Nucleolar localization of the human telomeric repeat binding factor 2 (TRF2)

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Summary
The telomeric repeat binding factor 2 (TRF2) specifically recognizes TTAGGG tandem repeats at chromosomal ends. Unexpectedly immunofluorescence studies revealed a prominent nucleolar localization of TRF2 in human cells, which appeared as discrete dots with sizes similar to those present in the nucleoplasm. The TRF2 dots did not overlap with dots stemming from the upstream binding factor (UBF) or the B23 protein. After treatment with a low concentration of actinomycin D (0.05 μg/ml), TRF2 remained in the nucleolus, although this condition selectively inhibited RNA polymerase I and led to a relocalization of UBF and B23. TRF2 was prominent in the nucleolus at G0 and S but seemed to diffuse out of the nucleolus in G2 phase. During mitosis TRF2 dispersed from the condensed chromosomes and returned to the nucleolus at cytokinesis. Treatment with low doses of actinomycin D delayed the release of TRF2 from the nucleolus as cells progressed from G2 phase into mitosis. With actinomycin D present TRF2 was detected in discrete foci adjacent to UBF in prophase, while in metaphase a complete overlap between TRF2 and UBF was observed. TRF2 was present in DNase-insensitive complexes of nucleolar extracts, whereas DNA degradation disrupted the protein-DNA complexes consisting of Ku antigen and B23. Following treatment with actinomycin D some of the mitotic cells displayed chromosome end-to-end fusions. This could be correlated to the actinomycin D-suppressed relocalization of TRF2 from the nucleolus to the telomeres during mitosis. These results support the view that the nucleolus may sequester TRF2 and thereby influences its telomeric functions.

Key words: Nucleolus, TRF2, UBF, B23, Ku antigen, Cell cycle, Ki-67, Telomere

Introduction
The nucleolus is a eukaryotic subnuclear organelle where ribosomal RNAs are transcribed, processed, modified and, finally, assembled into ribosomes. However, there is accumulating evidence pointing to further nucleolar activities that are not directly involved in ribosome metabolism (Pederson, 1998; Olson et al., 2002). In yeast the nucleolus seems to be important for cell cycle progression by sequestering the protein phosphatase Cdc14. This protein is released from the nucleolus only at anaphase to regulate the activity of Cdc28 at the mitotic exit (Visintin et al., 1999). It has been demonstrated that yeast Cdc14 binds to the rDNA-associated protein Net1 (nucleolar silencing establishing factor and telophase regulator) (Shou et al., 1999). Net1 also binds to a gene silencing protein named Sir2, which is an NAD-dependent histone deacetylase (Imai et al., 2000) associated with other Sir (silent information regulatory) proteins at telomeres and the silent mating-type (HM) loci (Straight et al., 1999). These proteins, Net1, Cdc14 and Sir2 together form a nucleolar complex termed RENT (regulator of nucleolar silencing and telophase). A yeast meiotic checkpoint protein named Pch2 seems to be an additional component of RENT, because this protein displays a Sir2-dependent localization to the nucleolus after meiotic arrest (San-segundo and Roeder, 1999). It should be noted that Net1 is required for maintaining a normal nucleolar structure (Straight et al., 1999) and RNA polymerase-I-catalyzed transcription (Shou et al., 2001). Most recently RENT has been found to be associated with the RNA polymerase I promoter and a non-transcribed region of rDNA that is prone to recombination (Huang and Moazed, 2003). These results suggest that RENT is a silencer of transcription and/or a suppressor of recombination at rDNA loci (Carmo-Fonseca et al., 2000).

