Structure, Subnuclear Distribution, and Nuclear Matrix Association of the Mammalian Telomeric Complex

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Abstract. Mammalian telomeres are composed of long arrays of TTAGGG repeats complexed with the TTAGGG repeat binding factor, TRF. Biochemical and ultrastructural data presented here show that the telomeric DNA and TRF colocalize in individual, condensed structures in the nuclear matrix. Telomeric TTAGGG repeats were found to carry an array of nuclear matrix attachment sites occurring at a frequency of at least one per kb. The nuclear matrix association of the telomeric arrays extended over large domains of up to 20–30 kb, encompassing the entire length of most mammalian telomeres. TRF protein and telomeric DNA cofractionated in nuclear matrix preparations and colocalized in discrete, condensed sites throughout the nuclear volume. FISH analysis indicated that TRF is an integral component of the telomeric complex and that the presence of TRF on telomeric DNA correlates with the compact configuration of telomeres and their association with the nuclear matrix. Biochemical fractionation of TRF and telomeric DNA did not reveal an interaction with the nuclear lamina. Furthermore, ultrastructural analysis indicated that the mammalian telomeric complex occupied sites throughout the nuclear volume, arguing against a role for the nuclear envelope in telomere function during interphase. These results are consistent with the view that mammalian telomeres form nuclear matrix-associated, TRF-containing higher order complexes at dispersed sites throughout the nuclear volume.

Mammalian telomeres have attracted considerable interest for their possible involvement in malignant transformation (for review see de Lange, 1995; Autexier and Greider, 1996). While many aspects of the structure and maintenance of telomeric DNA have been studied in detail, relatively little is known about the overall arrangement of the nucleoprotein complex at mammalian chromosome ends. This study represents a biochemical, immunocytochemical, and ultrastructural analysis of mammalian telomeres in interphase nuclei.

Mammalian chromosome ends carry telomeric tracts containing 2–50 kb of tandem TTAGGG repeats (Moyzis et al., 1988; de Lange et al., 1990; Kipling and Cooke, 1990). This sequence can be maintained by the telomere-specific RNP polymerase, telomerase (Morin, 1989; for review see Blackburn, 1993). In the absence of telomerase activity, telomeres shorten with cell divisions (Singer and Gottschling, 1994; McEachern and Blackburn, 1995), probably because the chromosomal replication strategy fails to duplicate DNA ends. In addition to recruiting telomerase, telomeric repeats are thought to mask the presence of natural chromosome ends from DNA damage checkpoints and repair activities (for review see Zakian, 1995).

Human somatic cells undergo programmed telomere shortening in a process that appears to involve repression of telomerase (Cooke and Smith, 1986; de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990; Counter et al., 1992; Kim et al., 1994). The progressive decline of somatic telomeres with cell divisions may constitute a tumor suppressor mechanism that limits the replicative potential of transformed cells. In agreement, telomerase is frequently activated in human and mouse tumors and restoration of telomere length is correlated with immortalization of human cells in vitro (Counter et al., 1992, 1994a, b; Kim et al., 1994; Blasco et al., 1996; Broccoli et al., 1996a).

There is mounting evidence that both the shielding of telomeric ends, as well as their interaction with telomerase, depend on the structural proteins in the telomeric complex. In yeast, telomere length homeostasis requires the binding of a protein along the telomeric tract (Lustig et al., 1990; Kyriacou et al., 1992; McEachern and Blackburn, 1995) and alterations in telomeric protein complex compromise the stability of chromosome ends (for review see...
Zakian, 1995). Consistent with a specific telomeric complex at human chromosome ends, an altered chromatin structure has been detected (Tommerup et al., 1994; Lejnine et al., 1995) and the telomeric TTAGGG repeats were found to associate with the nuclear matrix (de Lange, 1992). Since subtelomeric DNA is not tethered to the nuclear matrix and has a normal nucleosomal organization, the functional interactions appear to be confined to the TTAGGG repeat region. However, the molecular basis of telomeric protection of chromosome ends and the mechanism by which telomeres recruit and modulate telomerase are not known in mammals or other eukaryotes.

A human telomere-binding protein, the TTAGGG repeat binding factor (TRF), was recently identified and cloned (Zhong et al., 1992; Chong et al., 1995). hTRF is a novel Myb-related protein of ~60 kDa that binds to duplex telomeric DNA in vitro and has features expected for a protein that coats the telomeric tract along its length. The factor binds specifically to double-stranded telomeric repeat arrays and does not require a terminus for site recognition. Expression studies demonstrate TRF in the telomeric complex in interphase nuclei and in mitotic chromosomes. In agreement with an essential role for TRF at telomeres, de novo telomere formation in human cells requires sequences that can bind TRF in vitro (Hanish et al., 1994).

This report examines the complex formed between telomeric DNA and TRF, its subnuclear distribution, and the interaction of telomeres with the nuclear matrix. The results indicate that telomeres form discrete individual nucleoprotein complexes built up of telomeric DNA and TRF. These structures occupy dispersed positions throughout the nuclear volume and are strongly associated with the inner network of the nuclear matrix. Biochemical fractionation indicates that the telomeric complex is bound to the nuclear matrix through multiple interactions along the TTAGGG repeat array. This binding mode is consistent with tethering through TRF which is demonstrated to be a component of the nuclear matrix.

Materials and Methods

Cell Culture

HeLa-S and HeLa-L cells are described in Tommerup et al. (1994). HeLa-L cells stably transformed with a construct expressing a hemagglutinin (HA)-tagged mTRF were described in Chong et al. (1995). Human rhabdomyosarcoma RD cells (ATCC CCL 136) and mouse renal adenocarcinoma RAG cells (ATCC CCL 142) were grown in RPMI 1640 supplemented with 10% iron-supplemented bovine serum to 5 × 10^5 cells per ml. All media were prepared using Harlan Teklad no. 7092 basal Eagle's medium supplemented with 10% FCS to ~5 × 10^5 cells per ml. Mouse plasmacytoma J558 cells (ATCC TIB 6) were grown in suspension in Joklik's medium with 10% iron-supplemented bovine serum to 5 × 10^5 cells per ml. All media were supplemented with l-glutamine, nonessential amino acids, penicillin, and streptomycin.

