

Human telomeres are attached to the nuclear matrix

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This report shows that human telomeres are tightly associated with the nuclear matrix. Telomere attachment is observed in several cell types and in all stages of interphase. Mapping experiments show that telomeres are anchored via their TTAGGG repeats; a subtelomeric repeat located immediately proximal to the telomeric TTAGGG repeats is quantitatively released from the nuclear matrix by restriction endonuclease cleavage. TTAGGG repeats introduced at chromosome-internal sites by DNA transfection do not behave as matrix attached loci, suggesting that the telomeric position of the repeats is required for their interaction with the nuclear matrix. These findings are consistent with the idea that telomeres function as a nucleoprotein complex.

Key words: human chromosomes/nuclear matrix/telomere

Introduction

Eukaryotic telomeres are thought to form nucleoprotein complexes that protect chromosome ends from degradation, fusion and recombination. Telomeric DNA is composed of short tandem repeats that are usually G-rich in the strand that extends to the 3' end of the chromosome (for reviews see Zakian, 1989; Blackburn, 1991). The precise structure of the extreme end of the telomere is not known. In several species the G-strand protrudes 3' with two to three single stranded repeats (Klobutcher *et al.*, 1981; Pluta *et al.*, 1982; Henderson and Blackburn, 1989). The single stranded G-rich repeats can fold due to G–G (including Hoogsteen) interactions (Henderson *et al.*, 1987; Sen and Gilbert, 1988; Sundquist and Klug, 1989; Williamson *et al.*, 1989), but the existence of folded structures *in vivo* has not been demonstrated. Telomeric repeats are maintained by telomerase, a telomere-specific RNA-dependent DNA polymerase (for review see Blackburn, 1990). This enzyme is a ribonucleoprotein complex with a species-specific internal RNA template for the addition of the appropriate G-rich telomeric repeats to the 3' ends of the chromosome (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990; Yu *et al.*, 1990). Telomerase is thought to compensate for the inability of the general cellular replication machinery to replicate linear DNA ends.

The ends of human chromosomes contain 2–30 kb of the telomeric repeat TTAGGG (Moyzis *et al.*, 1988; Allshire *et al.*, 1988; de Lange *et al.*, 1990; Hanish and de Lange, unpublished observations). Three lines of evidence argue that the TTAGGG repeats are essential and sufficient for telomere function. First, the TTAGGG repeats are the only component of human chromosome ends known to be conserved during

vertebrate evolution (Meyne *et al.*, 1989). Second, human cells express a telomerase that adds TTAGGG repeats (Morin, 1989). Third, TTAGGG repeats are sufficient to heal broken chromosome ends into functional telomeres (Wilkie *et al.*, 1990). In addition to TTAGGG repeats human chromosome ends carry subtelomeric repetitive elements, some of which harbor sequences similar to TTAGGG repeats (Cheng *et al.*, 1989; Allshire *et al.*, 1989; de Lange *et al.*, 1990; Brown *et al.*, 1990). Subtelomeric DNA sequences are identical or similar at different chromosome ends but, unlike the TTAGGG repeats, none of the subtelomeric repetitive elements are associated with all telomeres and their DNA sequence is not conserved in mammals (Cheng *et al.*, 1989; Brown *et al.*, 1990; de Lange *et al.*, 1990). Functional interactions between telomeric DNA and other components of interphase nuclei are therefore expected to occur primarily in the region of the TTAGGG repeats.

Telomere function is thought to involve proteins that interact with telomeric repeats. In *Oxytricha* and *Euplotes*, the protruding G-rich repeats are bound by a telomere capping complex which protects telomeres from ligation and exonucleolytic attack *in vitro* (Lipps *et al.*, 1982; Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Price and Cech, 1987, 1989; Raghuraman *et al.*, 1989; Price, 1990). Telomere capping complexes have not been identified in other organisms, but genetic and biochemical experiments suggest that the telomeres of *Saccharomyces cerevisiae* interact with the repressor/activator protein RAP1 (Berman *et al.*, 1986; Buchman *et al.*, 1988; Longtine *et al.*, 1989; Conrad *et al.*, 1990; Lustig *et al.*, 1990). The function of the RAP1–telomere association is not known.

A potentially important interaction at telomeres is suggested by the position held by chromosome ends in interphase nuclei. Rabl (1885) first observed that the ends of prophase chromosomes have a peripheral position at one end of the nucleus. This topography would be expected if chromosomes maintain their telophase configuration in interphase. In agreement with this model, prematurely condensed chromosomes from muntjac, hamster and *Drosophila* cells often show the telophase orientation (Sperling and Ludtke, 1981; Cremer *et al.*, 1982; Foe and Alberts, 1985). Furthermore, direct analysis of chromosome topography by three-dimensional microscopy indicates that the ends of interphase chromosomes are close to the nuclear envelope in a wide variety of eukaryotes (Agard and Sedat, 1983; Mathog *et al.*, 1984; Hochstrasser *et al.*, 1986; Mathog and Sedat, 1989; van Dekken *et al.*, 1989; Chung *et al.*, 1990; Rawlins *et al.*, 1991). These cytological data suggest that telomeres could be attached to the nuclear envelope. However, direct evidence for this idea is not available.

In this report the subnuclear localization of telomeric DNA is examined by nuclear fractionation. The approach taken here employs the lithium diiodosalicylate (LIS) procedure (Mirkovitch *et al.*, 1984). LIS treatment of isolated nuclei

removes all histones in addition to other nuclear factors. The resulting 'halo' structures contain naked chromosomal DNA and an insoluble nuclear remnant (the nuclear matrix) that includes the nuclear envelope and internal proteins. While most of the halo DNA can be solubilized by restriction endonuclease cleavage, a minority of the restriction fragments remain attached to the nuclear matrix. In flies, mammals and yeast, nuclear matrix associated DNA has been shown to contain specific DNA sequences (matrix attachment regions or MARs) (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Amati and Gasser, 1988; see Gasser *et al.*, 1989 for review). The attachment is thought to reflect sequence-specific interactions between MAR DNA and unidentified non-histone proteins that are insoluble in the LIS extraction procedure. Using this technique it is demonstrated that human telomeres are tightly associated with the nuclear matrix. The results indicate that human telomeres form a nucleoprotein complex with non-histone proteins and are compatible with the suggestion that telomeres are attached to the nuclear envelope.

Results

Telomeric DNA is attached to the nuclear matrix

Sequence-specific associations of chromosome-internal DNA elements with the nuclear matrix have been observed previously in nuclei that were treated with LIS (see Gasser *et al.*, 1989 for review). In the experiment in Figure 1, this technique is used to examine the interaction of telomeres with the nuclear matrix of human cells. DNA in LIS-treated nuclei (halos) from three human cell lines was cleaved with *SryI* to disconnect sequences that are not attached to the nuclear matrix. The resulting fractions of solubilized and nuclear matrix-associated *SryI* fragments are analyzed by gel electrophoresis alongside corresponding amounts of total DNA digested with *SryI*. The ethidium bromide staining pattern of these DNAs (Figure 1a) shows that in all three cell lines *SryI* cleavage separates most of the chromosomal DNA from the nuclear matrix.