Besides its role in ribosome biogenesis, the mammalian nucleolus also contains proteins that are necessary for growth regulation and other non-nucleolar processes, such as p19Arf (a tumor suppressor protein) (Weber et al., 1999; Tao and Levine, 1999), nucleostemin (a p53-binding protein in human stem and cancer cells) (Tsai and Mckay, 2002), and c-Myc (a transcription factor and proto-oncogene) (Arabi et al., 2003). Mouse p19Arf interacts with Mdm2 in the nucleolus and thereby sequesters it away from the nucleoplasm, where Mdm2 regulates p53 by blocking its transactivating functions and by promoting its export to the cytoplasm for proteosomal degradation (Weber et al., 1999; Tao and Levine, 1999). In addition to its interaction with Mdm2, p19Arf has been shown to suppress pre-rRNA processing in the nucleolus (Sugimoto et al., 2003). Conversely, nucleostemin is stored in the nucleolus and probably (regulated by its N-terminal GTP-binding domain) shuttles into the nucleoplasm, where it reverses growth inhibition by p53 and thereby confers the higher growth potential of stem and cancer cells (Bernardi and Pandolfi, 2003). Differently, the nucleolar localization of c-Myc seems to cause an inactivating sequestration because the
c-Myc level in the nucleolus was increased after inhibiting protein degradation (Arabi et al., 2003). Previously it has been noted that the nucleolus harbors telomerase RNA, which shares a common H/ACA box with small nucleolar RNAs (snORNA) (Narayanan et al., 1999), and the telomerase reverse transcriptase (TERT) that prolongs telomeres by copying sequences from its template, i.e. the telomerase RNA (Etheridge et al., 2002; Yang et al., 2002). Particularly a nucleolar localization of TERT seems to be dependent on the progression of the cell cycle, because TERT is released from the nucleolus at late S and G2 phase, i.e. at a time that coincides with telomere elongation (Wong et al., 2002). These observations led to the idea that the nucleolus sequesters telomeric components and regulates their recruitment to the telomeres during the cell cycle. Interestingly, we identified by immunofluorescence that the telomeric DNA binding protein TRF2 is also a nucleolar protein in human cells, where it became released when cells progressed from interphase into mitosis. Presently it is still unknown whether TRF2 has any function in the nucleolus. However, after inhibition of RNA polymerase I by low doses of actinomycin D a delayed migration of TRF2 from the nucleolus into the nucleoplasm was observed. This was accompanied by chromosomal end-to-end fusions, which is in accordance with the view that the actinomycin-D-delayed recruitment of nucleolar TRF2 to the telomeres resulted in unprotected chromosomal ends. The nucleolar localization of human TRF2 in interphase and its pre-mitotic release into the nucleoplasm suggests an involvement of the nucleolus in a timely limited protein sequestration, with the consequence of regulating the function of a protein in the nucleus.

Materials and Methods
Antibodies
Rabbit polyclonal antibody against TRF2 (H-300) and B23 (H-106), a goat polyclonal antibody against TERT (L-20), and mouse monoclonal antibodies against UBF (F-9), Ku86 (B-1), Ku70 (A-9), and cyclin B1 (GNS1) were purchased from Santa Cruz (Santa Cruz, CA). A mouse monoclonal antibody against TRF1 binding protein Tin2 (Ab-1) was from Oncogene (San Diego). The monoclonal antibodies we used against B23 were clone FC82291 from Sigma (Saint Louis, MO) and FC61991 from Zymed (San Francisco, CA). A monoclonal antibody against Ki-67 protein (MIB-1) was purchased from DakoCytomation (Glostrup, Denmark). A mouse monoclonal antibody against bromodeoxyuridine (BrdU) was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

For testing the antigen-specificity of the rabbit polyclonal antibody against TRF2 (H-300), approximately 2 μg of purified recombinant TRF2 (containing amino acids 49-300, Santa Cruz) was immobilized on a nitrocellulose membrane. After washing with PBS, the membrane was incubated overnight with approximately 60 μg anti-TRF2 antibody (H-300) in 2 ml PBS at 4°C. Then the nitrocellulose membrane was washed with PBS and cut into small pieces. Antibodies retained by the TRF2 protein were eluted with 0.5 ml 100 mM glycine, pH 2.5, for 10 minutes at room temperature. After elution the antibody solution was adjusted to approximately pH 8 with 1 M Tris/HCl, pH 8.0.

Cell cultures
Human MCF-7 cells were maintained in RPMI supplemented with 10% fetal bovine serum. Human HeLa and mouse NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, C.C. Pro GmbH, Neustadt/Weinstrasse, Germany) supplemented with 10% fetal bovine serum (Gibco-BRL, Karlsruhe, Germany). The cells were cultured in an incubator with 10% CO2 at 37°C. For the selective inhibition of RNA polymerase I-catalyzed transcription, cells were treated with 0.05 μg/ml actinomycin D for 3 hours before measuring immunofluorescence.