1. Abbreviations used in this paper: EM-ISH, electron microscopical in situ hybridization; LIS, lithium diiodosalicylate; TRF, TTAGGG repeat binding factor.

Nuclear matrices were isolated as described previously (de Lange, 1992) following the lithium diiodosalicylate (LIS) extraction procedure of Mirkovic et al. (1984). A few minor modifications were made. Briefly, cells were washed twice with ice-cold PBS, twice with ice-cold CWB (50 mM KCl, 0.5 mM EDTA, 0.5% thiodiglycol, 0.05 mM spermine, 0.05 mM spermidine, 0.25 mM PMSF, 5 mM Tris-HCl [pH 7.4]), and suspended in CWB with 0.1% digitonin (Calbiochem, San Diego, CA) at a density of 2 × 10^6 cells per ml. Adherent human HeLa-S, HeLa-L, RD, and mouse RAG cells were lysed by passage through a needle as described (de Lange, 1992); nonadherent human SU-LyB-1 and mouse J558 cells were lysed by vortexing briefly. Resulting nuclei were centrifuged through a glycerol cushion (10% glycerol in CWB) and washed twice in CWB with 0.1% digitonin. The nuclei were stabilized by a 20-min incubation at 37°C in CWB with 0.1% digitonin and 0.5 mM CuSO_4 but without EDTA, and subsequently extracted by the addition of LIS buffer (10 mM LIS, 100 mM LiAc, 1 mM EDTA, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, 20 mM Hapes-KOH [pH 7.4]). Extraction was carried out for 10 min at room temperature at a density of 2 × 10^6 cells per ml. Resulting nuclear halos were collected by centrifugation and processed to yield nuclear matrices as described below.

For the isolation of nuclear shells, the same procedure was followed except that the incubation at 37°C in the presence of 0.5 mM CuSO_4 ions, which are used to stabilize the telomeric structure of the nuclear matrix (Lejeune et al., 1988; Ludèrus et al., 1992), was replaced by an incubation on ice without CuSO_4. LIS-generated nuclear shell halos were collected by centrifugation for 15 min at 4°C in an Eppendorf microcentrifuge at maximal speed.

DNAse I Mapping Experiments

Freshly isolated nuclear matrices, digested with restriction endonucleases, were collected by centrifugation for 5 min in an Eppendorf microcentrifuge at setting 7 (1,000 g), washed in 5 mM MgCl_2, 10 mM Tris [pH 7.4], and resuspended at a density of 10^7 cells per ml of the same buffer. DNAse I was added (final concentrations of 0.25, 50, 100, 250, or 500 ng/ml) to 50 μl samples, and digestion was carried out for 5 min at 37°C. Digestion was stopped by the addition of 8 mM EDTA at 0°C. The suspensions were centrifuged for 5 min at 4°C in an Eppendorf microcentrifuge at setting 7, and matrix-bound DNA and released DNA was purified as described above for the isolation of matrix-associated restriction fragments.

Genomic Blotting

Fractionation of DNA on agarose gels, transfer of the fractionated DNA to Hybond-N membrane, and annealing to labeled probes was performed as described by de Lange (1992). In human DNAs, TTAGGG sequences were detected with an 800-bp [TTAGGG]_135 fragment and subtelomeric sequences were detected with the insert of plasmid pTH2A (de Lange et al., 1990). To detect telomeric TTAGGG repeats on blots of mouse genomic DNA, the oligonucleotide AAAACTCGAC[TTAGGG]_135, sequence and subtelomeric sequences were detected with the insert of plasmid pTH2A (de Lange et al., 1990). To detect telomeric TTAGGG repeats on blots of mouse genomic DNA, the oligonucleotide AAAACTCGAC[TTAGGG]_135, end-labeled using [γ-32P]ATP (6,000 Ci/mmol) and T4 polynucleotide kinase, was used as probe. Blots were prehybridized for 30 min at 65°C in 0.5 M Na-phosphate buffer (pH 7.2), 1 mM EDTA, 7% SDS, 10% BSA (Church and Gilbert, 1984), and hybridized overnight in the same buffer at 37°C. Hybridization was stopped by the addition of 8 mM EDTA at 0°C. The suspensions were centrifuged for 5 min at 4°C in an Eppendorf microcentrifuge at setting 7, and matrix-bound DNA and released DNA was purified as described above for the isolation of matrix-associated restriction fragments.
Production of Anti-TRF Antibody

A 16-amino acid peptide (peptide I5) of sequence (NH2)-TSQDKPS-GNDVEMETC-(COOH), encompassing residues 273-287 of human TRF plus a COOH-terminal cysteine was synthesized (Rockefeller University peptide synthesis facility) and coupled to Maleimide-activated KLH (Pierce, Rockford, IL). A rabbit antiserum (serum #5) against this conjugate was produced commercially and affinity-purified using peptide I5 cross-linked to Suloflink Coupling gel (Pierce).

Western Blotting

Proteins from nuclei, nuclear matrices, and nuclear shells were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. A-type lamins were detected by monoclonal antibody (mAb) 41CC4 (Burke et al., 1983), hnRNP A2/B1 by mAb 7A9 (Piot-Bolma and Dreyfuss, 1991; McKay and Cooke, 1992b), the internal matrix protein p160 by mAb AM88 (de Graaf et al., 1991), and the anti-Jamin B mAb 101-B7 was purchased from Matiret (Cambridge, MA). Blots were blocked in 10% fat-free milk powder and 0.5% Tween-20 in PBS, and antibody incubations were performed in 0.1% milk powder and 0.1% Tween-20 in PBS (incubation buffer). Antibody-protein complexes were detected using the chemiluminescence ECL system (Amersham).

