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

M.E. Eva Ludérus, Bas van Steensel, Laura Chong, Ody C.M. Sibon,* Fons F.M. Cremers,* and Titia de Lange

Laboratory for Cell Biology and Genetics, The Rockefeller University, New York, NY 10021; and *Department of Molecular Cell Biology, Institute of Biomembranes, Universiteit Utrecht, 3584 CH Utrecht, The Netherlands

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; Feng et al., 1995; 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, 1994*a*, *b*; Kim et al., 1994; Blasco et al., 1996; Broccoli et al., 1996*a*).

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; Kyrion et al., 1992; McEachern and Blackburn, 1995) and alterations in telomeric protein complex compromise the stability of chromosome ends (for review see

Address all correspondence to T. de Lange, The Rockefeller University, Box 159, 1230 York Avenue, New York, NY 10021-6399. Tel.: (212) 327-8146. Fax: (212) 327-7147. E-Mail: delange@rockvax.rockefeller.edu

The current address of O.C.M. Sibon is the Department of Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794.

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)[1], was recently identified and cloned (Zhong et al., 1992; Chong et al., 1995). hTRF is a novel Myb-related protein of ~60 kD 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). Mouse TRF (mTRF) has considerable sequence similarity to hTRF, indicating that mammalian telomeres form a highly conserved nucleoprotein complex (Broccoli et al., 1996*b*).

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 $[HA]_2$-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 DMEM with 10% iron-supplemented bovine serum. Cells were harvested at 50–70% confluency. Suspension cultures of human nontransformed lymphoblastoid SU-LyB-1 cells (Saltman et al., 1993) were grown in RPMI 1640 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.

Isolation of Nuclear Matrices and Nuclear Shells

Nuclear matrices were isolated as described previously (de Lange, 1992) following the lithium diiodosalicylate (LIS) extraction procedure of Mirkovitch 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 Hepes-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 Cu^{2+} ions, which serves to stabilize the internal structure of the nuclear matrix (Izaurralde et al., 1988; Ludérus et al., 1992), was replaced by an incubation on ice without Cu^{2+}. LIS-generated nuclear shell halos were collected by centrifugation for 15 min at 4°C in an Eppendorf microcentrifuge at maximal speed.

Halo preparations were gently washed once in MWB (20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.4]) with 0.1% digitonin, twice in MWB, and twice in the appropriate restriction endonuclease digestion buffer. Nuclear halos were digested to completion with the appropriate restriction enzyme (as indicated in the text) and the resulting nuclear matrices were pelleted by centrifugation. To purify matrix/shell-bound and released restriction fragments from pellet and supernatant fraction, respectively, protein in both fractions was digested overnight at 37°C with 100 μg/ml proteinase K in 10 mM EDTA, 0.5% SDS, 10 mM Tris-HCl [pH 7.4]. Subsequently, the DNA was purified by phenol-chloroform extraction and isopropanol precipitation.

DNase I Mapping Experiments

Freshly isolated nuclear matrices, digested with restriction endonuclease, 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 matrices per ml of the same buffer. DNase I was added (final concentrations of 0, 25, 50, 100, 250, or 500 ng/ml) to 500 μl samples, and digestion was carried out for 15 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-attached 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 pTH2Δ (de Lange et al., 1990). To detect telomeric TTAGGG repeats on blots of mouse genomic DNA, the oligonucleotide AAAACTCGAC$[TTAGGG]_3$TTAGG, end-labeled using [γ-^{32}P]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 55°C. Genomic blots from J558 cells, which have telomeres between 50- and 150-kb long, were hybridized in the presence of a 10-fold molar excess of unlabeled probe oligonucleotide. Blots were washed in 4 × SSC, 0.1% SDS at room temperature and subsequently at 65°C. Hybridization signals were visualized by autoradiography at -80°C on Kodak XAR film using intensifying screens, and analyzed quantitatively with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Production of Anti-TRF Antibody

A 16–amino acid peptide (peptide I5) of sequence (NH_2)-TSQDKPSGNDVEMETC-(COOH), encompassing residues 273-287 of human TRF plus a COOH-terminal cysteine was synthesized (Rockefeller University peptide synthesis facility) and coupled to Imject 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 Sulfolink 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 A_2/B_1 by mAb 7A9 (Piñol-Roma and Dreyfuss, 1991; McKay and Cooke, 1992*b*), hnRNP D_1/D_2 by mAb 5B9 (Ishikawa et al., 1993), the internal matrix protein p160 by mAb AM88 (de Graaf et al., 1991), and the anti–lamin B mAb 101-B7 was purchased from Matritect (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).

For Western blotting of TRF, blots were probed for 16 h at 4°C with affinity–purified serum No. 5, diluted 1:200 in incubation buffer. Next, blots were washed three times for 10 min in 0.1% SDS, 0.5% deoxycholic acid, 1% NP-40 in PBS (wash buffer), incubated for 1–2 h at room temperature with horseradish peroxidase–conjugated goat anti–rabbit antibody (Amersham) in incubation buffer, and washed three times for 10 min in wash buffer, followed by detection by ECL. Similar results were obtained when ^{125}I-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 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. Procedures were essentially as described by Sibon et al. (1994).

For the in situ preparation of permeabilized nuclei, cells were washed twice with ice-cold PBS and once with ice-cold CSK buffer (100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 300 mM sucrose, 10 mM Pipes [pH 6.8]). Cells were extracted for 3 min on ice in CSK-buffer containing 0.5% (wt/vol) Triton X-100, washed with CSK buffer, and digested with 100 μg RNase A per ml CSK buffer for 10 min at 37°C. After two washes with CSK buffer at room temperature, the cells were fixed for 10 min at room temperature with 4% formaldehyde for light microscopy, or with 4% formaldehyde plus 0.05% glutaraldehyde for electron microscopy.

For the in situ preparation of nuclear halos and nuclear matrices, cells were washed twice in ice-cold PBS, twice in ice-cold CWB, and incubated in CWB with 0.1% digitonin plus 100 μg RNase A per ml for 15 min on ice. Permeabilized cells were then stabilized for 20 min at 37°C in CWB containing 0.1% digitonin and 0.5 mM $CuSO_4$, but without EDTA. Subsequently, they were extracted with 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 Hepes-KOH [pH 7.4]) for 10 min at room temperature, and washed several times in PvuII restriction endonuclease buffer. For the isolation of nuclear matrices, preparations were digested with PvuII plus HinfI for 2 h at 37°C. Nuclear matrices and nuclear halos were fixed as described above for permeabilized nuclei.