Immunofluorescence
Immunofluorescence measurements were performed as described before (Zhang et al., 1999). Primary antibodies were diluted at 1/200 for the rabbit polyclonal antibody against TRF2; 1/10 for the F-9 monoclonal antibody against UBF, the monoclonal antibodies against B23, the B-1 monoclonal antibody against Ku86 and the MIB-1 monoclonal antibody against Ki-67 protein, and 1/20 for the GNS1 monoclonal antibody against cyclin B1 and Tin2. The rhodamine-conjugated secondary antibodies against rabbit IgG and the FITC-conjugated secondary antibody against mouse or human IgG (Dianova, Jackson ImmunoResearch Laboratories, Germany) were all diluted at 1/100. For identifying S-phase cells BrdU was added to the medium of cultured cells to a final concentration of 50 μM, followed by an incubation period of 30 minutes. Treating with 1.5 N HCl for 30 minutes denatured DNA. Then DNA synthesis was visualized by subsequent incubation with a monoclonal antibody against BrdU and an FITC-conjugated secondary antibody. This was done after immunofluorescence of TRF2 in order to observe double-immunofluorescence of TRF2 and BrdU. Immunofluorescence was observed at ×100 magnification with a Zeiss Axiosvert 135 microscope in connection with a Sony CCD color video camera. For confocal microscopy we used a Zeiss LSM 510 laser scanning confocal microscope at ×63 magnification. All microscopic data were processed with Adobe Photoshop D1-4.0 and Canvas 6.0.

Isolation of nucleoli
HeLa cells (approximately 1 ml as pellet) were disrupted with a Teflon homogenizer in 2 ml RSB-100 buffer (10 mM Tris/HCl, pH 7.2, 10 mM NaCl, 1.5 mM MgCl2, including 5 μg/ml of each of the protease inhibitors aprotinin, leupeptin and pepstatin). The resulting extract was centrifuged at 3000 g for 5 minutes at 4°C. The nuclear pellet was resuspended in 2 ml 50 mM Tris/HCl, pH 7.8, 150 mM KCl, 5 mM MgCl2, 250 mM sucrose plus the protease inhibitors as described above. Then the suspension was sonicated by 6 pulses of 6 seconds each with a Branson sonifier at an output setting of 5. The sonicated suspension was centrifuged at 13,000 g for 15 minutes at 4°C through 0.88 M sucrose in the same buffer that was used for suspending the nuclei. The nucleoli were finally recovered in the pellet that migrated through the sucrose cushion.

Sucrose gradient centrifugation
The isolated nucleoli were extracted by shaking them for 45 minutes at 4°C with 350 mM NaCl in a buffer of 50 mM Tris/HCl, pH 8.0, 0.1 mM EDTA and the protease inhibitors, as used above. This step released salt-soluble nucleolar components that were separated from the insoluble remnants by centrifugation at 12,000 g for 5 minutes at 4°C. The supernatant (1 ml) was supplemented by CaCl2 to 1 mM and then divided into two equal parts. One aliquot remained untreated and the other one was treated with micrococcal nuclease (MNase) at 150 KU/ml (Amersham) for 30 minutes at room temperature. The two samples were then layered onto 10-40% sucrose gradients prepared in 30 mM potassium phosphate, pH 7.8, 100 mM KCl, 10 mM Na2S2O 5, 7 mM β-mercaptoethanol and 1 mM EDTA in SW40 tubes. Ultracentrifugation was performed in the SW40 rotor at 35,000 rpm for 4 hours at 4°C. After centrifugation samples (0.5 ml/fraction) were collected from the bottom of the tubes. The proteins were precipitated...
with 10% trichloroacetic acid and were finally dissolved in SDS-PAGE sample buffer for Western blotting.

Western blotting
After electrophoresis through a 10% SDS polyacrylamide gel, proteins were electro-transferred to a Hybond C extra nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) under semi-dry conditions. Immunodetection was performed using an enhanced chemiluminescence protocol as described by the supplier (ECL, Amersham Biosciences). Dilution of the primary antibody was 1/200 for the rabbit polyclonal antibodies against TRF2 or B23, and 1/1000 for the A-9 mouse monoclonal antibody against Ku70.