In Situ Hybridization Experiments

In Situ Preparation of Permeabilized Nuclei, Nuclear Halos, and Nuclear Matrices. In situ hybridization experiments were performed with HeLa-L cells and HeLa-L cells expressing a [HA]2-tagged mTRF (Chong et al., 1995). Cells were grown to 50-70% confluency on glass coverslips for light microscopy or on Thermanox coverslips (Lux) for electron microscopy. Cells and HeLa-L cells expressing a [HA]2-tagged mTRF (Chong et al., 1995) were extracted for 3 min on ice in CSK-buffer containing 0.5% NP-40 in PBS (wash buffer), incubated for 1-2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amer sham) in incubation buffer, and washed three times for 10 min in wash buffer, followed by detection by ECL. Similar results were obtained when i231-labeled protein A was used for detection (data not shown). As a control, in each experiment an identical blot was incubated in parallel with affinity-purified serum No. 5 that was preincubated for 30 min at room temperature with 1 mg/ml peptide I5.

In Situ Hyridization. DNA denaturation, hybridization, and washing conditions were identical to those described by Sibon et al. (1994). A [CCCUAA]4, repeat RNA probe was synthesized with SP6 polymerase in the presence of [35S]UTP (Boehringer Mannheim Corp., Indianapolis, IN) on HindIII-linearized plasmid pMTS (de Lange et al., 1990).

Detection of Hybrids by Immunofluorescence Microscopy. After the in situ hybridization and washing procedures, the preparations were incubated for 10 min in PBS with 50 mM glycine and for 15 min in PBG. Biotin-labeled RNA-DNA hybrids were detected using a rabbit anti-biotin antibody (Enzo) and a goat anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) (Jackson Immunoresearch Labs, West Grove, PA). For simultaneous visualization of telomeric DNA and [HA]2-tagged mTRF protein in stably transformed cells, HA epitopes were detected using mAb 12CA5, and a Cy3-coupled goat antibody to mouse IgG (Amer sham). All antibodies were diluted in PBG. DNA was stained with 0.2 μg/mL 4,6-diamidino-2-phenylindol-dihydrochloride (DAPI) (Boehringer) per mL. After the antibody incubations, preparations were washed extensively, first with PBG, then with PBS, and mounted in Mowiol (HOECHST) supplemented with 0.5% para-phenylenediamine (Johnson and De Neuge Araujo, 1984). Slides were examined using a Leitz Orthoplan microscope equipped with epifluorescence optics. Confocal laser scanning microscopy was performed with a 600 instrument on a Zeiss Axioplan microscope (BioRad Labs, Hercules, CA).

Results

Telomeres Are Attached to the Inner Nuclear Matrix

The nuclear matrix is the insoluble nonchromatin scaffolding of the interphase nucleus (for review see Berezney et al., 1995). It is isolated by removing histones, other loosely bound proteins, and most of the nuclear DNA and RNA (Berezney and Coffey, 1974). We have previously shown that the TTAGGG repeats at human telomeres are associated with the nuclear matrix (de Lange, 1992). An example of this binding is shown in Fig. 1 A, which represents HeLa-S nuclei extracted with LIS, a treatment that removes histones and other soluble proteins (Mirkovitch et al., 1984). The DNA in the resulting nuclear halos was cleaved with PvuII to disconnect DNA sequences that are not attached to the matrix. Matrix-attached and solubilized restriction fragments were separated by centrifugation and analyzed by gel electrophoresis and genomic blotting. A (TTAGGG)4 oligonucleotide was used to detect telomeric fragments and the signals were quantitated by Phosphorimage analysis. While a minor fraction of the bulk chromosomal DNA (left panel) is found in the nuclear matrix attached fraction, the majority (70%) of the telomeric restriction fragments are recovered in this fraction (Fig. 1 A, left panel).
The same fractionation pattern is observed for a subset of telomeres that carry a specific subtelomeric repetitive element that can be detected with the probe pTH2Δ (Fig. 1 A, right panel, and Fig. 1 C). Quantification revealed that ~70% of terminal fragments that carry the pTH2Δ sequence are recovered in the nuclear matrix (Fig. 1 A, right panel). This represents a considerable enrichment of telomeric fragments and is consistent with previous estimates for the relative retention of telomeric loci in the nuclear matrix preparations.

Ultrastructurally, the nuclear matrix contains the nuclear lamina, nuclear pore complexes, remnants of the nucleolus, and an internal fibrogranular network that contains hnRNP proteins (Fey et al., 1986; Jackson and Cook, 1988; He et al., 1990; Belgrader et al., 1991; Mattern et al., 1996). To address whether the attached telomeres are tethered to any one of these substructures, we examined the fractionation of telomeric DNA with nuclear shells. Isolated as nuclear matrices but without the heat-stabilization step, nuclear shells represent a peripheral substructure of the nuclear matrix that is highly enriched for lamins proteins (Izaurralde et al., 1988; Ludérus et al., 1992).

Nuclear matrices and nuclear shells were isolated in parallel from HeLa cells, the associated DNA fragments were cleaved with PvuII, and the released and bound DNA fractions were isolated. Both matrices and shells were found to contain only a minor fraction of the total chromosomal DNA (Fig. 1 B, left hand panel). Similar to the experiment shown in A of Fig. 1, nuclear matrices were found to contain 65% of the telomeric DNA (Fig. 1 B, right hand panel). In contrast, nuclear shells retained only ~5% of the telomeric restriction fragments (Fig. 1 B, right hand panel). These findings indicate that matrix-bound telomeres are predominantly associated with the internal nuclear matrix, rather than with a peripheral structure.

**Telomeres Contain Long Arrays of Nuclear Matrix Attachment Sites**

The attachment of telomeres to the nuclear matrix could either be mediated by the extreme end of the telomere or be due to interactions along the double-stranded telomeric repeat array (or both). To address this issue, our strategy was to use DNase I to cleave telomeres at multiple sites DNA gel (left), the hybridization pattern of a TTAGGG repeat probe (right), and the same Southern transfer probed with a pTH2Δ probe (middle). Positions of telomeric (t) and chromosome-internal (*) restriction fragments are indicated (de Lange et al., 1990). (B) Attachment of telomeric DNA to nuclear matrices and nuclear shells. Nuclear matrices and nuclear shells, both digested with PvuII, were isolated from HeLa-S cells. Matrix/shell-attached (P) and released (S) restriction fragments were fractionated on a 0.8% agarose gel. The left panel shows the ethidium/UV staining pattern of the DNA gel. The mean size of the DNA fragments in B is somewhat larger than in A due to slight partial digestion with PvuII in B. The right panel shows the hybridization pattern of a TTAGGG repeat probe to a Southern transfer of the same gel. (C) Physical map of HeLa-S telomeres that contain the pTH2Δ subtelomeric repeat (adapted from de Lange et al., 1990). The position of the PvuII restriction site is indicated.
within the double-stranded TTAGGG repeat stretch and to determine whether the resulting short telomeric subfragments are retained on the nuclear matrix.