To determine the fate of telomeric DNA in these experiments, a double stranded DNA probe containing ~130 tandemly arranged copies of the vertebrate telomeric repeat TTAGGG was used to detect human telomeres. Because long arrays of TTAGGG repeats are confined to chromosome ends in the human genome, this probe specifically detects telomere-derived restriction fragments (Moyzis *et al.*, 1988; Allshire *et al.*, 1989; Cross *et al.*, 1989; de Lange *et al.*, 1990). In most DNA preparations TTAGGG repeat probes hybridize to heterogeneously sized restriction fragments with an average molecular weight that varies in different cell lines and tissues. Variation in the length of telomeric restriction fragments has several causes. First, the physical map of subtelomeric DNA is variable (Brown *et al.*, 1990; de Lange *et al.*, 1990). Second, the telomeric repeat array at each chromosome end is heterogeneous (de Lange *et al.*, 1990). Finally, all human chromosome ends studied so far lose telomeric repeats during development and cellular proliferation (Cooke and Smith, 1986; Allshire *et al.*, 1988; de Lange *et al.*, 1990; Harley *et al.*, 1990; Hastie *et al.*, 1990). As a consequence of these properties, telomeric restriction fragments are readily identified in genomic blots. In Figure 1b, telomeric *SryI* fragments are visualized by hybridization to TTAGGG repeats. The telomeric repeat arrays in two of the cell lines (HL-60 and Raji) are fairly

short, yielding a broad zone of hybridization at ~6 kb. By contrast, the chromosomes of the HeLa cells used in this experiment end in a much longer telomeric repeat array (de Lange *et al.*, 1990) and yield *SryI* fragments that migrate at limiting mobility. Fractionation of *SryI*-digested halo DNA into released and nuclear matrix-associated restriction fragments shows that the majority of the telomeric *SryI* fragments are recovered in the attached DNA fraction. Densitometric quantification indicates that in each case at least 80% of the telomeric DNA is associated with the nuclear matrix. Because only ~5% of the total DNA was recovered as attached fragments in these experiments, telomeric DNA is highly enriched in nuclear matrix fractions.

In these experiments, isolation and digestion of halos involved incubations at 37°C, which potentially could give rise to heat induced artifacts (Evan and Hancock, 1987). For this reason telomere attachment was also examined in HeLa halos that were isolated at 4°C and digested with *PstI* at room temperature (20°C). This procedure gave identical results to the standard method performed in parallel (data not shown).

Telomeres are anchored via the TTAGGG repeats

Previously, we have described the structure of a subset of chromosome ends in the human genome that carry a similar telomere proximal sequence (de Lange *et al.*, 1990). These chromosome ends have well defined and nearly identical restriction maps (Figure 2b). The position of the nuclear matrix attachment site in these telomeres was mapped with a probe that contains part of the subtelomeric repeat (pTH2Δ). In *BglII*-digested DNA, pTH2Δ detects telomeric fragments that are readily identified based on their heterogeneous nature and variable average size. In Figure 2, two HeLa cell lines with different telomere length are analyzed. The adherent HeLa cells (HeLa I) have very long

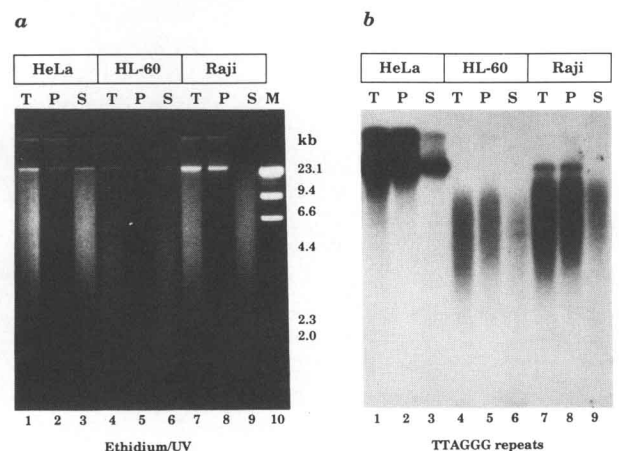


Fig. 1. Telomeres are attached to the nuclear matrix. Halo preparations from HeLa cells (lanes 1–3), HL-60 cells (lanes 4–6) and Raji cells (lanes 7–9) were digested with *SryI* and centrifuged to separate unattached (S, lanes 3, 6 and 9) and nuclear matrix-associated (P, lanes 2, 5 and 8) sequences. The samples were size-fractionated next to the corresponding *SryI*-digested total DNA samples (T, lanes 1, 4 and 7). The amount of DNA loaded from total, pellet and supernatant fractions of each experiment represents equivalent numbers of nuclei. **Panel a** shows the ethidium bromide/UV staining pattern of these DNAs. **Panel b** shows the hybridization pattern of a TTAGGG repeat probe to a Southern transfer of the gel shown in panel a. Lane 10 in panel a contains bacteriophage lambda DNA digested with *HindIII*.

telomeres that yield *Bgl*III fragments at the exclusion position of this agarose gel. By contrast, the telomeres of the non-adherent HeLa cells (HeLa II) contain a shorter array of telomeric repeats, resulting in a broad zone of hybridizing *Bgl*III fragments with an average size of ~6 kb. In addition, the subtelomeric repeat probe detects several chromosome-internal loci, which yield discrete bands of identical molecular weight in the two HeLa cell lines. Figure 2a (lanes 1–6) shows that the telomeric *Bgl*III fragments, but not the chromosome-internal fragments, are attached to the nuclear matrix. This maps the anchored telomeric DNA distal to the *Bgl*III site (Figure 2b).

Most chromosome ends that hybridize to pTH2Δ have a *Sty*I site at the boundary between the subtelomeric repeat and the telomeric repeat region and yield fragments of ~4 kb that contain the subtelomeric repeat but probably no TTAGGG repeats. The experiment in Figure 2a (lanes 7–12) shows that these subtelomeric fragments are released into the soluble fraction, as are several chromosome-internal fragments. A few chromosome ends lack the terminal *Sty*I site that delineates the transition between the telomeric repeat array and the subtelomeric element. Therefore, these chromosome ends yield telomeric *Sty*I fragments that are similar to the telomeric *Bgl*III fragments discussed above. As expected from the results with *Bgl*III-digested halos, these telomeric *Sty*I fragments are associated with the nuclear matrix (Figure 2a, lanes 7–12). These results, as well as additional mapping experiments with *Eco*RI, *Ava*II, *Pst*I and *Pvu*II (not shown), indicate that the subtelomeric repeat lacks attachment sites. Therefore chromosome ends are anchored

by a site(s) distal to the subtelomeric repeat in the region thought to be composed predominantly of TTAGGG repeats.

