Ub-Ub-Ub(T66C-Lucifer yellow)diol trimers, 8 nM PA700, and reaction buffer were incubated at 37 °C, stopped by the addition of 90 μl of 1 M acetic acid, and the products labelled with Lucifer yellow were separated by cation-exchange HPLC (TSK SP-NPR column eluted at 1.25 ml min^{-1} with 0.05–0.45 M NaCl in 25 mM NH_4OAc, pH 4.5) and quantified by their fluorescence (ex. 426 nm, em. 530 nm).

Degradation of Ub_n-α-globin conjugates. Gel-purified ^{125}I–α-globin and Ub_n–^{125}I-α-globin conjugates were prepared[30]. 26S proteasomes were assembled[25] by preincubation at 37 °C of 20S proteasomes (12 μg ml^{-1}) and PA700 (40 μg ml^{-1}) in 0.1 M Tris-HCl, pH 7.25, with 10 mM DTT, 40 μg ml^{-1} bovine serum albumin, 10 mM $MgCl_2$, and 60 μM ATP; after 40 min, some mixtures were supplemented with 4 μM Ubal, and the preincubation was continued for a further 5 min. Degradation reactions were initiated by mixing 10 μl of ^{125}I-labelled conjugates in 2 mM ATP with 10 μl of the 26S proteasomes; the final reaction mixtures contained 2–4 nM (~10^3 c.p.m.) of a conjugate. After incubation at 37 °C, substrate degradation was assayed as the fraction of ^{125}I-radioactivity that was soluble in cold 10% trichloracetic acid[8].

Received 15 October; accepted 19 December 1996.

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Acknowledgements. We thank J. Schaeffer for guidance in the production of the α-globin derivatives; S. Mikesell for the preparation of UbCN; C. Pickart for diubiquitin and $E2_{25K}$; D. Price for help with figure preparation; and M. Hochstrasser, C. Pickart and A. Robertson for discussions and comments on the manuscript. This work is in partial fulfillment of the Ph.D. in Biochemistry to Y.A.L. from the University of California at Los Angeles, and was supported by NIH grants to R.E.C. and G.N.D.

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Control of telomere length by the human telomeric protein TRF1

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Human telomeres, the nucleoprotein complexes at chromosome ends, consist of tandem arrays of TTAGGG repeats bound to specific proteins. In normal human cells, telomeres shorten with successive cell divisions[1,2], probably due to the terminal sequence loss that accompanies DNA replication. In tumours and immortalized cells, this decline is halted through the activation of telomerase[3–5], a reverse transcriptase that extends the telomeric TTAGGG-repeat arrays[6–7]. Telomere length is stable in several immortal human-cell lines[3], suggesting that a regulatory mechanism exists for limiting telomere elongation by telomerase. Here we show that the human telomeric-repeat binding factor TRF1 (ref. 8) is involved in this regulation. Long-term overexpression of TRF1 in the telomerase-positive tumour-cell line HT1080 resulted in a gradual and progressive telomere shortening. Conversely, telomere elongation was induced by expression of a dominant-negative TRF1 mutant that inhibited binding of endogenous TRF1 to telomeres. Our results identify TRF1 as a suppressor of telomere elongation and indicate that TRF1 is involved in the negative feedback mechanism that stabilizes telomere length. As TRF1 does not detectably affect the expression of telomerase, we propose that the binding of TRF1 controls telomere length *in cis* by inhibiting the action of telomerase at the ends of individual telomeres.