In Situ Hybridization. DNA denaturation, hybridization, and washing conditions were identical to those described by Sibon et al. (1994). A $[CCCUAA]_{27}$ repeat RNA probe was synthesized with SP6 polymerase in the presence of biotin-16-UTP (Boehringer Mannheim Corp., Indianapolis, IN) on HindIII-linearized plasmid pTH5 (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 (PBS with 0.1% cold water fish skin gelatin [Sigma] and 0.5% BSA). Biotin-labeled RNA-DNA hybrids were detected using a rabbit anti-biotin antibody (Enzo), and, subsequently, 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 (Amersham). All antibodies were diluted in PBG. DNA was stained with 0.2 μg 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-phenylendiamine (Johnson and De Nogeira 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 (Bio Rad Labs, Hercules, CA).

Detection of Hybrids by Immunoelectron Microscopy. After 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 ultrasmall gold particles (Aurion, Wageningen, The Netherlands), both diluted in PBG. The incubation with the gold-conjugated secondary antibody was performed for at least 16 h at room temperature. Coverslips were washed for 3 h in PBG and for 30 min in PBS. The samples were postfixed for 10 min with 1% glutaraldehyde in PBS, and the ultrasmall gold particles were enlarged by silver enhancement for 24 min (Danscher, 1981). The preparations were stained with 0.5% uranyl acetate in H_2O, dehydrated, and embedded in Epon. Nuclear preparations were cut parallel to the substratum in 0.28 μm sections using an Ultracut E (Reichert-Jung). Sections were examined in a Philips EM 420 electron microscope operated at 100 kV.

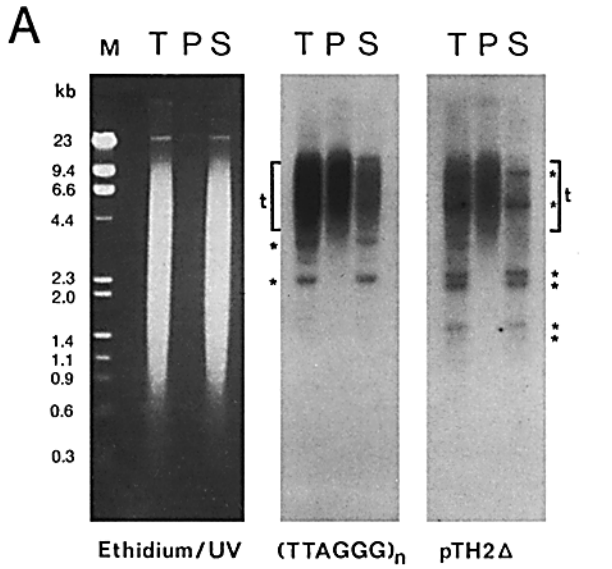
Analysis of the Subnuclear Distribution of Telomeric Loci

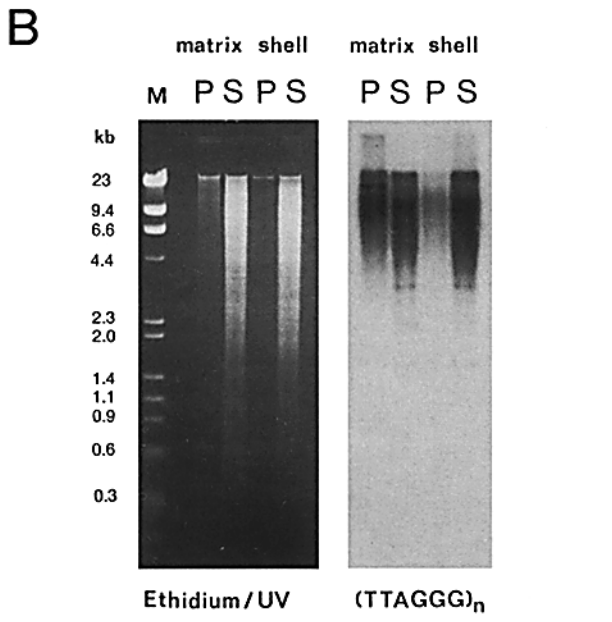
The analysis was performed on telomeric loci visualized by electron microscopical in situ hybridization (EM-ISH) in 22 nonsequential nuclear sections cut parallel to the substratum. Each nuclear section contained an average of 10 ± 3 telomeric signals. Outlines of nuclei were traced and their center was assigned as the origin of a two-dimensional XY coordinate system. For each telomeric signal the distance to the center relative to the local nuclear radius was measured. The nuclear surface was then subdivided into four concentric rings and the distribution of the telomeric signals over these respective segments was calculated for each section. Obtained distributions were averaged and the Students *t* test was used to determine significant deviations from a random distribution based on either surface area or volume.

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 Phosphorimager 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*,





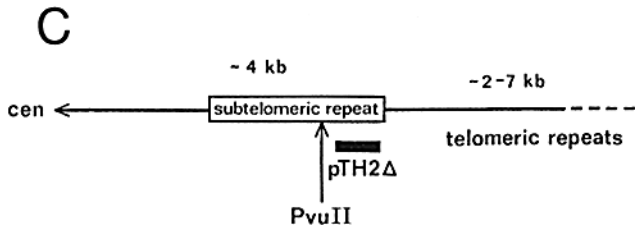


Figure 1. Telomeric DNA is predominantly attached to the internal nuclear matrix. (*A*) Quantitative comparison of matrix attachment of pTH2Δ telomeres with attachment of the total population of telomeres. Nuclear halos were isolated from HeLa-S cells and digested with PvuII. Matrix-attached (*P*) and released (*S*) PvuII fragments were separated by centrifugation and fractionated on a 0.8 % agarose gel alongside total PvuII-digested chromosomal DNA from nuclei that were not extracted with LIS (T). In each lane DNA samples derived from 10^6 nuclei was loaded. The panels show the ethidium/UV staining pattern of the DNA gel (*left*), the hybridization pattern of a TTAGGG repeat probe (*middle*), and the same Southern transfer probed with a pTH2Δ probe (*right*). 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.

middle 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*). Quantitation 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