This method was applied to four human cell lines with different telomere lengths. Two HeLa cell lines were tested, one with telomeres of 14–31 kb (HeLa-L) and one with telomeres of 2–7 kb (HeLa-S) (de Lange et al., 1990; Saltman et al., 1993; Tommerup et al., 1994). In addition,
RD rhabdomyosarcoma cells with 2–5 kb telomeres and a lymphoblastoid cell line (SU-LyB-1) with telomeres in the 10–15-kb range were examined (Saltman et al., 1993). Nuclear halos were isolated from each cell line and the associated DNA was first cleaved with PvulI. The resulting matrix preparations with bound PvulI fragments were isolated and subsequently cleaved with increasing concentrations of DNase I to create subfragments within the telomeric repeat array. The release of telomeric sequences was monitored by genomic blotting of attached and released DNAs. For each of the human cell lines, we found that the telomeric fragments remained quantitatively attached to the nuclear matrix, even when degraded to a median size of 1 kb (Fig. 2, A–D).

The quantitative attachment of 1 kb TTAGGG repeat stretches derived from telomeres that are 10–30 kb in length (HeLa-L and SU-LyB-1, Fig. 2, B and D), is incompatible with an attachment mode that is solely due to an interaction of the telomere terminus with the nuclear matrix. Similarly, the data cannot be explained by the presence of a single focal nuclear matrix attachment site within the telomeric stretch. Rather, it appears that the human telomeric tracts bind to the nuclear matrix along their length and that the attachment sites occur frequently, on the order of one site/kb. Furthermore, the results indicate that for human telomeres in the 2–30-kb length range, the whole telomeric tract has this type of interaction with the nuclear matrix.

The same mode of attachment was observed for mouse telomeres. The short telomeres of the mouse renal adenocarcinoma RAG cells (1.5–2.5 kb; Fig. 2, E and D; Kipling, D., personal communication) are bound to the nuclear matrix along approximately half of their length. The ratio between the matrix-bound and soluble matrix-bound telomeres of mouse J558 cells are attached to the nuclear matrix along approximately half of their length. The ratio between the matrix-bound and soluble fragments remained constant over the entire range of fragment sizes. This suggests that in J558 cells the TTAGGG repeats do not extend beyond this range. The telomeric TTAGGG repeat DNA at mouse and human telomeres interact with the inner nuclear matrix. Matrix-attached regions measure up to 30 kb, covering entire telomeres in this size range, and appear to be tethered by an array of anchorage sites occurring frequently along the telomeric tract.

**The Telomeric Protein TRF Is a Component of the Nuclear Matrix**

The finding that telomeres are bound to the matrix along their length implicates a double-stranded TTAGGG repeat binding factor in the attachment process. Since the telomeric protein TRF has this DNA binding specificity and is known to interact with mammalian telomeres in vivo (Zhong et al., 1992; Chong et al., 1995), we examined the presence of this protein in the nuclear matrix.

A rabbit polyclonal serum directed against a synthetic peptide representing amino acids 273-287 of the deduced sequence of hTRF was raised and subjected to affinity purification. This antiserum reacts with recombinant TRF and gives rise to a supershift when incubated with HeLa TRF in a gel-shift assay (data not shown). In Western blots of HeLa cell proteins, the antibody specifically detect a set of proteins of ~60 kD that are predominantly nuclear and can be extracted from chromatin by elution with 0.4 M KCl (Fig. 3). These polypeptides are not detected when the antibody with or without the addition of the peptide used for immunization.

**Figure 3.** The telomeric protein TRF is a nuclear protein and can be extracted from chromatin with 0.4 M KCl. HeLa-S cells were either brought up in Laemmli loading buffer (Total) or extracted by hypotonic lysis to yield cytoplasmic proteins (S100) and crude nuclei. The nuclear fraction was extracted with 0.4 M KCl yielding soluble (Nuclear Extract) and insoluble (Nuclear Pellet) proteins. A whole cell extract was prepared by hypotonic lysis of cells followed by extraction of nuclear proteins with 0.4 M KCl and removal of insoluble components (Zhong et al., 1992). Equal cell equivalents were fractionated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue (A). A Western transfer was probed with an anti-peptide antibody directed against human TRF (B). C shows nuclear extract proteins probed with the TRF antibody with or without the addition of the peptide used for immunization. TRF polypeptides migrate as a doublet of ~60 kD (asterisk). The nature of the protein band of ~70 kD (caret) is not known.
tibody is pretreated with excess peptide (Figs. 3 and 4). The apparent molecular weight of the detected proteins and their fractionation behavior is consistent with previous purification of HeLa TRF (Chong et al., 1995). The antiserum also detects a protein of ~70 kD. This band is not seen in all experiments (for instance, compare Figs. 3 and 4) and its relationship to TRF is unclear at this stage.

The database contains a partial cDNA (GenBank accession number X93512) with an open reading frame that shows considerable homology to the Myb domain of TRF. We have recently determined the open reading frame of a cDNA representing this protein and found it to lack the peptide used for the generation of the antiserum against TRF (Smogorzewska, A., D. Broccoli, and T. de Lange, unpublished observations). Therefore, it is unlikely that the bands detected by the TRF serum represent this TRF-related protein.