Results
TRF2 is localized in the nucleolus of human cells
Immunofluorescence studies with a rabbit polyclonal antibody against the telomere binding protein TRF2 revealed that this protein was situated in the nucleoli of human MCF-7 cells (Fig. 1A,B). However, with the same antibody we failed to visualize TRF2 in the nucleolus of mouse NIH 3T3 cells that instead stained some discrete dots in the nucleoplasm, which probably correspond to the telomeres (Fig. 1C,D). To confirm the antigen specificity of the applied antibody against TRF2 we immobilized recombinant TRF2 on nitrocellulose and used this membrane for affinity purification to obtain highly specific antibodies. As shown by western blotting the TRF2 antibody bound efficiently to recombinant TRF2 (Fig. 1E). The affinity-purified antibody only detected one band in whole cell extracts from MCF-7 or mouse 3T3 cell that had the same molecular weight as TRF2 (Fig. 1E). Moreover, these antibodies revealed a nucleolar staining of human HeLa cells together with some discrete nucleoplasmic dots that are supposed to be at the positions of telomeres (Fig. 1F,G). These results allow the conclusion that TRF2 is mainly situated in the nucleolus of human cells.

Nucleolar localization of human TRF2 is apparently unrelated to RNA polymerase-I-catalyzed transcription
Next we investigated whether the observed nucleolar localization of human TRF2 has any functional implication for ribosomal biosynthesis. To observe a possible co-localization between TRF2 and UBF, i.e. the upstream binding factor promoting RNA polymerase I transcription in the nucleolus, double-immunofluorescence studies were performed. Immunofluorescence of UBF revealed discrete dots that were confined to the nucleolus (Fig. 2A). It was already known that these UBF dots correspond to rDNA transcription sites. Although TRF2 also existed as discrete dots in the nucleolus, their distribution was apparently different from those of UBF (Fig. 2A versus B). This could be further resolved by confocal microscopy (Fig. 2C,C’). Immunofluorescence measurements (Fig. 2C”) revealed a non-overlapping pattern between most of the foci derived from TRF2 and UBF. A more striking difference between UBF and TRF2 was found by treating the cells with 0.05 μg/ml actinomycin D, a condition that specifically inhibits RNA polymerase I. After this treatment, UBF displayed a typical relocation towards the nucleolar periphery, which often appeared as a cap close to the tip of the nucleolus (Fig. 2D), indicating a clustering of UBF once transcription of rDNA was shut down (Jordan et al., 1997). The same treatment, however, did not lead to a relocation of TRF2. Instead, after actinomycin D treatment TRF2 remained in a central area of the nucleolus that was apparently devoid of UBF signals (Fig. 2E).

The nucleolar localization of TRF2 was also dissimilar to that of B23, i.e. a nucleolar protein involved in pre-rRNA synthesis or processing (Fig. 2F,H) (Olson et al., 2002).
Immunofluorescence revealed non-overlapping signals between TRF2 and B23 (Fig. 2H*). Moreover, actinomycin D led to an extrusion of B23 from the nucleolus (Fig. 2I), similarly as it has been observed for other proteins involved in ribosomal RNA processing (Olson et al., 2002). This again was in contrast to the persisting localization of TRF2 in the nucleolus after actinomycin D treatment (Fig. 2J).

The nucleolar localization of human TRF2 is cell cycle dependent

We further examined the cell-cycle-dependent localization of TRF2 in human cells. First we found that TRF2 displayed a predominant presence in the nucleolus at G0 phase. G0 cells were identified by the failure to immunostain the Ki-67 protein, i.e. a proliferation marker with a minimal expression at G0 phase and an increasing level in cycling cells (Fig. 3A,C) (Scholzen and Gerdes, 2000). Human TRF2 also remained in the nucleolus during S phase, as judged by BrdU incorporation (Fig. 3D,F). In contrast, TRF2 left the nucleolus when cyclin B1 became detectable. Cyclin B1 is a cell cycle marker that displays an expression peak at the G2/prophase transition. We observed that an increased staining of cyclin B1 always coincided with the dispersal of TRF2 from the nucleolus. In contrast, cells with a low level of cyclin B1 always displayed the nucleolar localization of TRF2 (Fig. 3G,I).

During mitosis the localization of TRF2 was compared with those of B23 and UBF (Fig. 4). In prophase TRF2 was dispersed from the nucleoli at an earlier time than B23 (Fig. 4A,C). Similar to B23 TRF2 was excluded from the highly condensed chromosomes in metaphase (Fig. 4D,G), but unlike B23 appeared at some discrete dots close to the surface of the condensed metaphase chromosomes (Fig. 4G*). These remaining TRF2 dots apparently did not overlap with those of UBF, neither in prophase (Fig. 4H-K,K*) nor in metaphase (Fig. 4L-O,O*), which excludes a c-localization of TRF2 with NORs (nucleolar organizer regions) during mitosis.