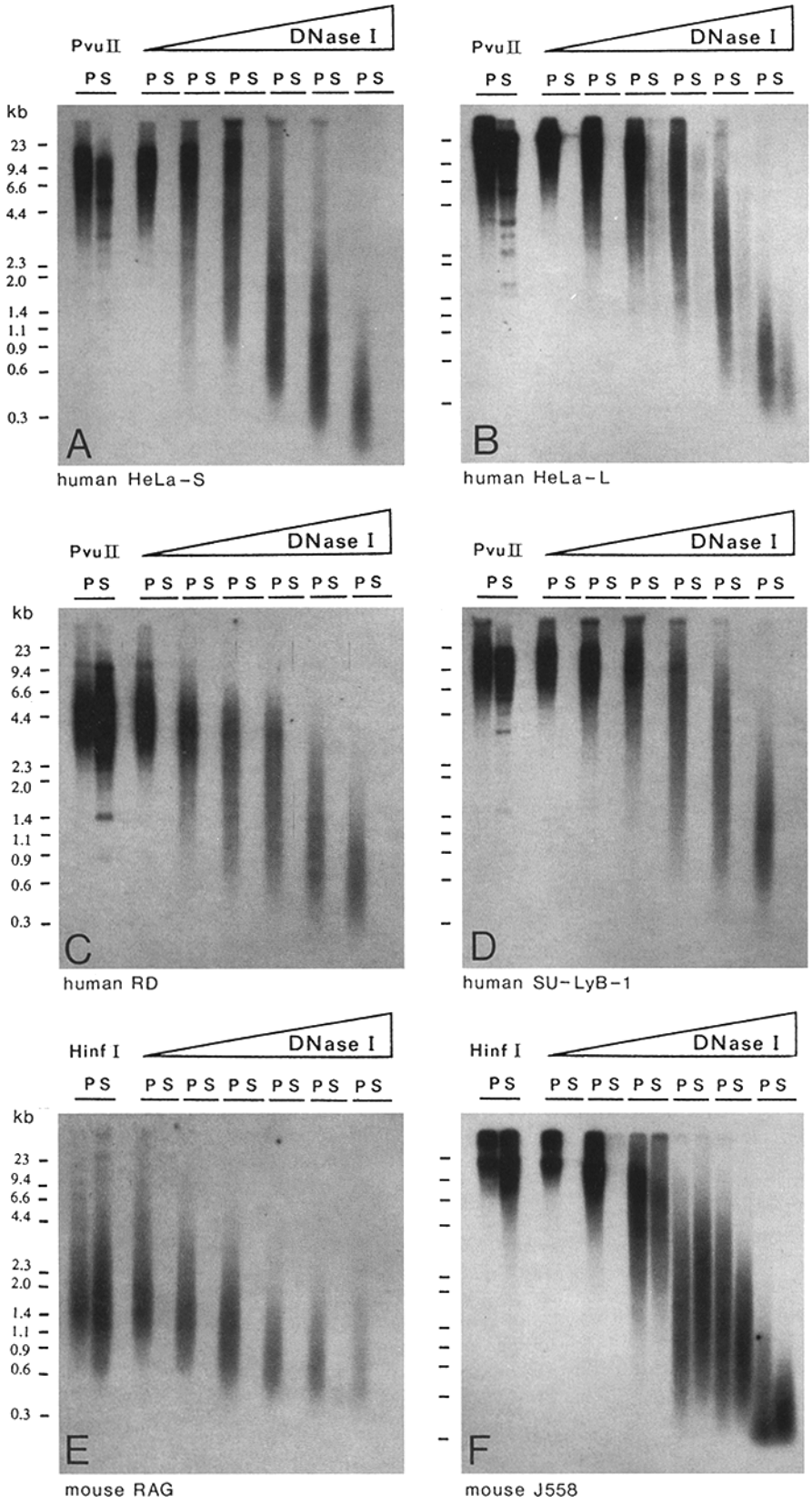


Figure 2. Telomeres contain long arrays of nuclear matrix attachment sites. Nuclear halos from human HeLa-S (*A*), HeLa-L (*B*), RD (*C*), and SU-LyB-1 cells (*D*) were digested with PvuII; nuclear halos from mouse RAG (*E*) and J558 cells (*F*) were digested with HinfI. Resulting nuclear matrices were collected by centrifugation to separate matrix-bound restriction fragments (*P*, PvuII; *P*, HinfI) from released restriction fragments (*S*, PvuII; *S*, HinfI). Samples of the sedimented nuclear matrices, containing matrix-bound telomeres, were digested with increasing amounts of DNase I (0, 25, 50, 100, 250, and 500 ng/ml). Digested matrices were spun down, and resulting fractions of matrix-bound (*P*) and DNase I–released (*S*) DNA fragments were fractionated on agarose gels alongside the original restriction enzyme-generated P and S fractions. In each lane, DNA derived from the same number of nuclei was loaded. To detect telomeric DNA, Southern transfers of the gels were hybridized with appropriate TTAGGG repeat probes, as described in Materials and Methods.

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 PvuII. The resulting matrix preparations with bound PvuII 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 and DNAse I digestion did not result in significant release of telomeric TTAGGG repeat DNA (Fig. 2 *E*). By contrast, the extremely long telomeres of BALB/c mouse plasmacytoma J558 cells, which carry on average greater than 50 kb of TTAGGG repeats (Kipling and Cooke, 1990; Tommerup et al., 1994; Broccoli, D., and M.E.E. Ludérus, unpublished observations), appeared to be only partially bound along their length. Severing of these telomeres into fragments shorter than 10 kb resulted in release of approximately half of the telomeric TTAGGG DNA from the matrix (Fig. 2 *F*). This release was telomere-specific, since no significant release of total matrix-bound chromosomal DNA was observed in these preparations (data not shown). We estimate that significant dissociation from the nuclear matrix occurs with fragments in the 10-kb size range.

The simplest explanation of the results in Fig. 2 *F* is that 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 regions are tethered to the nuclear matrix by multiple interactions, similar to what we observed for the shorter human telomeres and mouse RAG telomeres. These experiments demonstrate that long regions of double-stranded 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 an-