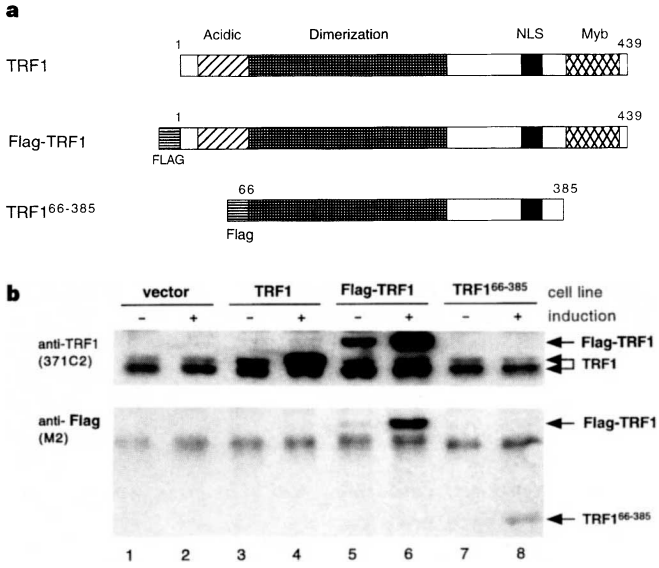

Figure 1 Tetracyclin-regulated expression of TRF1 proteins in HT1080 cells. **a**, Domain structure of TRF1 proteins used in this study. NLS, putative nuclear localization signal. **b**, Western analysis of inducible TRF1 expression in HTC75 clones. Whole-cell extracts from induced and uninduced cells were analysed using anti-TRF1 antibody 371C2 or anti-Flag monoclonal antibody M2. Endogenous TRF1 is represented by a doublet: the top band co-migrates with the transfected full-length protein (lane 4). The bottom band of the doublet probably represents an alternatively spliced form of TRF1 that lacks 20 amino acids in the non-conserved region of the protein (J. Karlseder and T.d.L., unpublished observations).

Mammalian telomeres show a species-specific length setting[9] which suggests that a regulatory mechanism exists to control telomere length in the germ line. Telomere-length control is also evident from the stability of telomeres in telomerase-expressing cell lines[3] and from the observation that newly formed telomeres acquire a length appropriate for the host cell[10,11]. The latter observation suggests that cells monitor and modulate the length of individual telomeres, a process that is likely to involve a protein that binds to the duplex telomeric repeat region at mammalian chromosome ends. A candidate for this function is TRF1, a duplex telomeric TTAGGG-repeat binding protein that is associated with human and mouse telomeres in interphase and in mitosis[8,12–15].

To investigate the role of TRF1 in the regulation of telomere length, we studied the effects of long-term overexpression of wild-type TRF1 and of a dominant-negative mutant on telomere length in a telomerase-positive human tumour-cell line with stable telo-

