMe in the adjacent domain. Furthermore, heterochromatin depletion, by knockdown of histone methyltransferase activity or inactivation of the retinoblastoma protein Rb, also allows the CENP-A chromatin to spread more extensively over the α-satellite DNA. This work raises interesting questions as to what mechanisms maintain these distinct chromatin domains, whether boundaries exist between the CENP-A chromatin and the flanking pericentromeric chromatin, and whether species differ in the organization of their centromeric chromatin domains.

Centromeres in evolution

The evolution of centromere plasticity has now been revealed by molecular cytogenetic studies. M. Rocchi (Bari, Italy) described evidence for the repositioning of centromeres during primate evolution, and the possibility that this could be explained by the appearance of neocentromeres in euchromatic regions (Ventura *et al*, 2004). These studies are now being extended into other mammals. He also described the frequent association of pericentromeric regions in the primate genome with extensive blocks of segmental duplication. In a few instances, clusters of duplications have been found in regions in which an ancestral centromere was located. Why pericentromeric chromosome regions should act as sinks for the accumulation and further mobilization of duplicated sequences remains a mystery.

Closing remarks

Many aspects of chromosome structure, how they function, their positions in the interphase nucleus and how (hinted at by the telltale footprints left in the genome sequence) they have evolved, remain to be explored and explained. The elegant Villa Mondragone, bathed in warm autumn sunshine and offering panoramic views of Rome, provided an enjoyable forum in which to explore these diverse topics.

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