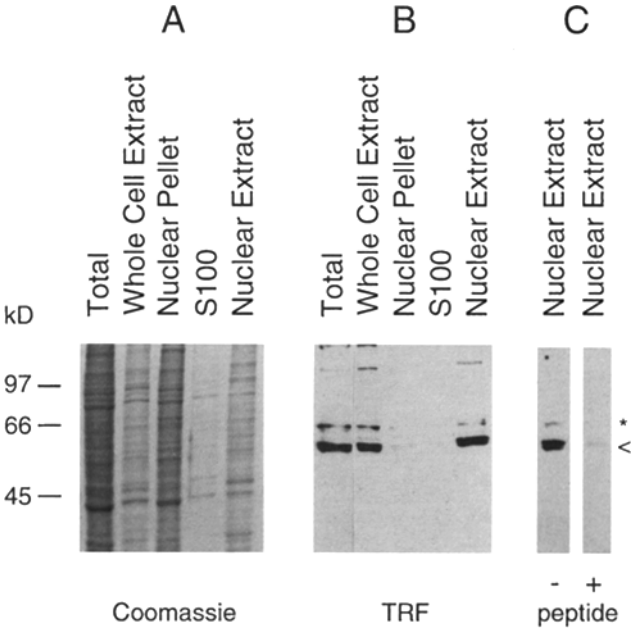


Figure 3. The telomeric protein TRF is a nuclear protein and can be extracted from chromatin with 0.4 M KCl. HeLa-S cells were either brought up in Laemmli loading buffer (*Total*) or extracted by hypotonic lysis to yield cytoplasmic proteins (*S100*) and crude nuclei. The nuclear fraction was extracted 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 cells 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 (*caret*). The nature of the protein band of ~70 kD (*asterisk*) 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

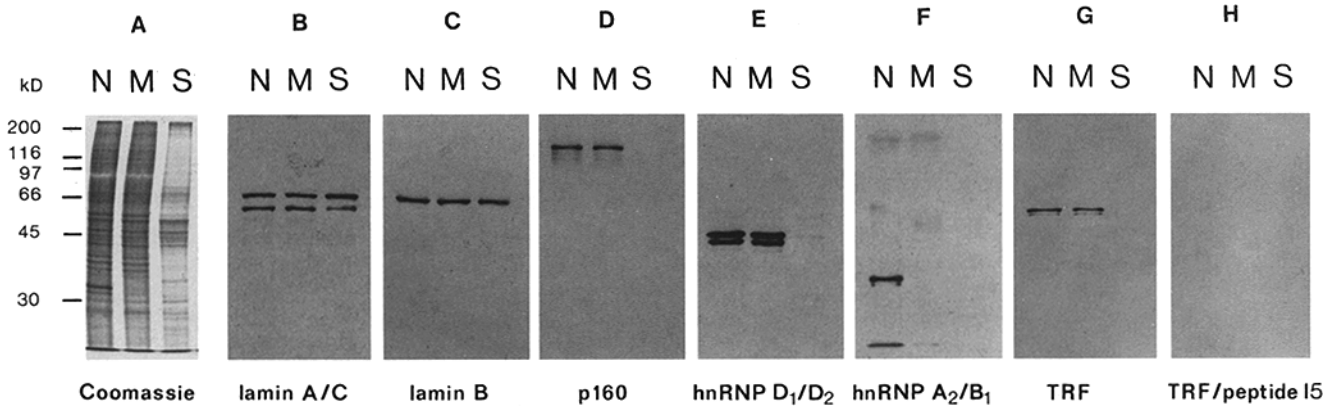


Figure 4. TRF is a component of the internal nuclear matrix. Proteins (equalized for cell number) from HeLa-S nuclei (*N*), nuclear matrices (*M*), and nuclear shells (*S*) were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue (*A*). The preparations also contained cytoskeletal intermediate filament proteins, which tend to persist in nuclear preparations of adherent tissue culture cells (Staufenbiel and Deppert, 1982; Payrastre et al., 1992). Western transfers of gels with identical samples were probed with antibodies recognizing lamins A and C (*B*), lamin B (*C*), internal matrix protein p160 (*D*), hnRNP D_1/D_2 (*E*), hnRNP A_2/B_1 (*F*), TRF (*G*), and antibodies to TRF in the presence of the peptide used for immunization (*H*).