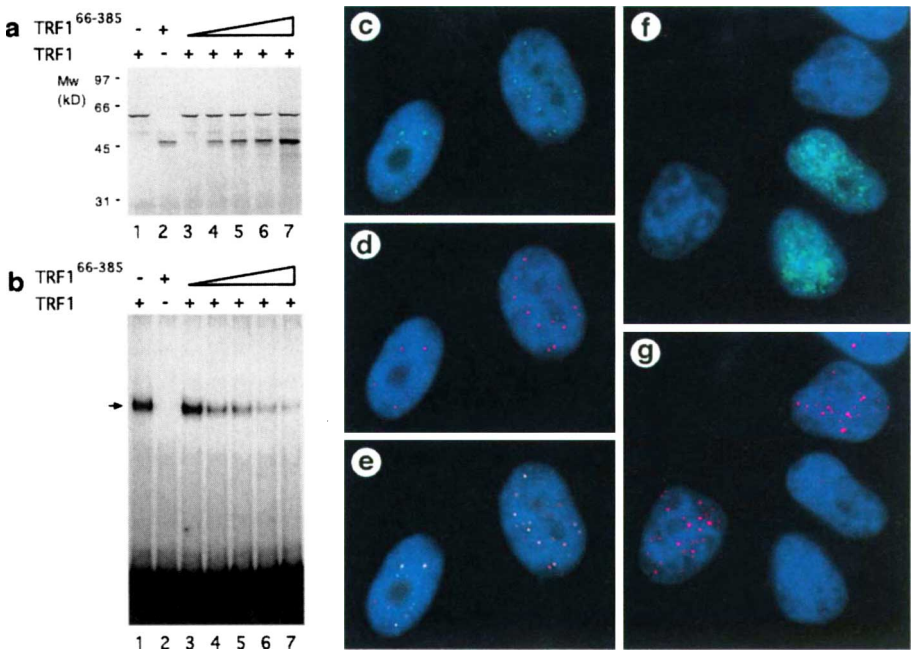

Figure 2 Characterization of the dominant interfering activity of TRF1$^{66-385}$. **a**, ^{35}S-Met-labelled translation of full-length TRF1 *in vitro* with increasing amounts of TRF1$^{66-385}$. **b**, TTAGGG-repeat binding activity (arrow) obtained with unlabelled proteins synthesized in parallel with **a**. **c**–**e**, Co-localization of endogenous TRF1 with telomeric DNA in HeLa interphase nuclei. **c**, TRF1 detected with antibody 371C2 (green); **d**, telomeric DNA visualized in the same nuclei with a $[CCCUAA]_{27}$ RNA probe (red); and **e**, superimposition of **c** and **d**. **f**, **g**, Overexpression of TRF1$^{66-385}$ inhibits binding of endogenous TRF1 to telomeres. HeLa cells transiently co-transfected with the tTA expression construct[16] and the TRF1$^{66-385}$ construct were stained in **f** for TRF1$^{66-385}$ using the Flag tag antibody (M2) (green) and in **g** for endogenous TRF1 with antibody 371C2 (red). DNA was stained with DAPI (4,6-diamidino-2-phenylindole) (blue).

meres. We established a tetracycline-controlled gene-expression system[16] in the human fibrosarcoma cell line HT1080 (resulting in cell line HTC75) and used it for inducible expression of full-length TRF1, a TRF1 protein containing an N-terminal Flag epitope (Flag-TRF1) and a TRF1-deletion mutant encompassing amino acids 66–385 (TRF1$^{66-385}$) (Fig. 1a). Analysis by western blotting showed that doxycyclin controlled expression of each of these TRF1 proteins in clonal HTC75 cell lines transfected with the constructs (Fig. 1b). Induced overexpression of full-length TRF1 and of Flag-TRF1 also resulted in a 10–30-fold increase in the TTAGGG-repeat binding activity[12] (data not shown). Expression of TRF1$^{66-385}$ protein was consistently low compared with other TRF1 proteins (Fig. 1b, and data not shown). Expression of wild-type or mutant TRF1 proteins did not affect the viability or growth rate of the cells (data not shown).

Based on the architecture of TRF1, the deletion mutant TRF1$^{66-385}$ was expected to act as a dominant-negative mutant. TRF1 binds telomeric DNA *in vitro* as a homodimer, using a large dimerization domain to position two identical Myb-related DNA-binding motifs on its telomeric recognition site[17]. TRF1$^{66-385}$ contains the dimerization domain and putative nuclear localization sequences, but lacks the DNA-binding motif (Fig. 1a), suggesting that it might inhibit wild-type TRF1 by forming an inactive heterodimer. We tested the ability of TRF1$^{66-385}$ to inhibit DNA binding of wild-type TRF1 in a gel-shift assay with *in vitro* translated proteins. As expected, wild-type TRF1 formed a complex with telomeric DNA, whereas TRF1$^{66-385}$ showed no DNA-binding activity (Fig. 2b, lanes 1 and 2). Co-translation of TRF1 and TRF1$^{66-385}$ (Fig. 2a, lanes 3–7) under conditions that allow dimerization[17] showed that TRF1$^{66-385}$ caused a dose-dependent inhibition of the DNA-binding activity of TRF1 (Fig. 2b, lanes 3–7), indicating that TRF1$^{66-385}$ is a dominant-negative mutant.