To determine whether TRF is a nuclear matrix protein, equivalent numbers of nuclei, nuclear matrices, and nuclear shells from HeLa-S cells were examined by Western blotting for TRF and other marker proteins. Compared to intact nuclei, nuclear matrices were depleted in histone proteins, but otherwise still had a complex protein composition, while the composition of nuclear shells was much simpler (Fig. 4 A). Western blotting revealed that, as expected, A- and B-type lamins were present in equal amounts in all fractions (Fig. 4, B and C), whereas p160, a known component of the internal nuclear matrix (de Graaf et al., 1991), was present in nuclei and nuclear matrices but absent from nuclear shells (Fig. 4 D). As further evidence for a specific differential fractionation of matrix and shell proteins, we found the hnRNP proteins D1/D2 in nuclei and nuclear matrices, but not in nuclear shells (Fig. 4 E) and the hnRNP A2/B1 proteins were only detectable in unextracted nuclei (Fig. 4 F). Incubation of the same blots with the anti-TRF antiserum revealed that TRF is a component of the internal matrix structure: the protein quantitatively cofractionated with nuclear matrices but could not be detected in nuclear shells (Fig. 4 G). TRF thus closely followed the fractionation behavior of telomeric DNA (Fig. 1 B). We note that even after extensive DNase I digestion, TRF was quantitatively recovered in the matrix fraction, indicating that persistence of TRF in the nuclear matrix does not require long regions of telomeric DNA (data not shown).

**TRF Colocalizes with Matrix-attached Telomeric DNA**

The association of TRF with telomeric DNA was further examined by microscopical analysis of nuclei, nuclear matrices, and nuclear halos, which represent nuclear matrices with all the genomic DNA still attached. Telomeric loci, detectable with a telomere-specific CCCUAA riboprobe (Fig. 5 C, inset), reveal a discrete punctate pattern in Triton-extracted HeLa cell nuclei (Fig. 5, A–C). As was previously noted for HeLa interphase nuclei (Chong et al., 1995), the telomeres appear distributed throughout the nuclear volume and are not strongly clustered. This is true for over 50 interphase nuclei we have examined in this and other studies, including several nuclei that contained double the number of telomeric signals and were therefore probably in G2 (data not shown). Thus, it appears that the dispersed distribution of telomeric loci is maintained throughout interphase. The only exception to this rule was noted in cells that were in late telophase or early G1, in which case a substantial proportion of telomeric signals were observed in a ring pattern at the nuclear periphery (data not shown). This peripheral localization may be expected for chromosomes just after telophase.

A dispersed punctate pattern of telomeric DNA is also seen with telomere specific FISH in nuclear halos generated by in situ extraction with LIS (Fig. 5, D–F) and in nuclear matrices (Fig. 5, G–I). In both histone-depleted preparations the telomeric loci appeared highly condensed and their distribution in the nucleus seemed unaltered compared with Triton-extracted nuclei, indicating that no major rearrangements have taken place during the extraction procedure. This conclusion was further corroborated by in situ analysis of HeLa nuclear matrices prepared by extraction with 2 M NaCl (Berezney and Coffey, 1974), which

![Figure 4](image-url)
Figure 5. Visualization of telomeric DNA in HeLa-L nuclei, nuclear halos, and nuclear matrices by FISH. Nuclei were permeabilized in situ with Triton X-100, digested with RNase A, and fixed with 4% formaldehyde (A–C). For the isolation of nuclear matrices (D–F) and nuclear halos (G–I), cells were permeabilized with digitonin, stabilized at 37°C in the presence of Cu²⁺ ions, and extracted with LIS. Resulting nuclear halos were either fixed directly with 4% formaldehyde or first digested with PvuII plus HinfI to yield nuclear matrices. Preparations were hybridized with a biotin-labeled [CCCUAA]₂₇ RNA probe. Hybrids were visualized by immunofluorescence using an anti-biotin primary antibody and a secondary antibody coupled to FITC (C, F, and J). The specificity of the hybridization signal was verified on spreads of metaphase chromosomes (C, inset). Chromosomal DNA was stained with DAPI (B, E, and H), and A, D, and G show corresponding phase contrast images. Arrows in F mark several elongated hybridization signals.
resulted in a telomeric pattern that was indistinguishable from that of LIS-matrices (data not shown).

To investigate the colocalization of TRF protein with telomeric DNA in the nuclear matrix, we used a HeLa cell line that was stably transformed with an epitope-tagged version of mouse TRF ([HA]2-mTRF) (Chong et al., 1995; Broccoli et al., 1996b). The [HA]2-mTRF protein can be detected with an anti-HA monoclonal antibody and was previously shown to localize specifically to the ends of metaphase chromosomes and to colocalize with telomeric DNA in interphase nuclei (Chong et al., 1995). Similarly, we found that TRF colocalizes with telomeric DNA in nuclear matrix preparations. Fluorescent double-labeling experiments were performed with HeLa-L cells that express the tagged TRF protein. Telomeric DNA was visualized by FISH, as described above, and TRF was detected by immunofluorescence using an anti-HA antibody. In nuclear matrices, isolated in vitro, most telomeric DNA foci colocalized with TRF (Fig. 6, A–C). Intriguingly, most matrices contained several clear TRF foci that did not coincide with detectable telomeric DNA. Since in unextracted nuclei every TRF signal coincides with telomeric sequences (Chong et al., 1995), this observation suggests that a minority of the telomeric tracts become dissociated from the matrix preparations. Biochemical fractionation also indicates that a small proportion of the telomeres are disconnected during the isolation of nuclear matrices (Figs. 1 and 2). Thus, it appears that some of the TRF foci remain bound to the nuclear matrix even when the telomeric DNA is removed. Similarly, TRF is retained in nuclear matrix preparations that have been treated with DNase I (not shown). The simplest interpretation of these observations is that telomeric DNA is tethered to the nuclear matrix by TRF.