**Fig. 2.** Comparison of nucleolar TRF2 with UBF and B23 in human MCF-7 cells. The colocalization of these proteins was probed by double-immunofluorescence of TRF2 and UBF (A-C) or B23 (F-H). The reorientation of TRF2, UBF, and B23 was measured after applying 0.05 µg/ml actinomycin D for 3 hours and following the signals of TRF2 and UBF (D-E) or TRF2 and B23 (I-J). The colocalization of TRF2 with UBF in C-C* or B23 in F-H was observed by laser scanning confocal microscopy. Red and green arrows in C* and H represent TRF2 and UBF or B23, respectively. The frames presented in A-B and D-E are the magnification of the arrow-indicated nucleoli of the same picture. Smaller arrows in the frames of D-E indicate the relocalization of UBF after actinomycin D treatment. Fluorescence measurements along the dashed lines in C* and H are presented in C** and H**.
Fig. 3. Nucleolar localization of human TRF2 in interphase. The colocalization of TRF2 in human MCF-7 cells with Ki-67 protein (A-C), BrdU (D-F), and cyclin B1 (G-I) are shown at different stages of interphase. Arrows in A-C indicate a G0 phase cell that lacks a positive staining of Ki-67. Arrows in D-F indicate S-phase cells displayed by BrdU incorporation. Arrows in G-I indicate G2 phase cells with a high expression of cyclin B1.

Fig. 4. Localization of TRF2, B23 and UBF in prophase and metaphase. The immunofluorescence of TRF2 and B23 or UBF is shown in mitotic human MCF-7 cells during prophase (A-C and H-K) and metaphase (D-G and L-O). Panels A-C were observed by light microscopy and panels D-O were observed by laser-scanning confocal microscopy. Panels G', K' and O' depict the fluorescent measurements along the indicated lines in G, K and O, respectively.
When mitotic cells progressed into early telophase, an initial re-entry of TRF2 or B23 into the areas of decondensing chromosomes was seen (Fig. 5A,a-c). As chromosomes further decondensed in telophase (Fig. 5A,d-f), B23 partially returned to the resuming rDNA transcription sites (NOR) and appeared as prenucleolar body (PNB) near the NORs or the reforming nuclear boundary. Both these structures were known before to correlate with the sites of rDNA transcription and the recruitment of components involved in pre-rRNA processing to the newly formed nucleoli at telophase (Olson et al., 2002). But neither was found together with TRF2 signals above the background level. A discernible nucleolar signal of TRF2 only appeared in late telophase (Fig. 5A,g-i) when cells were exiting from mitosis into interphase. At this stage B23 already returned into the nucleolus, which now displayed a similar staining as that observed in normal interphase cells (Fig. 2F-H).

Ku antigen (Ku86/70) is another nucleolar protein that undergoes redistribution during mitosis (Li and Yeh, 1992). Here we observed that Ku86 remained associated with nucleolar regions at early prophase when chromosomes had already started to condense, which was in contrast to an apparent extrusion of TRF2 from the nucleolus (Fig. 5B,a-c). However, at the end of telophase TRF2 returned to the nucleolus at a higher level than Ku86 (Fig. 5B,d-f). Apparently, TRF2 and Ku86 behave differently with regard to their nucleolar migration kinetics.

Actinomycin D delayed the release of TRF2 from the nucleolus during mitosis
When the cell-cycle-dependent localization of TRF2 was followed in the presence of actinomycin D, it turned out that the telomere binding protein became stalled in the nucleolus in G2 phase (Fig. 6A-C), which was verified by the expression of cyclin B1 that, in the absence of actinomycin D, correlated with a reduced nucleolar concentration of TRF2 (Fig. 3G-I). At the beginning of prophase, when condensation of the chromosomes had just begun, TRF2 was still in the dispersing nucleolus in actinomycin D-treated cells, whereas B23 (Fig. 6D-F) and Ku antigen (Fig. 6G-I) were already excluded from the nucleolus. This is in contrast with the above results, where B23 and Ku antigen remained associated with nucleolar structures in normal prophase cells that had not been treated with actinomycin D (Fig. 4A,a-c and 5B,a-c). Moreover, the remaining nucleolar association of TRF2 after actinomycin treatment gave rise to discrete dots in prophase that partially overlapped with those of UBF (Fig. 7A-C). When mitosis proceeded into metaphase TRF2 and UBF even displayed a complete colocalization (Fig. 7D-F). A possible reason for these findings could be that a maximal chromosomal condensation in metaphase resulted in a minimized distance between the nucleolar sites that sequestrate TRF2 and NORs containing UBF, which in turn led to an apparent colocalization.