Evidence was obtained that this dominant-negative effect on DNA binding resulted in loss of TRF1 from telomeres *in vivo*. Immunofluorescent labelling using a rabbit polyclonal antibody affinity-purified against the acidic domain of TRF1 (antibody 371C2; see 7Methods), combined with fluorescent *in situ* hybridization (FISH) using a telomere-specific probe, showed that endogenous TRF1 in HeLa cells was distributed in a speckled pattern which coincided with telomeric repeat DNA (Fig. 2c–e). This indicated that endogenous TRF1 was primarily located at telomeres in interphase nuclei. In contrast, immunolocalization of transiently transfected TRF1$^{66-385}$ in HeLa cells using a mouse monoclonal anti-Flag antibody revealed that this protein was distributed throughout the nucleus, regardless of its level of expression (Fig. 2f, and data not shown). Thus, as expected from the deletion of the Myb-homology region, TRF1$^{66-385}$ did not accumulate at telomeres. To test whether the mutant protein affected the localization of the endogenous TRF1 to telomeres, we carried out dual-labelling immunofluorescence with antibody 371C2 to detect endogenous wild-type TRF1 and with an anti-Flag monoclonal antibody to detect TRF1$^{66-385}$. The distribution of endogenous TRF1 was drastically altered in cells that expressed TRF1$^{66-385}$ (Fig. 2f, g). In transfected cells expressing large amounts of TRF1$^{66-385}$, the endogenous TRF1 protein did not show a punctate pattern, indicating that TRF1 was dislodged from the telomeres (Fig. 2g). In general, we observed an inverse correlation between the expression level of TRF1$^{66-385}$ and the staining intensity of endogenous TRF1 on telomeres (data not shown). We presume that displaced TRF1 was present throughout the nucleus, but in amounts too low to reveal a dispersed pattern of immunostaining. These results demonstrated that expression of TRF1$^{66-385}$ reduced the amount of TRF1 detectable on telomeres *in vivo*. Conversely, overexpression of full-length TRF1 in HeLa cells increased TRF1 detectable on the telomeres (data not shown).

Changes in the level of TRF1 expression affected telomere length in HTC75 cells. A control HTC75 cell line transfected with the vector had stable telomeres over 124 population doublings and