These observations were extended by similar double-labeling experiments on nuclear halos isolated in situ (Fig. 6, D–F). Also, in these preparations most telomeric DNA foci coincided with TRF. In nuclei, as well as in nuclear matrices and halos, the telomeric signals and the TRF

![Figure 6. Matrix-attached telomeric DNA colocalizes with TRF. Confocal laser scanning microscopic projections of optical sections of a nuclear matrix, isolated in vitro (A–C), and nuclear halos, isolated in situ (D–F), from HeLa-L cells that were stably transformed with a [HA]2-tagged mTRF gene. Telomeric DNA was detected by FISH using a biotin-labeled [CCUAA]27 RNA probe, an anti-biotin antibody and a secondary antibody coupled to FITC (A and D). Simultaneously, [HA]2-mTRF protein was visualized by immunofluorescence using an anti-HA antibody and a secondary antibody coupled to Sy-3 (B and E). The merged image of the two fluorochromes in A and B is shown in C, and that of D and E is shown in F. The arrow in D–F marks a partially decondensed telomere that extends out of the residual nucleus. The x-symbol in D–F marks a nuclear halo from a cell that does not express the [HA]2-mTRF protein.](image-url)
staining foci are very discrete (Figs. 5 and 6; Chong et al., 1995). This suggests that telomeres are highly condensed and that, despite the fact that the majority of histone proteins are extracted by LIS, no unfolding occurs during processing of the nuclei. However, in a number of nuclei a few telomeres display an alternate structure, revealing long linear tracts of telomeric sequences that extend outwards to the periphery of the halos (see arrowheads in Figs. 5 F and 6 F). Since this linear configuration is never observed in Triton-extracted nuclei, these strings probably represent experimental decondensation of previously folded telomeres. It is noteworthy that the linear telomeric signals were never coated with TRF (Fig. 6 and data not shown). In many cases a TRF spot could be detected at the base of the fluorescent string inside the residual nucleus and there appeared to be a strong correlation between the condensation of telomeres, their interaction with the nuclear matrix, and colocalization with TRF.

Electron Microscopy of Telomeric Loci in Interphase Nuclei

To investigate the ultrastructure of interphase telomeres and their location with respect to known nuclear structures, telomeric DNA was visualized by pre-embedding electron microscopical in situ hybridization (EM-ISH). Nuclear extraction with Triton X-100 and annealing of telomeric riboprobe were executed identically to the FISH experiments. Biotin-labeled hybrids were detected using antibodies coupled to ultrasmall gold particles and subsequently enlarged by silver enhancement. After the detection, samples were embedded and sectioned. Hybridization signals consisting of fewer than ten clustered gold particles appeared not specific for telomeric TTAGGG repeats, since they were also present outside the nucleus and in mock hybridized preparations (data not shown).

Consistent with the light microscopy of interphase telomeres in Figs. 5 and 6, telomeric loci appear highly condensed in electron micrographs. EM analysis also confirmed the presence of telomeres throughout the nuclear volume of permeabilized interphase nuclei (Fig. 7, A and B). While telomeres were sometimes observed near the nuclear envelope (5–10% of the signals), none of these telomeric loci appeared to make extensive contact with the nuclear lamina (Fig. 7 C). This result is consistent with the lack of enrichment of telomeric DNA in nuclear shell preparations. No specific association of telomeric DNA with nucleoli or clusters of interchromatin granules was observed.

The ultrastructural data in Fig. 7 are also consistent with our inference from biochemical fractionation that telomeric DNA is bound to the inner nuclear matrix. In EM analysis of nuclear matrices, telomeric gold clusters were associated with the internal granular material (Fig. 7 E), but we could not discern underlying telomere-specific protein structures. A notable feature of the telomeric gold clusters was their frequent occurrence in doughnut- or horse shoe–shaped configurations that may indicate that telomeres have a coiled conformation. This configuration was notably enhanced upon prolonged extraction of nuclei with Triton X-100 (for instance, Fig. 7 D). Whether these structures represent a specific conformation of the telomeric domains in vivo remains to be determined.

We determined the average number of telomeric hybridization signals per EM section, and calculated that per interphase nucleus 152 ± 39 (n = 22) telomeric signals are detected. Since HeLa-L cells contain 76 ± 6 (n = 10) chromosomes (Chong et al., 1995; this work), this number suggests that telomeres in these cells occur largely nonclustered. The subnuclear distribution of telomeres in the horizontal plane was analyzed on nonsequential EM sections cut parallel to the substratum. We found no evidence for a preferential peripheral position of the telomeric signals. In fact, the signal density in the outer 25% radial segment was somewhat lower than expected for a random distribution based on surface area or volume (Table I). It should be stressed that this type of analysis does not give any information about the distribution of telomeres in the vertical plane.

These data demonstrate that human telomeres are condensed elements that predominantly occupy nonperipheral sites in interphase nuclei, and are associated with the granular structure of the nuclear matrix.

Discussion

Mammalian chromosomes offer a unique opportunity for the structural analysis of telomeres because their telomeric TTAGGG repeat tracts are both well-defined and exceedingly long. As a result, their presence in interphase nuclei is readily detectable with telomere-specific hybridization techniques allowing for unequivocal identification and localization not afforded by other systems. The present study on the structure and localization of mammalian telomeres has revealed the close association of telomeric DNA with the telomeric protein, TRF, and the presence of these complexes in the internal network of interphase nuclei and nuclear matrix preparations. Telomeres appear to form compact structures that are attached to the nuclear matrix through multiple interactions along the TTAGGG repeat array, possibly through binding of TRF to the nuclear matrix.

Characterization of TRF on Telomeres

TRF is the major duplex telomeric DNA-binding activity in mammalian cells and the only telomeric specific protein so far identified in vertebrates (Zhong et al., 1992; Chong et al., 1995). In agreement with results obtained during purification of TRF from HeLa cells, Western analysis indicates that human TRF is a nuclear protein of ~60 kD that can be extracted from nuclei with 0.4 M KCl. TRF displays a strong association with the nuclear matrix, specifically with its inner fibrous network. TRF protein was not detectable in preparations enriched for nuclear envelope lamin proteins. These results are in agreement with immuno staining experiments in which epitope-tagged exogenous TRF is found in the internal compartment of the nucleus.