Nucleolar TRF2 exists in high molecular weight complexes resistant to DNase digestion
The clustered appearance of human TRF2 in the nucleolus suggested the binding of TRF2 to higher molecular weight complexes (Fig. 1). For an estimation of the complex size nucleoli were isolated and subsequently extracted by 0.35M NaCl. The resulting components then were ultracentrifuged through a sucrose density gradient (10-40%). Western blot analysis of the collected fractions showed that TRF2 sedimented to the bottom of the gradient (Fig. 8).
Treatment with micrococcal nuclease (MNase) hardly resolved these TRF2-containing high molecular weight complexes. Conversely, Ku antigen only ran into the upper half of the gradient, although a low amount of Ku antigen was also seen at the bottom. In contrast to TRF2, the Ku-containing complex running to the middle of the sucrose gradient could be disrupted by MNase, suggesting that Ku antigen was associated with actively transcribed rDNA, which is more sensitive to DNA digestion. Similar to Ku antigen B23 complexes from the middle part of the gradient were found to be sensitive to MNase digestion, although this protein, like TRF2, also existed in MNase-insensitive complexes that sedimented to the bottom of the gradient.

Actinomycin D treatment led to chromosome-end fusion in mitosis, possibly by preventing the recruitment of TRF2 to telomeres

The observed delayed release of TRF2 from the nucleolus of
actinomycin D treated cells, particularly in G2 and early prophase, may limit the recruitment of this protein to telomeres. This increases the chance that unprotected chromosome ends become fused to each other, an event that happens also to DNA double-strand breaks generated by DNA damage. Indeed, in actinomycin D-treated samples some mitotic cells displayed a chromosomal bridge in telophase that was obviously due to the fusion of chromosome ends (Fig. 9A). Immunofluorescence studies revealed the presence of TRF2 at the junction of these fused chromosomes (Fig. 9B,B') that in turn favors a telomeric bridge. This chromosome end-to-end bridge even lasted until the end of cytokinesis, where the fusion point still overlapped with some TRF2 dots (Fig. 9C). When these cells were observed under Nomarski contrast (DIC, differential-interference contrast) a cleavage furrow (indicated by arrow in Fig. 9D,E) could be seen at the cell junction where the two chromosomes still remained fused. To assess whether the chromosome fusion might have been due to an insufficient amount of TRF2 at telomeres, the telomeric localization of TRF2 was compared with that of the TRF1 binding protein Tin2 by immunofluorescence (Fig. 9F-L). The results show that TRF2 in actinomycin D-treated cells (Fig. 9L'), as compared with control cells (Fig. 9H',H''), indeed displayed an intensified nuclear signal and, conversely, a reduced amount at telomeres in the nucleoplasm. In contrast to TRF2, Tin2 displayed enhanced signals at telomeres after actinomycin D treatment, which suggests that actinomycin D induced different responses to the localization of different telomere-associated proteins. This observation supports the idea that actinomycin D increased the accumulation of TRF2 in the nucleolus, probably as a result of inhibited transcription mediated by RNA polymerase I. Accordingly, the amount of TRF2 at telomeres must have been reduced under this condition assuming that the total amount of TRF2 has not been substantially changed. This seems to be the case, because western blots (Fig. 9N) show only a slight decrease (probably due to partial degradation) rather than an increase of total TRF2 in actinomycin-D-treated cells compared with untreated control cells. Therefore, we suppose that a defective function of TRF2, either due to a reduced amount at, or a delayed recruitment to, the telomeres, is the reason for the observed chromosome fusions at telomeres. Moreover, it should be pointed out that the observed chromosome fusion occurred shortly (3.5 hours) after treatment by a very low dose (0.05 μg/ml) of actinomycin D, which is conditionally different from similar chromosome changes observed earlier after prolonged incubation of cells treated with a much higher concentration (1 μg/ml) of actinomycin D (Hsu et al., 1978).