Nuclear matrix association of telomeric DNA was observed in all other cell types that were examined: normal peripheral blood lymphocytes, two histiocytic lymphoma cell lines and HL-60 cells induced to differentiate with dimethylsulfoxide (data not shown). In each case the nuclear matrix attachment mapped to the TTAGGG repeat region.

Densitometric quantification of the results in Figures 1 and 2 and other similar experiments indicate that 80–95% of the telomeric restriction fragments are attached. This result is reproducible and does not depend on the cell type, the length of the telomeres or the restriction enzyme used to cleave the halo DNA. By contrast, the extent to which other sequences are attached to the nuclear matrix, as determined by Hoechst fluorescence of pellet and supernatant fractions, was found to be somewhat variable. For example, after *Bgl*III digestion of HeLa halos the nuclear matrix retained ~5% of the DNA in the experiment shown in Figure 3, whereas in a similar experiment (Figure 4) 20% of the *Bgl*III-digested DNA remained attached. Yet in both experiments 80–95% of the telomeric restriction fragments are associated with the nuclear matrix. As a result, the enrichment factor of telomeres in nuclear matrix fractions varies between 4- and 20-fold.

To characterize the nature and stability of telomere anchorage, nuclear remnants of *Bgl*III-digested HeLa cell halos were incubated separately with each of the following agents: 0.1% Triton X-100, 2 M NaCl, 10 mM β-mercaptoethanol, 10 mM *o*-phenanthroline, 10 mM EDTA,

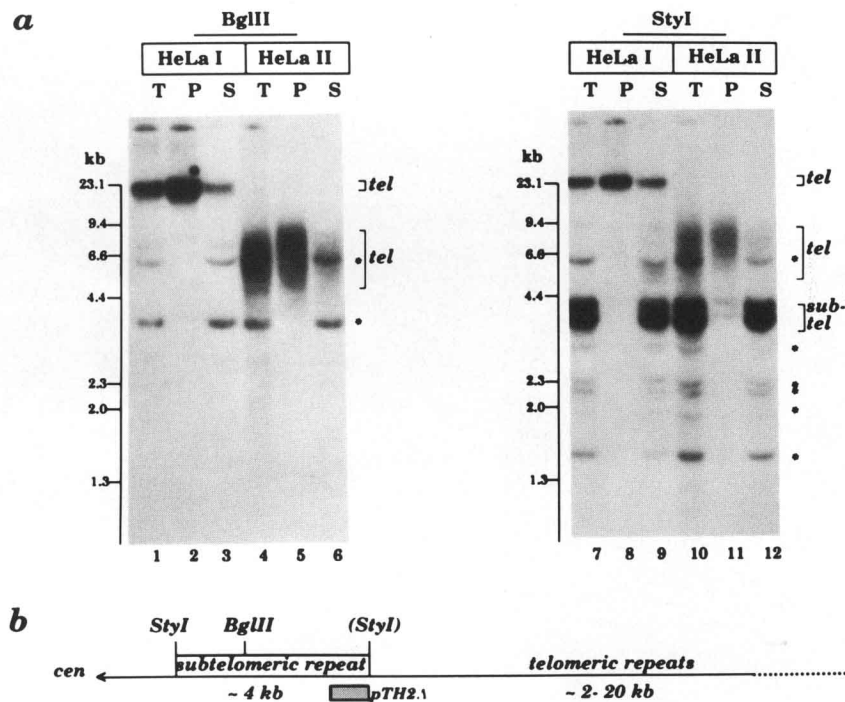


Fig. 2. Telomere anchorage maps to the TTAGGG repeat region. Halos derived from an adherent HeLa cell line with long telomeres (HeLa I, lanes 1–3 and 7–9) and a non-adherent HeLa cell line with short telomeres (HeLa II, lanes 4–6 and 10–12) were digested with either *Bgl*III (lanes 1–6) or *Sty*I (lanes 7–12) and fractionated into attached (P, lanes 2, 5, 8 and 11) and unattached (S, lanes 3, 6, 9 and 12) sequences. Proportional amounts of these DNAs (representing identical numbers of nuclei) were size-fractionated next to the corresponding total DNAs (T, lanes 1, 4, 7 and 10) and hybridized to a probe for a human subtelomeric repeat (pTH2Δ, de Lange *et al.*, 1990). The position of telomeric (tel), subtelomeric (subtel) and chromosome-internal (*) restriction fragments are indicated on the right side of the autoradiographs in **panel a**. In **panel b**, the physical map of the telomeres that are detected in panel a is presented (from de Lange *et al.*, 1990); the positions of the subtelomeric repeat, the telomeric (TTAGGG) repeat region, and the probe (pTH2Δ) are indicated. See text for discussion of the length variation at telomeres. The most terminal *Sty*I site is drawn in parentheses to indicate its absence from some of the telomeres detected in panel a.

10 mM EGTA, 20 μ g RNase A per ml, 50 μ g proteinase K per ml and 0.1% SDS. The stability of the nuclear matrix association of telomeres under these conditions was analyzed with pTH2 Δ as a probe. With the exception of proteolytic degradation and solubilization with SDS, none of these treatments released the telomeres or other attached DNA fragments (Figure 3 and data not shown). In addition, incubation at 4°C with 1% Triton X-100 followed by 1 M NaCl did not release telomeres from the nuclear matrix (Figure 3, lanes 8–11). These data are consistent with attachment of telomeres to a proteinaceous component of the nuclear matrix. The attachment is probably not mediated by a covalently linked protein since telomeric DNA can be recovered quantitatively by extraction of nuclear matrix-associated DNA with phenol without prior treatment with proteinase K (data not shown).

Telomeres are anchored in G₁, S and G₂ phase

Since unsynchronized cell populations were used in the experiments described above, it remained possible that the minor fraction of telomeres that are released from the nuclear matrix derive from cells in a particular stage of the cell cycle. To address this question, G₁, S and G₂ phase HeLa cells were isolated by centrifugal elutriation. The DNA content of the isolated fractions and the unsynchronized starting population was assessed by flow cytometry (Figure 4a). The association of telomeres with the nuclear matrix was determined using *Bgl*III to cleave the halo DNA and a subtelomeric repeat probe (pTH2 Δ) to detect telomeric fragments. A representative of three independent experiments shown in Figure 4 demonstrates that telomeres from G₁, S and G₂ phase cells are attached to the nuclear matrix to the same extent. Thus, there is no indication that the association

of human telomeres with the nuclear matrix changes during interphase. Although the G₂ fraction probably contains mitotic cells, the question of whether telomeres detach during M phase cannot be answered by this procedure.