The present results indicate that TRF is predominantly associated with telomeric DNA. In fractionation experiments, TRF and telomeric sequences are both recovered in the inner nuclear matrix. Neither the telomeric protein nor the telomeric DNA is found to be enriched in nuclear shells which represent a peripheral substructure of nuclei.
Figure 7. Visualization of telomeric DNA in HeLa-L nuclei and nuclear matrices by EM-ISH. For the isolation of permeabilized nuclei (A–D), cells were extracted in situ with Triton X-100 for either 3 (A–C) or 10 min (D), digested with RNase A, and fixed with 4% formaldehyde plus 0.05% glutaraldehyde. For the isolation of nuclear matrices (E) cells were permeabilized with digitonin, stabilized at 37°C in the presence of Cu²⁺ ions, and extracted with LIS. Resulting nuclear halos were digested with PvuII plus HinfI and fixed with 4% formaldehyde plus 0.05% glutaraldehyde. Preparations were hybridized with a biotin-labeled [CCCUAA]₂₇ RNA probe, and hybrids were visualized by immunolabeling using an anti-biotin primary antibody and a secondary antibody coupled to ultrasmall gold particles. Gold particles were enlarged by silver enhancement, and preparations were embedded and cut into 280-nm thick sections. N, nucleus; Nu, nucleolus; C, cytoskeletal element; Ig, interchromatin granule cluster. Bars: (A, B, and E) 1 μm; (C and D) 0.2 μm.
Anchoring of the Telomeric Complex in the Nuclear Matrix

A minor fraction of the telomeric DNA can become dislodged from the nuclear matrix and is recovered in the soluble fraction. Since this fraction appears devoid of TRF, it is possible that the disconnected telomeric fragments represent telomeric complexes that were disrupted by the isolation procedure.

Immunostaining experiments further demonstrate the colocalization of TRF with telomeres. In nuclei, as well as in nuclear matrices, double labeling for TRF protein and telomeric DNA results in overlapping patterns in which the majority of the signals coincide. The only exceptions are found in nuclear matrix preparations where occasional TRF foci appear to lack telomeric DNA. Most likely these sites represent previous attachment points of the minority of telomeric DNA fragments that become disconnected during isolation of nuclear matrices. Thus, the results further corroborate the view that TRF is a structural component of all telomeres and that most TRF in cells is actually complexed with telomeric DNA.

The immunostaining experiments presented here relied on the expression of an epitope-tagged version of mouse TRF. We have recently been able to demonstrate endogenous human TRF in association with telomeric DNA in interphase nuclei (Schäfer, M., B. van Steensel, and T. de Lange, unpublished data). In an examination of endogenous human TRF in cells transfected with the epitope-tagged mouse TRF, we have been unable to discern any differences in the localization of these two proteins or a change in localization compared to untransfected cells. In addition, it should be noted that the overexpression of the epitope-tagged TRF from the construct used here is moderate (approximately fivefold; Schäfer, M., B. van Steensel, and T. de Lange, unpublished data), making it unlikely that this system introduces major artifacts due to overexpression of the protein.

Anchoring of the Telomeric Complex in the Nuclear Matrix

Human and mouse telomeres are attached to the nuclear matrix. The present work establishes that this interaction involves multiple matrix attachment sites along the double-stranded TTAGGG repeat region. We estimate that a nuclear matrix binding site occurs at least once in every kb of the telomeric tract. Large regions of telomeric DNA display this type of interaction. According to rough estimates, human telomeres with up to 20–30 kb of TTAGGG repeats are decorated along their entire length with such dispersed nuclear matrix interaction sites. From these observations a picture emerges in which most mammalian telomeres have frequent multiple interactions with the nuclear matrix over a large domain that encompasses the whole telomere at most chromosome ends. Thus, telomeres are not only repetitive in their DNA sequence but also have reiterated properties in terms of their interactions in the nuclear matrix.

Extended regions that are not bound to the nuclear matrix were detected in one mouse cell line with telomeric tract of >50 kb. In these telomeres, approximately half of the telomeric DNA can be released as long soluble fragments. The remainder of the telomeric sequences behave as attached domains in which nuclear matrix attachment sites occur every 1–2 kb. Whether these two domains coexist within individual telomeres is not clear at present. In addition, since this particular configuration was only observed in a single cell line, it remains to be seen whether a domain of unattached telomeric DNA is a general property of very long telomeres.

The array of nuclear matrix attachment sites along the duplex telomeric DNA tract can be explained if a double-stranded TTAGGG repeat binding factor is responsible for the tethering. Since TRF binds duplex telomeric DNA and fractionates to the nuclear matrix, it is a likely candidate for the anchorage factor. If TRF binds the telomeric tract to the nuclear matrix, its abundance must be sufficient to provide at least one tethering site per kb of telomeric DNA. TRF activity was previously estimated at ~5–10 binding units per telomere based on the abundance of the binding factor in nuclear extracts of HeLa cells. If TRF binds at dispersed sites along the TTAGGG repeat array, this expression level would represent sufficient TRF to anchor telomeric tracts once every kb. A dispersed binding mode for TRF is consistent with the fact that TRF does not display strong cooperative interactions in vitro (Zhong et al., 1992; Chong, L., and T. de Lange, unpublished data). In addition to TRF, the anchorage of telomeres may well involve other, as yet unidentified, telomere-associated proteins. An obvious candidate to be considered is the TRF-related protein that emerged from genome sequencing efforts (Genbank X93512; Bilaud et al., 1996). Whether this protein, like TRF, binds telomeric DNA in vivo remains to be determined.