**Discussion**

In mammalian cells there exist two telomeric sequence (TTAGGG)-specific DNA-binding proteins termed TRF1 and TRF2 that protect the integrity of the telomeres (Broccoli et al., 1997). TRF2 promotes the formation of a telomeric t-loop structure by folding back the 3´ chromosomal end and inserting it into an upstream region of the telomeric tandem repeat duplex (de Lange, 2002). Both TRF1 and TRF2 contain a C-terminal Myb domain for specific binding to the telomeric DNA sequence and a central TRFH (TRF homology) domain for homodimerization (Fairall et al., 2001). However, the N-terminus of TRF2 is highly basic while the same region of TRF1 comprises acidic amino acids. Importantly, after removing both the basic N-terminus and the Myb domain a dominant negative effect of the resulting TRF2 mutants has been observed. This caused an immediate loss of telomeric protection, leading to chromosomal end fusion and/or apoptosis mediated by ATM and p53 (Karlseder et al., 1999).

So far TRF2 has been known to physically interact with several proteins. These include a human ortholog to the yeast telomeric protein scRap1p (Li et al., 2000), Ku antigen (Ku70) (Song et al., 2000), the WRN and Bloom syndrome DNA helicases involved in premature aging (Opresko et al., 2002), and poly(ADP-ribose) polymerase 2 (PARP-2) (Dantzer et al., 2004).

Here we show that human TRF2 also localizes to the nucleolus. The nucleolar situation of TRF2 is dissimilar to that of UBF or B23, both of which are involved in ribosomal biogenesis. After inhibiting RNA polymerase I with low doses of actinomycin D TRF2 remained in the nucleolus, while UBF and B23 redistributed to the nucleolar periphery or nucleoplasm. Nevertheless, TRF2 left the nucleolus when cells progressed from G2 phase into mitosis, while actinomycin D diminished and delayed this cell-cycle-dependent release of TRF2. Moreover, telomeric chromosome end-to-end fusions showed up in cells treated with actinomycin D. Similar fusions were previously observed by replacing wild-type TRF2 with its dominant negative mutant (Smogorzewska and de Lange, 2002). Taken together these data led to the conclusion that the nucleolus may sequester TRF2 and that its timely recruitment to the telomeres is critical for chromosomal integrity.
Fig. 9. Actinomycin D treatment led to chromosome end-to-end fusions in mitotic cells. In response to actinomycin D treatment some telophase MCF-7 cells displayed chromosome end-to-end fusions (A) as well as the presence of TRF2 at the junctions of the observed chromosomal arcs (B). B' represents the magnified area framed in B. Chromosome fusion from cells undergoing cytokinesis is also shown (C-E). Co-localization of TRF2 with the telomere-binding protein Tin2 is shown in untreated (F-I) and actinomycin D-treated (J-M) MCF-7 cells. H', H'' and L' present profiles of fluorescence measurements along the indicated red lines shown in the same panel. Large arrows (F-M) indicate nucleoli and small arrows chromosome fusions (A-E) or telomeres (F-I and J-M). Total amounts of TRF2 were determined by western blotting of lysates of untreated and actinomycin D-treated MCF-7 cells (N). The lysates were prepared by directly dissolving cells on culture dishes into equal volumes of SDS-PAGE loading buffer.
Presently it remains unclear why TRF2 is found in the nucleolus of human cells while in mouse cells the nucleolus contains hardly detectable levels of TRF2. This may suggest some species-related differences of telomere metabolism as already noted before (Smogorzewska and de Lange, 2002). Previously we observed that a nucleic acid helicase named NDH II (nucleolar DNA helicase II) or RNA helicase A (RHA) displayed a nucleolar localization in mouse cells whereas it is apparently confined to the nucleoplasm in a variety of human cell lines (Zhang et al., 1999). This phenomenon can be explained by the different RNA polymerase I transcription kinetics between mouse and human cells, because NDH II is actively involved in transcription. Possibly, the species-specific nucleolar compositions, as observed here for TRF2, reflects the dynamics of ribosomal metabolism optimized for individual species and cell types. Importantly, a species-specific nucleolar localization comparable to that of TRF2 has also been observed for the Werner syndrome helicase (WRN), which was found in the nucleolus of human, but not of murine, cells (Marciniak et al., 1998). Interestingly, the different subnuclear localization of human and mouse WRN has been ascribed to a local protein domain responsible for nucleolar targeting (von Kobbe and Bohr, 2002). Analogously, a still unidentified protein domain may be responsible for the nucleolar localization of TRF2.