Integrated transfected TTAGGG repeat arrays are not attached to the nuclear matrix

It seemed important to know whether the telomeric position of the TTAGGG repeats is a requirement for association with the nuclear matrix. To address this question, HeLa cells were transfected with a construct in which two arrays of TTAGGG repeats enclose a 4.4 kb *Cla*I fragment from bacteriophage lambda, which serves as a molecular tag (see Figure 5a). This DNA was co-transfected with a neomycin resistance marker (pMC1neo poly A) and a pool of ~200 G418 resistant colonies was amplified. Halo structures from these cells were digested with either *Bgl*III or *Eco*RI and fractionated into solubilized and attached sequences. Both enzymes yield fragments composed of λ DNA and one of the TTAGGG repeat arrays (Figure 5a). Hybridization of the 4.4 kb λ *Cla*I fragment in Figure 5c shows that the *Eco*RI and *Bgl*III fragments are not attached to the nuclear matrix. The integrity of the fragments in Figure 5c was verified by *Bgl*III + *Cla*I and *Eco*RI + *Cla*I double digestion. In both cases *Cla*I shortened the detected fragments by ~800 bp as expected if the *Bgl*III and *Eco*RI fragments each contain an 800 bp TTAGGG repeat array (not shown).

The successful fractionation of nuclear matrix attached DNA in these experiments is suggested by the ethidium bromide staining pattern of bulk DNA shown in Figure 5b. To verify this point further, two control experiments were performed. First, hybridization of a TTAGGG repeat probe to these DNA fractions showed that the *Bgl*III and *Eco*RI

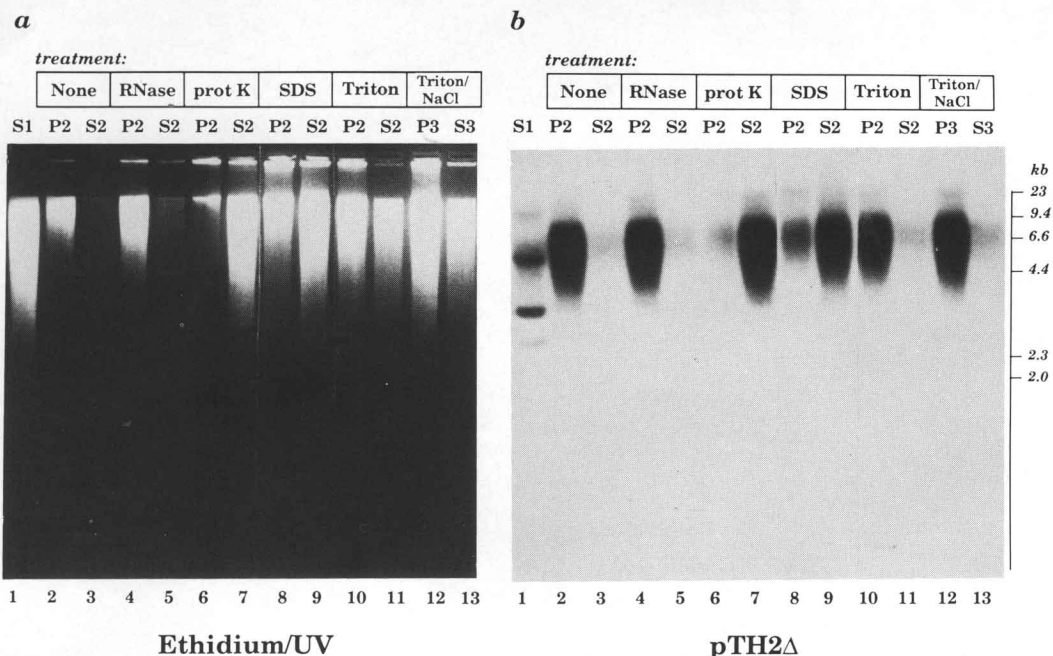


Fig. 3. Biochemical characterization of telomere–nuclear matrix association. *Bgl*III-digested HeLa halos, from which the unattached DNA fragments (fraction S1, lane 1) was removed, were incubated separately with each of the agents listed below and subsequently centrifuged to separate released *Bgl*III fragments (fraction S2) from the DNA that remained attached to the nuclear matrix (fraction P2). Lanes 2 and 3, control incubation; lanes 4 and 5, RNase A; lanes 6 and 7, proteinase K; lanes 8 and 9, SDS; lanes 10 and 11, 1% Triton X-100; lanes 12 and 13, 1% Triton X-100, followed by 1 M NaCl resulting in tertiary pellet and supernatant fractions P3 and S3 (see Materials and methods for experimental details). Equal proportions of each fraction (representing the same number of nuclei) were electrophoretically fractionated (**panel a**) and hybridized to pTH2 Δ to detect telomeric fragments (**panel b**).

fragments from the HeLa chromosome ends were preferentially recovered in the attached DNA fraction, as expected (not shown). Second, hybridization of the same DNAs to a *neo* probe showed that several *neo*-containing *Bgl*II and *Eco*RI fragments specifically fractionated with the nuclear matrix (Figure 5d). The precise composition of the nuclear matrix attached sequence within these DNAs was not investigated further.

These data indicate that a chromosome-internal array of ~130 TTAGGG repeats does not become attached to the nuclear matrix. Since many previously described matrix attachment regions are much shorter than 800 bp (Gasser *et al.*, 1989), this result is probably due to a functional difference between chromosome-internal and telomeric TTAGGG repeats rather than an effect of the length of the TTAGGG repeat array.

Discussion

Unlike random DNA ends, the natural termini of chromosomes are resistant to exonucleolytic degradation, ligation and recombination. This stability is the consequence of telomeric DNA, which is hypothesized to stabilize

chromosome ends by forming a protective nucleoprotein complex. As part of a search for factors that interact with mammalian telomeres, this report demonstrates that telomeres are tethered to the nuclear matrix of human cells. The nuclear matrix is an operationally defined fraction of the nucleus that includes the nuclear envelope and numerous other nuclear proteins. The retention of human telomeres in this structure is most likely due to the interaction of human telomeric DNA with a non-histone protein in the nuclear matrix. Thus, the data provide evidence for the existence of a nucleoprotein complex at human chromosome ends.