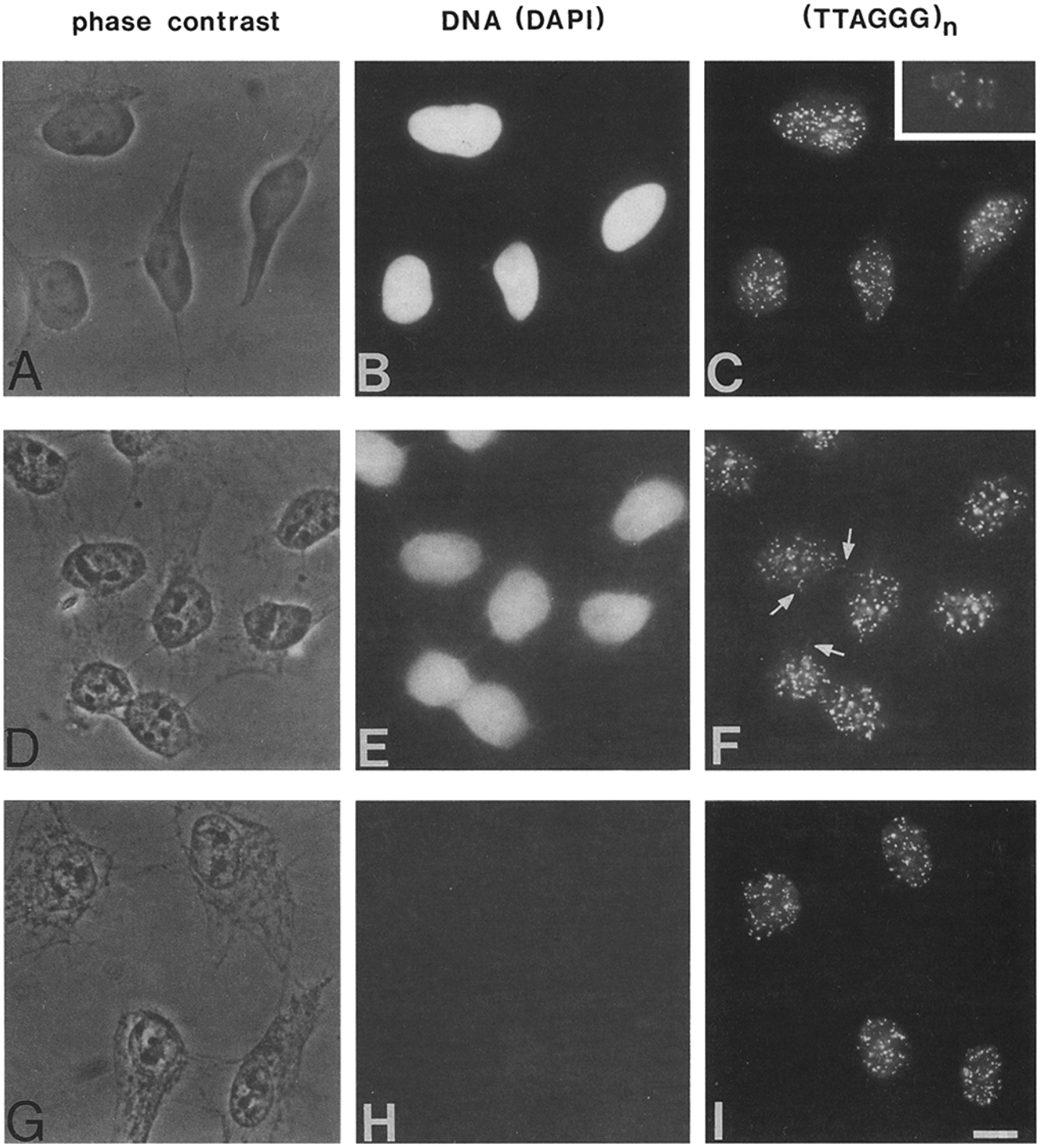


Figure 5. Visualization of telomeric DNA in HeLa-L nuclei, nuclear halos, and nuclear matrices by FISH. Nuclei were permeabilized in situ with Triton X-100, digested with RNase A, and fixed with 4% formaldehyde (*A–C*). For the isolation of nuclear matrices (*D–F*) and nuclear halos (*G–I*), cells were permeabilized with digitonin, stabilized at 37°C in the presence of Cu^{2+} 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., 1996*b*). 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

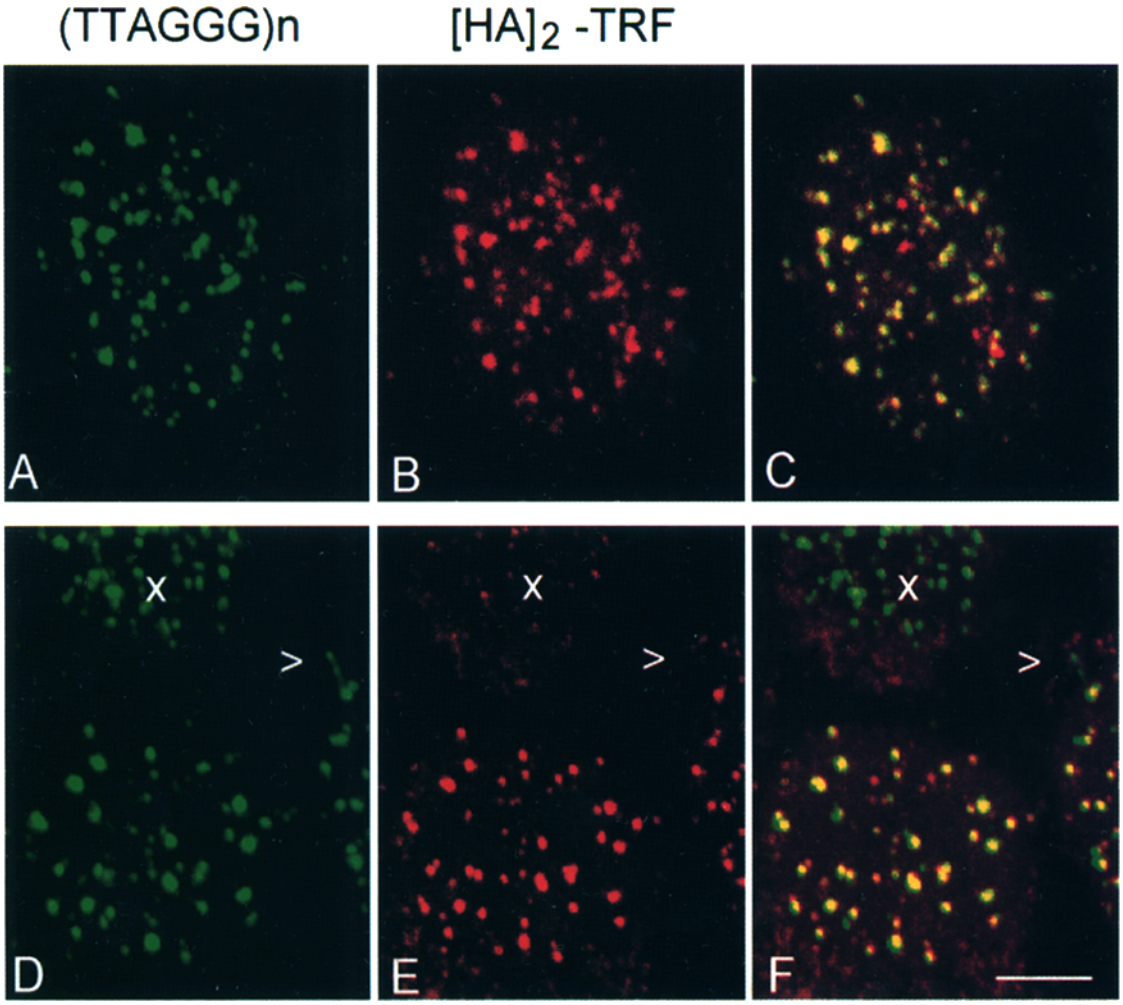


Figure 6. Matrix-attached telomeric DNA colocalizes with TRF. Confocal laser scanning microscopic projections of optical sections of a nuclear matrix, isolated in vitro (*A–C*), and nuclear halos, isolated in situ (*D–F*), from HeLa-L cells that were stably transformed with a $[HA]_2$-tagged mTRF gene. Telomeric DNA was detected by FISH using a biotin-labeled $[CCCUAA]_{27}$ 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.

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 substructures, 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 immunostaining 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.