In yeast the telomeres and the nucleolus share some similarities because both are positioned near the nuclear membrane and are associated with Sir proteins that function as gene silencers (Carmo-Fonseca et al., 2000). Yeast Sir complexes are comprised of Sir 2, 3 and 4 proteins, where the nucleolar localization of Sir2 is accomplished by its direct binding to Net1 (Straight et al., 1999). Sir3 appears in the nucleolus of prematurely aged yeast cells due to a mutation of the yeast RecQ DNA helicase Sgs1, which is an ortholog of the human WRN and Bloom syndrome DNA helicases (Sinclair et al., 1997). The Sir complexes are important for maintaining the heterochromatin structure at telomeres and for suppressing rDNA recombination in the nucleolus (Johnson et al., 1998). It has been shown that a recruitment of yeast Sir proteins to the telomeres is mediated by Rap1, which is a telomere-bound protein that directly interacts with Sir 4 (Luo et al., 2002). Notably in yeast there is no TRF2 ortholog and its function apparently has been taken over by Rap1. On human telomeres Rap1 interacts with TRF2 (Li et al., 2000) indicating an evolutionary link between human TRF2 and yeast Sir proteins. Hence, the localization we observed of human TRF2 at telomeres and in the nucleolus obviously parallels that of the yeast Sir proteins.

It is likely that the nucleolar situation of TRF2 depends on a protein interaction network as observed for yeast Sir2 that requires the presence of Net1 in the nucleolus (Straight et al., 1999). Net1 represents an rDNA-bound protein that is stably associated with the nucleolus throughout the cell cycle, whereas Sir2 and Cdc14 are components of the RENT complex that is transiently released from the nucleolus in mitosis (Straight et al., 1999). It remains to be shown whether the DNA-binding protein TRF2 is also associated with nucleolar rDNA, albeit with a lower affinity than with telomeric DNA. Intriguingly, in the presence of actinomycin D TRF2 dissociated with delayed kinetics from the nucleolus and now occurred at positions that overlapped with UBF at NORs of metaphase chromosomes. This indicates very similar or the same nucleolar localization of TRF2 and UBF, where the latter remained on rDNA even after RNA polymerase I is arrested in metaphase (Gebrane-Younes et al., 1997). However in the interphase of actinomycin D treated cells TRF2 was localized at the center of the nucleolus, whereas UBF was confined to the nucleolar boundary and away from TRF2. Moreover this effect excludes a longitudinal binding of TRF2 to rDNA, because actinomycin D not only led to the dissociation of UBF (Jordan et al., 1997) but also to a retraction of rDNA fibers to the nucleolar periphery (Schofer et al., 1996). Therefore, rather than spreading on route along rDNA fibers, TRF2 may help to build up complexes that abut on rDNA or its associated proteins, although spreading on route may occur on the TTAGGG telomeric sequence repeats. This may be accomplished by dimer formation via the TRFH domain (Fairall et al., 2001), or even by homo-multimerization.

Although TRF2 may bind adjacent rather than directly to rDNA, its influence on rRNA synthesis should not be neglected. For example, sequestering TRF2 to the nucleolus can be advantageous for facilitating rDNA silencing, which in turn should suppress illegitimate recombination at these highly repetitive loci. In this aspect nucleolar TRF2 may act as TRF2 at telomeres, which is involved in the formation of compact telomeric structures reassembling heterochromatin (Chan and Blackburn, 2002). Moreover, TRF2 interacts with WRN helicase (Opresko et al., 2002) and Ku antigen (Song et al., 2000) that are involved in DNA repair and recombination. Both WRN (Opresko et al., 2002) and Ku antigen (Hsu et al., 1999) are also required for the maintenance of telomeres, possibly by preventing illegitimate DNA recombination. Therefore, there seems to exist a functional relationship between human telomeres and rDNAs that has been already recognized in yeast (Johnson et al., 1998), or even an interplay between these two loci that may be important for aging processes and the life span of eukaryotic cells.

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