The most compelling argument that telomere attachment to the nuclear matrix reflects an association present *in vivo*, derives from cytological studies of chromosome topography. Prematurely condensed G₁ and G₂ chromosomes from mammalian cells are in the telophase orientation with their ends positioned at the periphery of the nucleus (Sperling and Ludtke, 1981; Cremer *et al.*, 1982). *In situ* hybridization

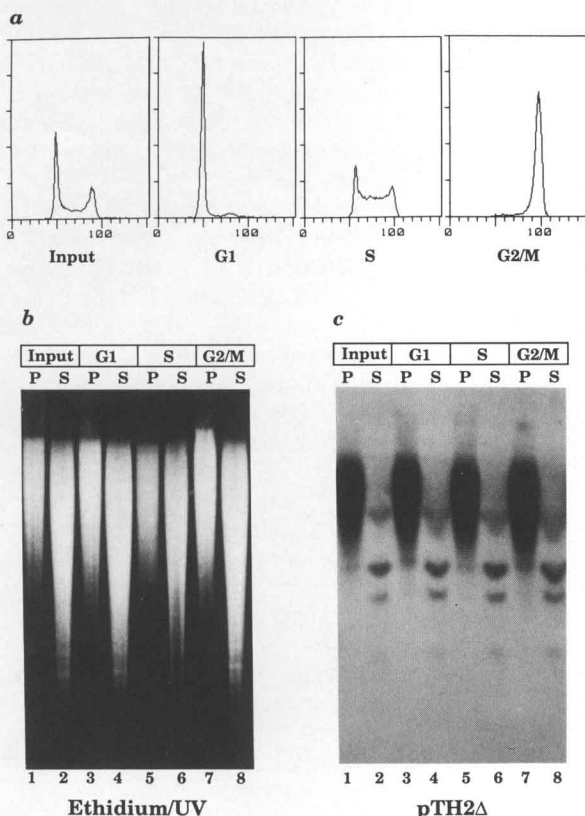


Fig. 4. Telomeres are attached throughout interphase. **Panel a** shows the DNA content of G₁, S and G₂/M-phase cells that were isolated by elutriation of an unsynchronized population of non-adherent HeLa cells (indicated by 'input'). The gel shown in **panel b**, contains proportional amounts of unattached (S, lanes 2, 4, 6 and 8) and nuclear matrix-associated (P, lanes 1, 3, 5 and 7) DNA fragments obtained by *Bgl*II cleavage of halos derived from the cell populations shown in panel a (lanes 1 and 2, input; lanes 3 and 4, G₁; lanes 5 and 6, S; lanes 7 and 8, G₂/M). In **panel c**, telomeric DNA fragments are identified in a Southern transfer of the gel shown in panel b by hybridization to a probe for the subtelomeric repeat (pTH2Δ, see Figure 2).

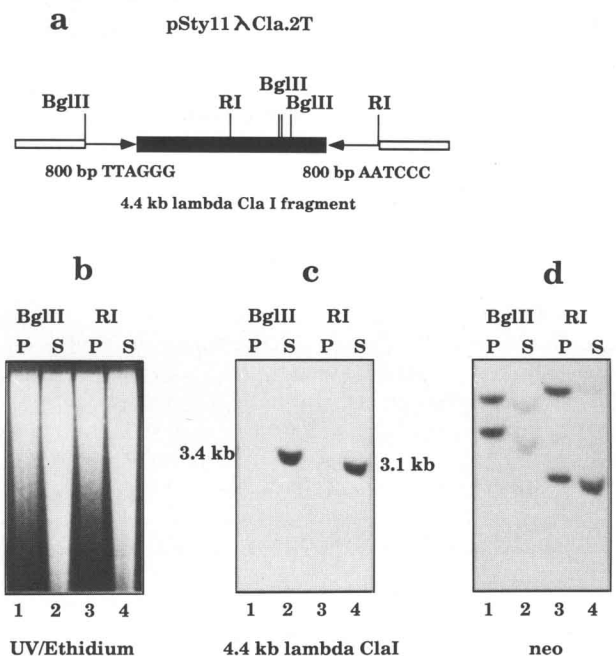


Fig. 5. Chromosome-internal TTAGGG repeats in transfected HeLa cells are not associated with the nuclear matrix. **Panel a** shows the construct pSty11.λCla.2T which was inserted in the genome of HeLa cells by co-transfection with pMC1neo poly A (Stratagene) and selection for G418 resistant colonies. The pSty11.λCla.2T plasmid is a pSP73 (open boxes) derivative containing a 4.4 kb *Cla*I fragment from bacteriophage lambda (black box) flanked at either side by a 800 bp TTAGGG repeat array (arrows). In a pool of transfected cells the subnuclear localization of integrated copies of pSty11.λCla.2T was examined. **Panel b** shows proportional amounts of unattached (S, lanes 2 and 4) and nuclear matrix associated (P, lanes 1 and 3) DNA from halos cleaved with either *Bgl*II (lanes 1 and 2) or *Eco*RI (lanes 3 and 4). After the isolation all DNAs shown in panel b were digested with *Dpn*I to exclude detection of unintegrated plasmids. In **panel c** a Southern transfer of the gel shown in panel b is hybridized to the 4.4 kb λ *Cla*I fragment. The position of the relevant *Bgl*II and *Eco*RI fragments that contain both lambda DNA and TTAGGG repeat arrays is indicated. In **panel d** the same membrane is hybridized to a probe for the neomycin resistance gene (*neo*). This probe detects several fragments which, based on their MW, could be due to occasional co-integration pMC1neo poly A and pSty11.λCla.2T. The majority of pMC1neo poly A plasmids in these cells appear to have unique integration sites and are not detected in digests with enzymes, such as *Bgl*II and *Eco*RI, that cleave pMC1neo poly A at a unique site. These additional pMC1neo poly A copies can be detected in DNA that is digested with *Pst*I which cleaves twice in the *neo* gene.

locates telomeric loci near the surface of mammalian interphase nuclei (van Dekken *et al.*, 1989). These and similar observations in other systems suggest that the ends of eukaryotic chromosomes are located close to the nuclear envelope (Agard and Sedat, 1983; Mathog *et al.*, 1984; Foe and Alberts, 1985; Hochstrasser *et al.*, 1986; Mathog and Sedat, 1989; Chung *et al.*, 1990; Rawlins *et al.*, 1991). This positioning throughout interphase in organisms as diverse as flies, trypanosomes, plants and mammals is likely to be a consequence of specific anchorage of telomeric DNA at the nuclear periphery. Thus, the association of telomeres with the nuclear matrix, which includes the nuclear envelope, could reflect the peripheral anchorage of telomeres observed cytologically.

The attachment of chromosome ends to the nuclear matrix must involve at least two components: the telomeric DNA and an anchorage factor. Mapping experiments implicate the telomeric repeat region as the DNA site that is bound. Subtelomeric DNA is not detectably associated with the nuclear matrix unless it is physically linked to the telomeric repeat region. As the sequence TTAGGG is the most abundant and conserved sequence at human chromosome ends, it seems likely that these repeats mediate the anchorage of telomeres. However, the possibility cannot be excluded that the DNA end itself, independent of its sequence, is captured by a nuclear matrix factor.

Previously described matrix attachment regions are generally A/T-rich and contain sequences identical or similar to the cleavage site of topoisomerase II, a major component of the nuclear matrix and chromosomal scaffold (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Berrios *et al.*, 1985; Earnshaw *et al.*, 1985; Gasser *et al.*, 1986). By contrast, human telomeric repeats are G-rich and although topoisomerase II cleavage of telomeric repeats has not been tested, the repeat sequences do not conform to the topoisomerase II consensus site (GTN^A/_TAC^C/_TATTNATNN^A/_G). Although the functional significance of the A/T-rich boxes and topoisomerase II sites in MARS has not been assessed by mutagenesis, the absence of these sequence motifs in telomeres raises the possibility that these two kinds of elements are attached in different ways.