Previous work showed no nuclear matrix attachment for a 0.8-kb stretch of TTAGGG repeats that was integrated at a chromosome-internal site by transfection (de Lange, 1992). Similarly, we found that a stretch of 1.6 kb of internal TTAGGG repeats was not bound to the nuclear matrix (Lundérus, M.E.E., and T. de Lange, unpublished results). One possibility is that the TTAGGG repeats require the proximity of a telomere terminus (or a terminus-associated factor) to initiate or maintain interaction with the matrix. A second possibility, raised by the current work, is that the chromosome-internal TTAGGG repeat fragments fail to capture TRF. If binding of TRF is a limiting factor in the attachment process, a 1–2-kb internal

### Table 1. Horizontal Distribution of Telomeres in HeLa-L Nuclei

| Fraction of nuclear radius* | Telomeric signals† Based on volume | Based on surface area |
|-----------------------------|-----------------------------------|----------------------|
|                             | %                                 | %                    |
| 0–0.25                      | 8.0 ± 7.1                         | 1.6                  | 6.25                 |
| 0.25–0.50                   | 24.7 ± 10.1                       | 10.9                 | 18.75                |
| 0.50–0.75                   | 33.8 ± 13.2                       | 29.7                 | 31.25                |
| 0.75–1                      | 33.3 ± 8.9                        | 57.8                 | 43.75                |

*Given as relative distance from the nuclear center.
†The horizontal location of telomeric signals was scored in 22 nonsequential nuclear EM sections as a fraction of the local nuclear radius. Each section contained in the order of 10 signals. The relative distribution of telomeric signals over four concentric nuclear rings is expressed as mean ± SD.
‡Since the HeLa nuclei used in these experiments have a flattened shape, the random distribution is represented by values in between those calculated based on volume and based on surface area.
TTAGGG repeat array may fail to compete for TRF with ~500-kb of telomeric tracts at the telomeres in the transfected cells.

Condensed Telomeric Loci

Ultrastructurally, telomeres appear as compact nucleoprotein structures. EM-ISH detection revealed individual telomeres as tight clusters of gold particles. The apparent compressed configuration was also obvious in FISH analysis of interphase nuclei. Cytological evidence was obtained that the interaction of human telomeres with the nuclear matrix contributes to their condensation. Fluorescent labeling of telomeric DNA in nuclear halos from HeLa cells revealed that the majority of the telomeres remained tightly condensed as discrete spots within the nuclear matrix. In addition, there was a minor population of unfolded telomeres which were labeled as fluorescent strings extending out of the residual nucleus. These decondensed telomeres are likely to represent the minority of telomeric fragments that are released from the nuclear matrix in vitro (de Lange, 1992; this report, Fig. 1).

A correlation between the level of DNA packaging and the interaction with the nuclear matrix has also been reported for nontelomeric sequences. By fluorescence hybridization to nuclear halos, Gérases et al. (1994) reported that a given sequence either remained condensed as a single spot within the residual nucleus or produced a fluorescent string on the extended portion of the DNA halo. In most of these cases, the observed configuration correlated with the functional status of the sequence; only replicating DNA and transcriptionally active sequences were tightly packaged and matrix-attached. These observations extend earlier findings that many key nuclear processes, including DNA replication (Dijkwel et al., 1979; Pardoll et al., 1980; Nakayasu and Berezney, 1989; Hózak et al., 1993) and RNA synthesis and processing (Ciejek et al., 1982; Jackson and Cook, 1985; Xing and Lawrence, 1991), are matrix-bound (for review see van Driel et al., 1995). Thus, it will be of interest to examine whether telomere replication activities, such as telomerase, are similarly associated with the nuclear matrix.

Subnuclear Distribution of Human Telomeres

Our cytological observations failed to reveal strong clustering of telomeres in interphase. Although we cannot exclude that telomere dimers occur, our data argue against wide-spread occurrence of higher order clusters. In situ hybridization with telomere specific probes or the application of more sensitive detection techniques are required to further address the question of telomere-telomere associations in interphase. In addition, it will be of interest to determine whether telomere clustering occurs in other cell types.

The telomeric signals were generally detected throughout the nuclear volume. EM-ISH showed that matrix-attached telomeres are embedded in the granular material of the internal nuclear matrix. No significant association of telomeric loci with nucleoli or interchromatin granules clusters was observed. EM-ISH analysis of the horizontal distribution of telomeric loci in interphase nuclei revealed that these appear to be located significantly more inter-

Comparison with Yeast Telomeres

It is of interest to compare our findings on the mammalian telomeric complex with observations on the telomeres of yeast, which like mammalian telomeres are known to complex with a duplex telomeric DNA-binding protein (Buchman et al., 1988; Longtine et al., 1989). Although the yeast telomeric protein, Rap1p, is not a homologue of the mammalian TRF proteins, these factors have a number of shared properties. Both recognize telomeric sequences using a Myb-type three helix bundle, bend DNA, and bind along the length of the telomeric tract (for review see Smith and de Lange, 1997). Similar to the requirement for TRF binding sites for telomere formation (Hanish et al., 1994), binding sites for Rap1p promote efficient telomere healing in yeast (Lustig et al., 1990). The present work reveals an additional similarity of these factors, their association with the nuclear matrix. Although it has not been shown that yeast telomeres bind to the nuclear matrix, Rap1p fractionates with this structure (Hofmann and Gasser, 1989).

However, prominent differences between the yeast telomeric complex and what is reported here for mammalian telomeres should be noted as well. Specifically, the subnuclear distribution of yeast telomeres has been inferred to be radically different. The subnuclear distribution of yeast telomeric loci was first inferred based on Rap1p staining patterns (Klein et al., 1992) and recently verified by in situ hybridization with subtelomeric sequences (Gotta et al., 1996). These data suggest that unlike mammalian telo-
meres, yeast telomeres are highly clustered in groups of four. Furthermore, while yeast telomeres do not associate directly with the nuclear envelope, they do occur predominantly in the outer 50% volume segment of yeast nuclei. No such preferential distribution at the outskirts of nuclei was noted for human telomeres in this study. Thus, while peripheral positioning and clustering of yeast telomeres may be important for their function, this aspect of telomere biology is unlikely to be conserved in somatic mammalian cells.

**Telomerase and the Telomeric Complex**

Mammalian chromosomes terminate in a conserved large complex of telomeric DNA and telomere-associated proteins, including TRF. This complex functions to protect chromosome ends and plays an important role in the replication of telomere termini. Mounting evidence suggests that changes in telomere length maintenance, in particular the activation of telomerase, play a role in malignant transformation. It will be of interest to study the subnuclear localization of telomerase in normal and in tumorigenic mammalian cells vis-à-vis the telomeric complex we have described here.

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