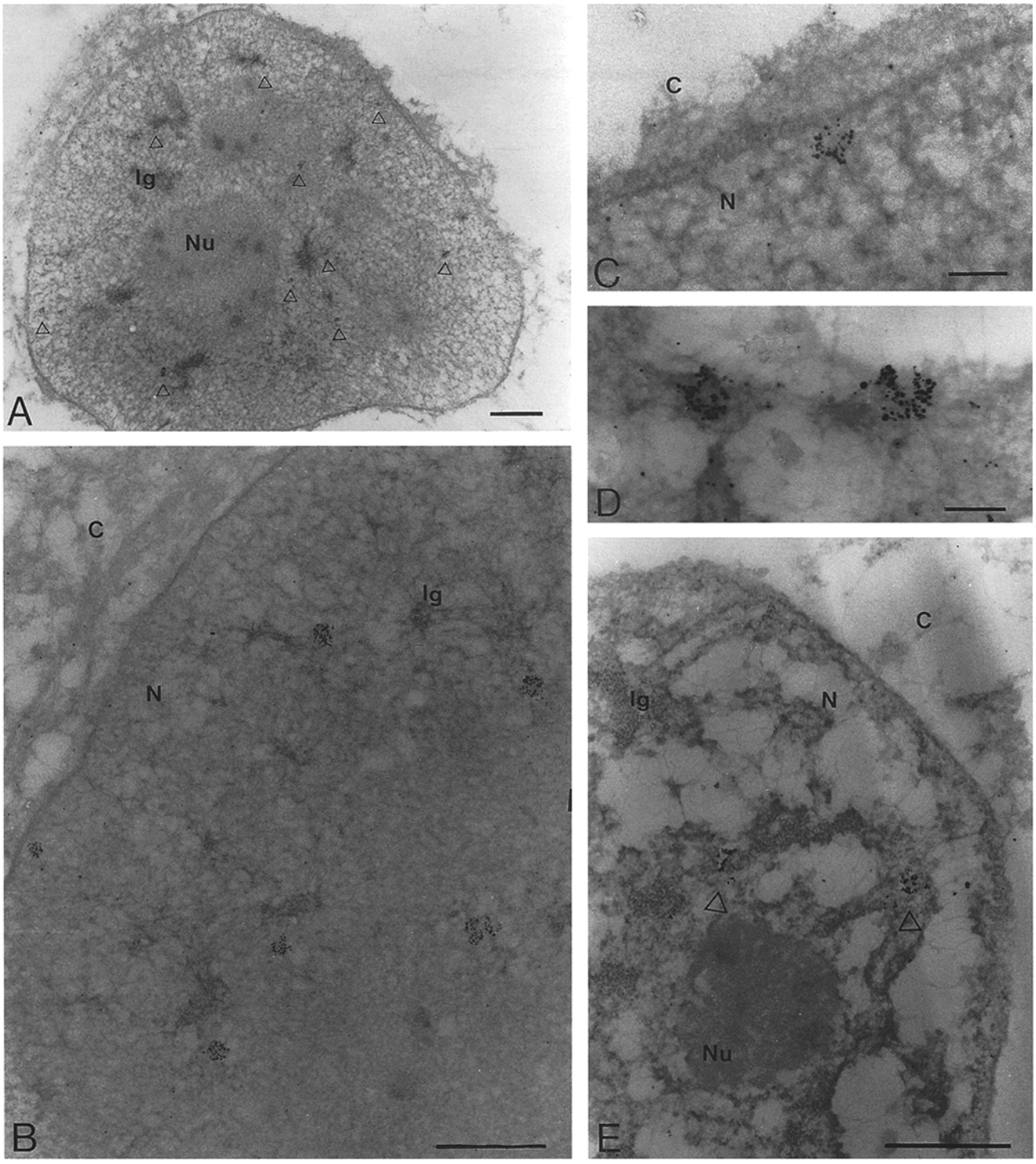


Figure 7. Visualization of telomeric DNA in HeLa-L nuclei and nuclear matrices by EM-ISH. For the isolation of permeabilized nuclei (*A–D*), cells were extracted in situ with Triton X-100 for either 3 (*A–C*) or 10 min (*D*), digested with RNase A, and fixed with 4% formaldehyde plus 0.05% glutaraldehyde. For the isolation of nuclear matrices (*E*) cells were permeabilized with digitonin, stabilized at 37°C in the presence of Cu^{2+} 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.

Table I. Horizontal Distribution of Telomeres in HeLa-L Nuclei

Fraction of nuclear radius*	Telomeric signals‡	Random distribution§	
		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.

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

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, Gerdes 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 internally than would have been expected from a random distribution. This finding is similar to the subnuclear distribution of telomeres reported for G1 phase mouse lymphocytes (Vourc'h et al., 1993) but contrasts the situation in plants (Rawlins et al., 1991) and Drosophila (Mathog et al., 1984; Hochstrasser et al., 1986) where chromosome ends are preferentially positioned near the nuclear envelope. By EM-ISH we sometimes observed telomeres at the periphery of the HeLa-L nuclei, but these did not appear to make extensive contact with the nuclear lamina. In agreement, our biochemical analysis indicates that only a minor fraction of the telomeric DNA is associated with nuclear shells, peripheral substructures enriched for the nuclear lamins.

Previous in vitro DNA binding studies have indicated that isolated lamin proteins (*A*, *C*, and *B*) can bind telomeric sequences. Initial studies demonstrated weak interaction of lamin polymers with short telomeric oligonucleotides (Shoeman and Traub, 1990). Further analysis indicated a specific, length-dependent, high affinity interaction between G-rich telomeric repeat arrays and mammalian lamin aggregates. However, these interactions depend on the presence of single stranded telomeric DNA within the binding probes (Ludérus, M.E.E., unpublished data). The binding affinity of lamin polymers for single stranded DNA has been noted before (Ludérus et al., 1994). In light of the finding that mammalian telomeres have no extensive contacts with the lamina in interphase of mitotic cells, the significance of the propensity of lamins to associate with single-stranded telomeric DNA remains to be determined.

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.

We are indebted to Willem Hage (Hubrecht Laboratorium, Utrecht, The Netherlands) for expert assistance with confocal scanning laser microscopy. Lisette Verspui (Universiteit Utrecht, Utrecht, The Netherlands) is thanked for excellent photographical work. We are grateful to Dr. Gideon Greyfuss (University of Pennsylvania, Philadelphia, PA) and Dr. Brian Burke (Harvard Medical School, Boston, MA), and Dr. Roel van Driel (University of Amsterdam, The Netherlands) for generous gifts of anti-hnRNP antibodies 7A9 and 5B9, and anti-lamin antibody 41CC4, and anti-p160 antibody AM88, respectively. Art Lustig (Memorial Sloan-Kettering Cancer Center), Richard Lang (New York University), and members of the de Lange lab are thanked for discussion of this work.

This study was funded by a fellowship to M.E.E. Ludérus from the Charles H. Revson Foundation, a fellowship to B.v. Steensel from the Human Frontier Science Program, and by grants to T. de Lange from the National Institutes of Health and the Lucille P. Markey Charitable Trust.

Received for publication 26 June 1996 and in revised form 20 August 1996.

References

Autexier, C., and C.W. Greider. 1996. Telomerase and cancer: revisiting the telomere hypothesis. *Trends Biol. Sci.* In press.

Belgrader, P., A.J. Siegel, and R. Berezney. 1991. A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. *J. Cell Sci.* 98:281–291.

Berezney, R., and D.S. Coffey. 1974. Identification of a nuclear protein matrix. *Biochem. Biophys. Res. Commun.* 60:1410–1417.