A unique mechanism of telomere attachment is also suggested by the fact that non-telomeric (transfected) TTAGGG repeats do not associate with the nuclear matrix. One interpretation of this result is that the DNA sequence itself is not sufficient to initiate or maintain nuclear matrix association, i.e. attachment of the TTAGGG repeats to the nuclear matrix shows a position effect. By contrast, MARS can become bound to the nuclear matrix during *in vitro* add-back experiments (Cockerill and Garrard, 1986), indicating that their nuclear matrix association is not dependent on genomic context. A factor that recognizes telomeric repeats specifically at a DNA terminus could explain the observation that the activity of telomeric repeats is dependent on their position in the genome. Such a factor might directly affect nuclear matrix association or recruit additional factors that determine the subnuclear position of telomeric DNA. While in *Oxytricha* and *Euplotes* a protein complex that binds the 3' overhang of telomere termini has been identified (Lipps *et al.*, 1982; Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Price and Cech, 1987, 1989; Raghuraman *et al.*, 1989; Price, 1990), a similar activity remains to be demonstrated in mammalian cells.

Telomere mediated position effects have previously been documented in flies and yeast (Biesmann and Mason, 1988; Levis, 1989; Gottschling *et al.*, 1990; Aparicio *et al.*, 1991). In yeast, genes located within a few kilobases of a telomere are reversibly repressed. Since chromosome-internal telomeric repeats fail to manifest this silencer activity, some telomere attribute other than telomeric DNA itself must be involved (Gottschling *et al.*, 1990). Gene silencing at yeast telomeres is dependent on three *SIR* genes (*SIR2*, 3 and 4) which were first identified as repressors of the silent mating type loci (Aparicio *et al.*, 1991). Interestingly, *SIR4* encodes a protein with sequence similarity to the polymerization domain of the nuclear lamins (Diffley and Stillman, 1989), suggesting that silencing at yeast telomeres is mediated by a protein network such as the nuclear envelope. The attachment of human telomeres to the nuclear matrix could similarly reflect an association of the telomeric nucleoprotein complex with a lamin-like factor. The nuclear lamins A and C, but not lamin B, bind human telomeric DNA *in vitro* (Shoeman and Traub, 1990). However, the interactions are rather weak and in addition, several human cell lines only express lamin B (Shoeman and Traub, 1990; Guilly *et al.*, 1987; David Saltman and T.de Lange, unpublished observations). We have found that nuclear envelopes are not enriched for telomeric DNA (Maria Cardenas and T.de Lange, unpublished observations). However, the relevant interaction may have been perturbed, since the isolation of nuclear envelopes includes incubation in 2 M NaCl. The issue of direct anchorage of telomeres to the nuclear envelope will have to be addressed with additional biochemical fractionation techniques and sensitive electron microscopic analysis.

The mechanism of telomere function is generally thought to be highly conserved. This expectation is inspired by the structural conservation of telomeric DNA and by the fact that telomere healing in yeast occurs on DNA ends with heterologous telomeric repeats (see Zakian, 1989 and Blackburn, 1991 for reviews). According to this view important telomere-protein interactions are expected to be highly conserved as well. While several telomere binding activities have been identified in unicellular organisms, none of these interactions have been shown to be a conserved so far. The only exception is telomerase, whose importance for telomere maintenance is underscored by the presence of telomerase activity in ciliates as well as in human cells. The association of human telomeres with the nuclear matrix represents an additional example of an interaction that may be of general importance for telomere function. It will be of interest to determine whether telomeres in other systems are similarly attached to the nuclear matrix. If the peripheral position of telomeres is an indication of their attachment to the nuclear matrix, it is expected that this property of telomeres is conserved in eukaryotes.

Materials and methods

Cell lines

Raji, a Burkitt's lymphoma cell line, and HL-60, a promyelocytic cell line, were grown in roller bottles in RPMI 1640. HL-60 cells were induced to differentiate by the addition of dimethylsulfoxide to 1.25% to a culture with a density of 1×10^5 cells/ml and the cells were incubated for 3 days. Non-adherent HeLa, a cervical carcinoma cell line, were grown in spinner flasks or roller bottles in Joklik's. Adherent HeLa cells were grown in Dulbecco's modified Eagle medium. All media were supplemented with 10% fetal calf

serum, non-essential amino acids, glutamine and antibiotics. Cells were harvested at 50% confluence.

DNA transfection

Adherent HeLa cells were transfected with 1 μ g pMC1neo poly A (Stratagene) and 5 μ g pSty11. λ Cla. 2T (see Figure 5) using the calcium phosphate co-precipitation protocol of Land *et al.* (1983). Approximately 200 G418 resistant (400 μ g/ml) colonies were pooled three weeks after transfection and amplified.

Isolation of LIS-generated halo structures

The procedure used is a modification of the protocol of Dijkwel and Hamlin (1988); the LIS technique was originally developed by Mirkovitch *et al.* (1984). All procedures were carried out at 4°C unless indicated otherwise. Centrifuge steps were carried out in a Sorvall RT6000. About 1×10^8 cells were centrifuged at 1000 r.p.m. and washed twice with 50 ml phosphate-buffered saline and twice with CWB (50 mM KCl, 0.5 mM EDTA, 0.05 mM spermine, 0.05 mM spermidine, 0.5% thiodiglycol, 0.25 mM PMSF, 5 mM Tris-HCl pH 7.4). The cells were suspended in 50 ml CWB with 0.1% digitonin (Calbiochem) and lysed by forcing the suspension four times through a 19.5 gauge needle. Lysis was monitored by phase contrast microscopy. The suspension was layered onto six 5 ml glycerol cushions (CWB with 10% glycerol) and centrifuged at 800 r.p.m. for 10 min. The nuclei were washed twice in 50 ml CWB with 0.1% digitonin and suspended in 5 ml CWB with 0.1% digitonin and 0.5 mM CuSO₄ but without EDTA. The nuclei were incubated for 20 min at 37°C. 95 ml of LIS (10 mM lithium diiodosalicylate, 100 mM LiAc, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, 20 mM HEPES-KOH pH 7.4) was added gradually and the nuclei were incubated for 10 min at room temperature. Subsequent steps were performed at room temperature. The resulting halos were collected by centrifugation at 1000 r.p.m. for 10 min and washed once with 50 ml MWB (20 mM KCl, 70 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl pH 7.4) with 0.1% digitonin, twice with 50 ml MWB and twice with 10 ml of the appropriate restriction endonuclease digestion buffer (as listed by New England Biolabs 1988–1989 catalogue under the assay conditions for each enzyme). Before the last centrifugation step the halos were counted and suspended at 1×10^8 /ml in restriction endonuclease buffer.