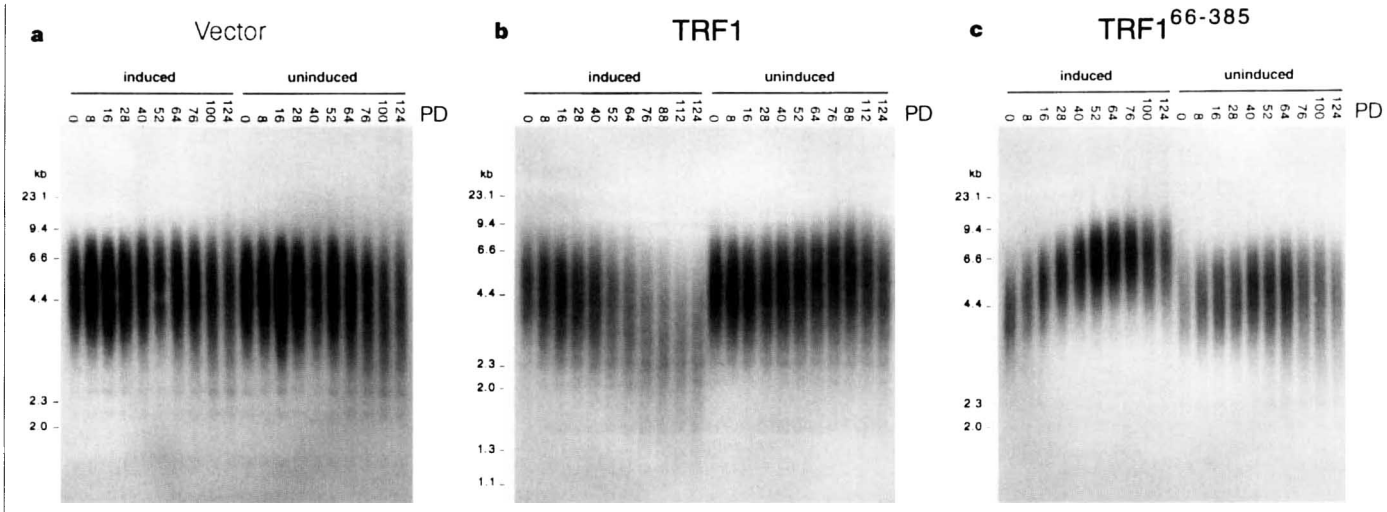

Figure 3 Telomere length changes in response to TRF1. **a**–**c**, Southern blots of *Hin*fI/*Rsa*I-digested genomic DNA from three clonal HTC75 cell lines (**a**, B6; **b**, D4; **c**, K10) expressing the indicated TRF1 genes. Each cell line was passaged for 124 PD in the presence (uninduced) or absence (induced) of doxycyclin and DNA samples were analysed at the indicated PDs. Blots were probed with a TTAGGG-repeat probe to detect telomeric restriction fragments.

there was no effect of doxycyclin on telomere length (Fig. 3a and Table 1). In contrast, cells overexpressing TRF1 showed gradual and progressive telomeric decline when grown under inducing conditions (Fig. 3b). The loss of telomeric sequences was evident from the shortening of the terminal restriction fragments and from a reduction in the TTAGGG repeat signal. Telomeres shortened in 4 out of 5 cell lines overexpressing either full-length or Flag-tagged TRF1 (Table 1). In three clones (D4, D16, and C20), the decline was strongly dependent on the absence of doxycyclin. One clone (C14), which expressed Flag-tagged TRF1 in considerable amounts and independently of induction (data not shown), showed a moderate loss of telomeric DNA both in the induced and non-induced state. These results implicated TRF1 in the regulation of telomere length. The rate of telomere shortening varied in the different cell lines from 3 to 11 base pairs (bp) per population doubling (Table 1).

In contrast, HTC75 cells expressing the dominant-negative mutant TRF1$^{66-385}$ showed a gradual increase in telomere length. In the K10 clone (Fig. 3c), telomeres showed progressive elongation over ~80 population doublings at a rate of ~35 bp per population doubling. Eventually the telomeres stabilized, suggesting that a feedback mechanism was operating, like that in budding yeast[18–20]. At this stage in the K10 culture, telomere length remained under the control of the dominant-negative form of TRF1 because repression of the gene at PD104 with doxycyclin induced a gradual shortening of the telomeres (data not shown).

Telomere elongation was also induced in four additional independent HTC75 clones expressing TRF1$^{66-385}$ (Table 1). In each case, telomere elongation was enhanced in the absence of doxycyclin, indicating that the effect is due to expression of the TRF1$^{66-385}$ protein. The altered dynamics appeared to affect all telomeres to about the same degree, leading to a gradual uniform elongation. After extensive growth of the cell lines (88 population doublings), the telomeres had elongated by 0.5–2.9 kilobases (kb) (Table 1). We estimated that the terminal restriction fragments visualized by genomic blotting (Fig. 3) harboured ~1.5 kb of subtelomeric DNA (see 7Methods). On the basis of this estimate, we infer that the telomere alterations in the TRF1$^{66-385}$-expressing cell lines represent a 20–105% increase in the length of the telomeric repeat array. There was a commensurate increase in the TTAGGG repeat signal (Fig. 3c, and data not shown).