Berezney, R., M.J. Mortillaro, H. Ma, X. Wei, and J. Smarabandu. 1995. The nuclear matrix: a structural milieu for genomic function. *Int. Rev. Cytol.* 162A:1–65.

Bilaud, T., C.E. Koering, E. Binet-Brasselet, K. Ancelin, A. Pollice, S.M. Gasser, and E. Gilson. 1996. The telobox, a Myb-related telomeric DNA binding motif found in proteins from yeast, plants, and human. *Nucleic Acids Res.* 24: 1294–1303.

Blackburn, E. 1993. Telomerase. *In* The RNA World. R. Gesteland and J. Atkins, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 557–576.

Blasco, M.A., M. Rizen, C.W. Greider, and D. Hanahan. 1996. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nature Genet.* 12:200–204.

Broccoli, D., L.A. Godley, L.A. Donehower, H.E. Varmus, and T. de Lange. 1996*a*. Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol. Cell. Biol.* 16:3765–3772.

Broccoli, D., L. Chong, S. Oelmann, A.A. Fernald, N. Marziliano, B. van Steensel, D. Kipling, M.M. Le Beau, and T. de Lange. 1996*b*. Comparison of the human and mouse genes encoding the telomeric protein, TRF: chromosomal localization, expression, and conserved protein domain. *Hum. Mol. Genet.* In press.

Buchman, A.R., W.J. Kimmerly, J. Rine, and R.D. Kornberg. 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 8:210–225.

Burke, B., J. Tooze, and G. Warren. 1983. A monoclonal antibody which recognizes each of the nuclear lamin polypeptides in mammalian cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:361–367.

Chong, L., B. van Steensel, D. Broccoli, H. Erdjument-Bromage, J. Hanish, P. Tempst, and T. de Lange. 1995. A human telomeric protein. *Science (Wash. DC).* 270:1663–1667.

Church, G.M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* 81:1991–1995.

Ciejek, A.M., J.L. Nordstrom, M.-J. Tsai, and B.W. O'Malley. 1982. Ribonucleic acid precursors are associated with the chick oviduct nuclear matrix. *Biochemistry.* 21:4945–4953.

Cooke, H.J., and B.A. Smith. 1986. Variability at the telomeres of the human X/Y pseudo-autosomal region. *Cold Spring Harbor Symp. Quant. Biol.* 51: 213–219.

Counter, C.M., A.A. Avilion, C.E. LeFeuvre, N.G. Stewart, C.W. Greider, C.B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO (Eur. Mol. Bio. Organ.) J.* 11:1921–1929.

Counter, C.M., F.M. Botelho, P. Wang, C.B. Harley, and S. Bacchetti. 1994*a*. Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. *J. Virol.* 68:3410–3414.

Counter, C.M., H.W. Hirte, S. Bacchetti, and C. Harley. 1994*b*. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA.* 91:2900–2904.

Danscher, G. 1981. Localization of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry.* 71:81–88.

de Graaf, A., P.M.P. van Bergen en Henegouwen, A.M.L. Meijne, R. van Driel, and A.J. Verkleij. 1991. Ultrastructural localization of nuclear matrix proteins in HeLa cells using silver-enhanced ultra-small gold probes. *J. Histochem. Cytochem.* 39:1035–1045.

de Lange, T. 1992. Human telomeres are attached to the nuclear matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:717–724.

de Lange, T. 1995. Telomere dynamics and genome instability in human cancer. *In* Telomeres. E.H. Blackburn and C.W. Greider, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 265–295.

de Lange, T., L. Shiue, R.M. Myers, D.R. Cox, S.L. Naylor, A.M. Killery, and H.E. Varmus. 1990. Structure and variability of human chromosome ends. *Mol. Cell. Biol.* 10:518–527.

Dijkwel, P.A., L.H.F. Mullenders, and F. Wanka. 1979. Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase nuclei. *Nucleic Acids Res.* 6:219–230.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Feng, J., W.D. Funk, S.-S. Wang, S.L. Weinrich, A.A. Avilion, C.-P. Chiu, R.R. Adams, E. Chang, R.C. Allsopp, J. Yu et al. 1995. The RNA component of human telomerase. *Science (Wash. DC).* 269:1236–1241.

Fey, E.G., G. Krochmalnik, and S. Penman. 1986. The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102:1654–1665.

Gerdes, M.G., K.C. Carter, P.T. Moen Jr., and J.B. Lawrence. 1994. Dynamic changes in the higher-level chromatin organization of specific sequences revealed by in situ hybridization to nuclear halos. *J. Cell Biol.* 126:289–304.

Hanish, J.P., J. Yanowitz, and T. de Lange. 1994. Stringent sequence requirements for telomere formation in human cells. *Proc. Natl. Acad. Sci. USA.* 91: 8861–8865.

Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature (Lond.).* 345:458–460.

Hastie, N.D., M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green, and R.C. Allshire. 1990. Telomere reduction in human colorectal carcinoma and with ageing. *Nature (Lond.).* 346:866–868.

He, D., J.A. Nickerson, and S. Penman. 1990. Core filaments of the nuclear matrix. *J. Cell Biol.* 110:569–580.

Hochstrasser, M., D. Mathog, Y. Gruenbaum, H. Saumweber, and J.W. Sedat. 1986. Spatial organization of chromosomes in the salivary gland nuclei of *Drosophila melanogaster. J. Cell Biol.* 102:112–123.

Hofmann, J.F.-X., and S.M. Gasser. 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus HML. *Cell.* 57:725–737.

Hozák, P., A.B. Hassan, D.A. Jackson, and P.R. Cook. 1993. Visualization of replication factories attached to a nucleoskeleton. *Cell.* 73:361–373.

Gotta, M., T. Laroche, A. Formenton, L. Maillet, H. Scherthan, and S.M. Gasser. 1996. Cytological evidence for the clustering of telomeres and their colocalization with Rap1p, Sir3 and Sir4 proteins in wild-type *Saccharomyces cerevisiae. J. Cell Biol.* In press.

Ishikawa, F., M.J. Matunis, G. Dreyfuss, and T.R. Cech. 1993. Nuclear proteins that bind the pre-mRNA 3′ splice site sequence r(UUAG/G) and the human telomeric DNA sequence $d(TTAGGG)_n$. *Mol. Cell. Biol.* 13:4301–4310.

Izaurralde, E., J. Mirkovitch, and U.K. Laemmli. 1988. Interaction of DNA with nuclear scaffolds in vitro. *J. Mol. Biol.* 200:111–125.