Restriction endonuclease cleavage and isolation of halo DNA

All centrifuge steps were performed for 5 min in an Eppendorf microcentrifuge at setting 4. Approximately 5×10^7 halos were cleaved in 0.5 ml with 1000 units of enzyme for 1.5 h at 37°C. The suspension was centrifuged and the supernatant was removed and incubated at 37°C for an additional hour. The pellet was suspended in 0.5 ml buffer and digested with 500 units of enzyme for 1 h at 37°C. This suspension was centrifuged and the pellet was washed with 0.5 ml buffer. The pellet was suspended in 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.5% SDS and proteinase K was added to 100 μ g/ml. The combined supernatants were brought to the same concentrations of EDTA, SDS and proteinase K and both fractions were incubated overnight at 37°C. The DNA was purified by extraction with phenol-chloroform and precipitation with isopropanol. Both fractions were dissolved in 0.5 ml 10 mM Tris-HCl pH 7.4. DNA concentrations were determined by Hoechst fluorescence in a Hoefer fluorometer using calf thymus DNA standards.

Biochemical characterization

The details of the experiment shown in Figure 3 were as follows. Cells (1×10^8) were processed according to the LIS protocol described above and the resulting halos were cleaved with *Bgl*III. The nuclear remnants and associated DNA were isolated by centrifugation and suspended in 4 ml MWB. Aliquots (200 μ l) were brought to 1 mM EDTA and incubated for 30 min at 37°C with the following agents added separately: no addition (control); Triton X-100 to 0.1%; NaCl to 2 M; β -mercaptoethanol to 10 mM; *o*-phenanthroline to 10 mM; EDTA to 10 mM; EGTA to 10 mM; RNase A to 20 μ g/ml; proteinase K to 50 μ g/ml; SDS to 0.1%. An additional aliquot was brought to 0.1 mM MgCl₂ and 1% Triton X-100 and incubated on ice for 10 min and centrifuged. The supernatant was kept for analysis and the pellet was suspended in MWB with 0.1 mM MgCl₂ and 1 M NaCl and incubated on ice for 10 min. After incubation all samples were centrifuged. Pellet and supernatant DNAs were isolated as described in the preceding paragraph. The activity of RNase A in this experiment was apparent from the degradation of endogenous nuclear RNA in the LIS-treated nuclei.

Cell elutriation

Non-adherent HeLa cells (4×10^9) were fractionated by centrifugal cell elutriation at 4°C in PBS in a Beckman J-6M using a JE5.0 rotor. The DNA content of the cells in each fraction was determined by DAPI

fluorescence using a Becton-Dickinson FACScan. Fractions with the appropriate fluorescence profiles, containing $\sim 1 \times 10^8$ cells in G₁, S or G₂/M phase were processed alongside an aliquot of the unfractionated input material according to the LIS protocol described above. The enzyme used to dissociate DNA fragments from the nuclear matrix was *Bgl*III.

Genomic blotting

For Southern blotting analysis, equal volumes representing DNA from identical numbers of halos ($\sim 2 \times 10^6$ per lane) were fractionated side by side on 0.7% agarose gels. RNase A was added to the DNA samples to a concentration of 20 μ g/ml before loading. Gels were run in the presence of ethidium bromide at 1–2 V/cm in TAE buffer (0.04 M Tris-acetate pH 8.3, 1 mM EDTA). The fractionated DNA was depurinated *in situ* by a 20 min incubation in 0.25 N HCl, subsequently denatured and nicked with 0.5 M NaOH, 1.5 M NaCl (2 \times 20 min) and neutralized in 0.5 M Tris-HCl pH 7.5, 3 M NaCl (2 \times 20 min). The DNA was transferred to Hybond-N membrane (Amersham) in 20 \times SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) in 3 h and cross-linked by UV exposure in a Stratelinker (Stratagene). After a 20 min prehybridization the membranes were hybridized overnight at 65°C in 0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 7% SDS, 1% BSA (Church and Gilbert, 1984) with 10 ng labelled DNA/ml. The insert of pTH2 Δ (de Lange *et al.*, 1990), the 4.4 kb *Cla*I fragment from bacteriophage lambda, and a 1.1 kb *Sal*I-*Xho*I fragment from pMC1neo poly A which contains the *neo* gene were labelled using the hexamer labelling technique of Feinberg and Vogelstein (1983). The TTAGGG repeat probe is a 800 bp fragment composed entirely of TTAGGG repeats (T.de Lange, unpublished) which was labelled by the method of Feinberg and Vogelstein (1983) using a 5'CCCTAACCCCTAA3' primer. Post-hybridization washes were performed in 40 mM Na-phosphate buffer pH 7.2/1 mM EDTA/1% SDS (Church and Gilbert, 1984) at 65°C. Various exposures were made at -70°C on Kodak XAR film using intensifying screens and the signals were analyzed quantitatively using a digital densitometer kindly supplied by Biological Vision.

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References

- Agard, D.A. and Sedat, J.W. (1983) *Nature*, **302**, 676–681.
- Allshire, R.C., Gosden, J.R., Cross, S.H., Cranston, G., Rout, D., Sugawara, N., Szostak, J.W., Fantes, P.A. and Hastie, N.D. (1988) *Nature*, **332**, 656–659.
- Allshire, R.C., Dempster, M. and Hastie, N.D. (1989) *Nucleic Acids Res.*, **17**, 4611–4627.
- Amati, B.B. and Gasser, S.M. (1988) *Cell*, **54**, 967–978.
- Aparicio, O.M., Billington, B.L. and Gottschling, D.E. (1991) *Cell*, **66**, 1–20.
- Berman, J., Tachibana, C.Y. and Tye, B.-K. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3713–3717.
- Berrios, M., Osheroff, N. and Fisher, P.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4142–4146.
- Biessmann, H. and Mason, J.M. (1988) *EMBO J.*, **7**, 1081–1086.
- Blackburn, E.H. (1990) *Science*, **249**, 489–490.
- Blackburn, E.H. (1991) *Nature*, **350**, 569–573.
- Brown, W.R.A., MacKinnon, P.J., Villasante, A., Spurr, N., Buckle, V.J. and Dobson, M.J. (1990) *Cell*, **63**, 119–132.
- Buchman, A.R., Kimmerly, W.J., Rine, J. and Kornberg, R.D. (1988) *Mol. Cell. Biol.*, **8**, 210–225.
- Cheng, J.-F., Smith, C.L. and Cantor, C.R. (1989) *Nucleic Acids Res.*, **17**, 6109–6127.
- Chung, H.-M.M., Shea, C., Fields, S., Taub, R.N., van der Ploeg, L.H.T. and Tse, D.B. (1990) *EMBO J.*, **9**, 2611–2619.
- Church, G.M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.

- Cockerill, P.N. and Garrard, W.T. (1986) *Cell*, **44**, 273–282.
- Conrad, M.N., Wright, J.H., Wolf, A.J. and Zakian, V.A. (1990) *Cell*, **63**, 739–750.
- Cooke, H.J. and Smith, B.A. (1986) *Cold Spring Harbor Symp. Quant. Biol.*, **51**, 213–219.
- Cremer, T., Cremer, C., Baumann, H., Luedtke, E.-K., Sperling, K., Teuber, V. and Zorn, C. (1982) *Hum. Genet.*, **60**, 46–56.
- Cross, S.H., Allshire, R.C., McKay, S.J., McGill, N.I. and Cooke, H.J. (1989) *Nature*, **338**, 771–774.
- de Lange, T., Shiue, L., Myers, R.M., Cox, D.R., Naylor, S.L., Killery, A.M. and Varmus, H.E. (1990) *Mol. Cell. Biol.*, **10**, 518–527.
- Diffley, J.F.X. and Stillman, B. (1989) *Nature*, **342**, 24.
- Dijkwel, P.A. and Hamlin, J.L. (1988) *Mol. Cell. Biol.*, **8**, 5398–5409.
- Earnshaw, W.C., Halligan, B., Cook, C.A., Heck, M.M.S. and Liu, L.F. (1985) *J. Cell Biol.*, **100**, 1706–1715.
- Evan, G.I. and Hancock, D.C. (1987) *Cell*, **43**, 253–261.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, **133**, 6–13.
- Foe, V.E. and Alberts, B.M. (1985) *J. Cell Biol.*, **100**, 1623–1636.
- Gasser, S.M. and Laemmli, U.K. (1986) *EMBO J.*, **5**, 511–518.
- Gasser, S.M., Laroche, T., Falquet, J., Boy de la Tour, E. and Laemmli, U.K. (1986) *J. Mol. Biol.*, **188**, 613–629.
- Gasser, S.M., Amati, B.B., Cardenas, M.E. and Hofman, J.F.-X. (1989) *Int. Rev. Cytol.*, **119**, 57–96.
- Gottschling, D.E. and Cech, T.R. (1984) *Cell*, **38**, 501–510.
- Gottschling, D.E. and Zakian, V.A. (1986) *Cell*, **47**, 195–205.
- Gottschling, D.E., Aparicio, O.M., Billington, B.L. and Zakian, V.A. (1990) *Cell*, **63**, 751–762.
- Greider, C.W. and Blackburn, E.H. (1989) *Nature*, **337**, 331–337.
- Guilly, M.N., Bensussan, A., Bourge, J.F., Bornens, M. and Courvalin, J.C. (1987) *EMBO J.*, **6**, 3795–3799.
- Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) *Nature*, **345**, 458–460.
- Hastie, N.D., Dempster, M., Dunlop, M.G., Thompson, A.M., Green, D.K. and Allshire, R.C. (1990) *Nature*, **346**, 866–868.
- Henderson, E.R. and Blackburn, E.H. (1989) *Mol. Cell. Biol.*, **9**, 345–348.
- Henderson, E.R., Hardin, C.C., Walk, S. K., Tinoco, I.Jr. and Blackburn, E.H. (1987) *Cell*, **51**, 899–908.
- Hochstrasser, M., Mathog, D., Gruenbaum, Y., Saumweber, H. and Sedat, J.W. (1986) *J. Cell Biol.*, **102**, 112–123.
- Klobutcher, L.A., Swanton, M.T., Donini, P. and Prescott, D.M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3015–3019.
- Land, H., Parada, L.F. and Weinberg, R.A. (1983) *Nature*, **304**, 596–601.
- Levis, R.W. (1989) *Cell*, **58**, 791–801.
- Lipps, H.J., Gruissem, W. and Prescott, D.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2495–2499.
- Longtine, M.S., Maxfield Wilson, N., Petruseck, M.E. and Berman, J. (1989) *Curr. Genet.*, **16**, 225–239.
- Lustig, A.J., Kurtz, S. and Shore, D. (1990) *Science*, **250**, 549–553.
- Mathog, D. and Sedat, J.W. (1989) *Genetics*, **121**, 293–311.
- Mathog, D., Hochstrasser, M., Gruenbaum, Y., Saumweber, H. and Sedat, J. (1984) *Nature*, **308**, 414–421.
- Meyne, J., Ratliff, R.L. and Moyzis, R.K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7049–7053.
- Mirkovitch, J., Mirault, M.-E. and Laemmli, U.K. (1984) *Cell*, **39**, 223–232.
- Morin, G.B. (1989) *Cell*, **59**, 521–529.
- Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L. and Wu, J.-R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6622–6626.
- Pluta, A.F., Kaine, B.P. and Spear, B.B. (1982) *Nucleic Acids Res.*, **10**, 8145–8154.
- Price, C.M. (1990) *Mol. Cell. Biol.*, **10**, 3421–3431.
- Price, C.M. and Cech, T.R. (1987) *Genes Dev.*, **1**, 783–793.
- Price, C.M. and Cech, T.R. (1989) *Biochemistry*, **28**, 769–774.
- Rabl, C. (1885) In Gegenbaur, C. (ed), *Morphologisches Jahrbuch*. Vol. 10, pp. 214–330.
- Raghuraman, M.K., Dunn, C.J., Hicke, B.J. and Cech, T.R. (1989) *Nucleic Acids Res.*, **17**, 4235–4253.
- Rawlins, D.J., Highett, M.I. and Shaw, P.J. (1991) *Chromosoma*, **100**, 424–431.
- Sen, D. and Gilbert, W. (1988) *Nature*, **334**, 364–366.
- Shippen-Lentz, D. and Blackburn, E.H. (1990) *Science*, **247**, 546–552.
- Shoeman, R.L. and Traub, P. (1990) *J. Biol. Chem.*, **265**, 9055–9061.
- Sperling, K. and Luedtke, E.-K. (1981) *Chromosoma*, **83**, 541–553.
- Sundquist, W.I. and Klug, A. (1989) *Nature*, **342**, 825–829.
- van Dekken, H., Pinkel, D., Mullikin, J., Trask, B., van den Engh, G. and Gray, J. (1989) *J. Cell Sci.*, **94**, 299–306.
- Wilkie, A.O.M., Lamb, J., Harris, P.C., Finney, R.D. and Higgs, D.R. (1990) *Nature*, **346**, 868–871.
- Williamson, J.R., Raghuraman, M.K. and Cech, T.R. (1989) *Cell*, **59**, 871–880.
- Yu, G.-L., Bradley, J.D., Attardi, L.D. and Blackburn, E.H. (1990) *Nature*, **344**, 126–132.
- Zakian, V.A. (1989) *Annu. Rev. Genet.*, **23**, 579–604.

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