Jackson, D.A., and P.R. Cook. 1985. Transcription occurs at a nucleoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:919–925.

Jackson, D.A., and P.R. Cook. 1988. Visualization of a filamentous nucleoskeleton with a 23 nm axial repeat. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3667–3677.

Johnson, G.D., and G.M. De Nogueira Araujo. 1984. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Meth.* 43:349–350.

Kim, N.W., M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, and J.W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science (Wash. DC).* 266:2011–2015.

Kipling, D., and H.J. Cooke. 1990. Hypervariable ultra-long telomeres in mice. *Nature (Lond.).* 347:400–402.

Klein, F., T. Laroche, M.E. Cardenas, J.F.-X. Hofmann, D. Schweizer, and S.M. Gasser. 1992. Localization of RAP1 and topoisomerase II in nuclei and meiotic chromosomes of yeast. *J. Cell Biol.* 117:935–948.

Kyrion, G., K.E. Boakye, and A.J. Lustig. 1992. C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 12:5159–5173.

Lejnine, S., V.L. Makarov, and J.P. Langmore. 1995. Conserved nucleoprotein structure at the ends of vertebrate and invertebrate chromosomes. *Proc. Natl. Acad. Sci. USA.* 92:2393–2397.

Ludérus, M.E.E., A. de Graaf, E. Mattia, J.L. den Blaauwen, M.A. Grande, L. de Jong, and R. van Driel. 1992. Binding of matrix attachment regions to lamin B_1. *Cell.* 70:949–959.

Ludérus, M.E.E., J.L. den Blaauwen, O.J.B. de Smit, D.A. Compton, and R. van Driel. 1994. Binding of matrix attachment regions to lamin polymers involves single-stranded regions and the minor groove. *Mol. Cell. Biol.* 14: 6297–6305.

Lustig, A.J., S. Kurtz, and D. Shore. 1990. Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. *Science (Wash. DC).* 250:549–553.

Longtine, M., N. Wilson, M. Petracek, and J. Berman. 1989. A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. *Curr. Genet.* 16:225–239.

Mathog, D., M. Hochstrasser, Y. Gruenbaum, H. Saumweber, and J.W. Sedat. 1984. Characteristic folding pattern of the polytene chromosomes in *Drosophila* salivary gland nuclei. *Nature (Lond.).* 308:414–421.

Mattern, K.A., B.M. Humbel, A.O. Muijsers, L. de Jong, and R. van Driel. 1996. HnRNP proteins and B23 are the major proteins of the internal matrix of HeLa S3 cells. *J. Cell. Biochem.* In press.

McEachern, M.J., and E.H. Blackburn. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature (Lond.).* 376:403–409.

McKay, S.J., and H. Cooke. 1992*b*. HnRNP A_2/B_1 binds specifically to single stranded vertebrate telomeric repeat $TTAGGG_n$. *Nucleic Acids Res.* 20: 6461–6464.

Mirkovitch, J., M.-E. Mirault, and U.K. Laemmli. 1984. Organization of the higher order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell.* 39:223–232.

Morin, G.B. 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell.* 59:521–529.

Moyzis, R.K., J.M. Buckingham, L.S. Cram, M. Dani, L.L. Deaven, M.D. Jones, J. Meyne, R.L. Ratliff, and J.-R Wu. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA.* 85:6622–6626.

Nakayasu, H., and R. Berezney. 1989. Mapping replication sites in the eucaryotic cell nucleus. *J. Cell Biol.* 108:1–11.

Pardoll, D.M., B. Vogelstein, and D.S. Coffey. 1980. A fixed site of DNA replication in eucaryotic cells. *Cell.* 19:527–536.

Payrastre, B., M.G. Nievers, J. Boonstra, M. Breton, A.J. Verkleij, and P.M.P. van Bergen en Henegouwen. 1992. A differential location of phosphoinositide kinases, diacylglycerol kinases and phospholipase C in the nuclear matrix. *J. Biol. Chem.* 267:5078–5084.

Piñol-Roma, S., and G. Dreyfuss. 1991. Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. *Science (Wash. DC).* 253:312–314.

Rawlins, D.J., M.I. Highett, and P.J. Shaw. 1991. Localization of telomeres in plant interphase nuclei by in situ hybridization and 3D confocal microscopy. *Chromosoma.* 100:424–431.

Saltman, D., R. Morgan, M.L. Cleary, and T. de Lange. 1993. Telomeric structure in cells with chromosome end associations. *Chromosoma.* 102:121–128.

Shoeman, R.L., and P. Traub. 1990. The in vitro DNA-binding properties of purified nuclear lamin proteins and vimentin. *J. Biol. Chem.* 265:9055–9061.

Sibon, O.C.M., B.M. Humbel, A. De Graaf, A.J. Verkleij, and A.F.M. Cremers. 1994. Ultrastructural localization of epidermal growth factor (EGF)-receptor transcripts in the cell nucleus using pre-embedding in situ hybridization in combination with ultra-small gold probes and silver enhancement. *Histochemistry.* 101:223–232.

Singer, M.S., and D.E. Gottschling. 1994. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science (Wash. DC).* 266:404–409.

Smith, S., and T. de Lange. 1997. TRF1, a mammalian telomeric protein. *TIG.* In press.

Staufenbiel, M., and W. Deppert. 1982. Intermediate filament systems are collapsed onto the nuclear surface after isolation of nuclei from tissue culture cells. *Exp. Cell Res.* 138:207–214.

Tommerup, H., A. Dousmanis, and T. de Lange. 1994. Unusual chromatin in human telomeres. *Mol. Cell. Biol.* 14:5777–5785.

van Driel, R., D.G. Wansink, B. van Steensel, M.A. Grande, W. Schul, and L. de Jong. 1995. Nuclear domians and the nuclear matrix. *Int. Rev. Cytol.* 162A:151–189.

Vourc'h, C., D. Taruscio, A.L. Boyle, and D.C. Ward. 1993. Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. *Exp. Cell Res.* 205:142–151.

Xing, Y., and J.B. Lawrence. 1991. Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by in situ hybridization coupled with biochemical fractionation. *J. Cell Biol.* 112:1055–1063.

Zakian, V.A. 1995. *Saccharomyces* telomeres: function, structure and replication. *In* Telomeres. E.H. Blackburn and C.W. Greider, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 107–138.

Zhong, Z., L. Shiue, S. Kaplan, and T. de Lange. 1992. A mammalian factor that binds telomeric TTAGGG repeats in vitro. *Mol. Cell. Biol.* 13:4834–4843.