Telomere length in human cell lines can be maintained in two ways: either by telomerase-mediated elongation[6,7] or in a telomerase-independent pathway that may involve recombination (known as alternative lengthening of telomeres, or ALT[21]). The gradual, rather than sudden, elongation of telomeres in cells expressing TRF1$^{66-385}$ is consistent with telomerase-mediated telomere maintenance. Furthermore, whereas the ALT pathway results in telomeres that are heterogeneous in length, often extending over a range of 30 kb, telomeres in the TRF1$^{66-385}$-expressing cell lines maintain roughly the same length heterogeneity as the starting population (Fig. 3c). These results suggest that TRF1 regulates the telomerase-dependent pathway of telomere-length maintenance.

We investigated whether TRF1 controls the expression of telomerase by using a polymerase chain reaction(PCR)-based TRAP assay[5,22]. In this assay, soluble telomerase in cell extracts extends a non-telomeric single-stranded substrate with arrays of TTAGGG repeats. As TRF1 only binds to duplex telomeric DNA[12], neither the TRAP-assay substrate nor the telomerase products will be associated with TRF1. The TRAP assay data therefore represent an approximation of the soluble telomerase present in the cell and do not reflect any interaction between telomerase and TRF1 at telomeres. Similar telomerase activity was detected in each of the cell lines shown in Table 1 and no difference was found between cells grown in the presence or absence of doxycyclin (data not shown). These results eliminated the possibility that TRF1 modulates telomere dynamics through a major effect on either the expression of telomerase or its activity in the TRAP assay.

Taken together, our findings showed that one function of TRF1 is to control telomere length, revealing an interesting parallel with the yeast telomeric proteins Rap1 and Taz1 (refs 23–26). The proposed role of Rap1 is to regulate telomere-length in budding yeast[23,25,27,28], and TRF1 likewise is inferred to have a negative effect on telomere-length maintenance by telomerase. As TRF1 affected telomere length without altering telomerase activity in cell extracts, we propose that the TRF1 provides a negative feedback signal to telomerase at individual telomeres. Although TRF1 may function less directly, we favour the possibility that TRF1 controls telomere maintenance by binding to telomeres. Such a *cis*-acting mechanism can explain how cells can monitor and regulate the length of individual telomeres[10,11]. According to this view, longer telomeres (elongated by telomerase) would recruit more of the telomerase

Table 1 Telomere-length control by TRF1

HTC75 clone	TRF1 construct	Δ Telomere length at PD88	
		+Dox	− Dox
B6	Vector	− 0.1 kb	0.0 kb
D4	Full-length TRF1	+0.2 kb	− 1.0 kb
D16	Full-length TRF1	− 0.1 kb	− 0.6 kb
D20	Full-length TRF1	+0.2 kb	+0.7 kb
C14	Flag-TRF1	− 0.3 kb	− 0.3 kb
C20	Flag-TRF1	+0.2 kb	− 0.6 kb
K4	$TRF1^{66-385}$	− 0.8 kb	+0.5 kb
K10	$TRF1^{66-385}$	+0.1 kb	+2.4 kb
K15	$TRF1^{66-385}$	+0.1 kb	+2.8 kb
K16	$TRF1^{66-385}$	+0.7 kb	+2.9 kb
K17	$TRF1^{66-385}$	+0.1 kb	+1.0 kb

PD, population doubling; Dox, doxycyclin.

inhibitor TRF1 than shorter telomeres would, causing elongated telomeres to exert a stronger negative feedback on telomerase and inhibit the enzyme. Such telomeres will shorten with successive rounds of replication until they no longer bind sufficient TRF1 to inhibit telomerase. The resulting telomere length homeostasis is a dynamic process governed by the amount of TRF1 on the telomere and the activity of telomerase. However, it is not excluded that TRF1 modulates telomere length by altering the rate at which telomeres are shortened. As telomere dynamics have been implicated in human ageing and cancer[2–5,29,30], it will be interesting to see how TRF1 contributes to changes in the length of human telomeres in normal, ageing and malignant cells. □

Methods

Inducible gene expression system. HT1080 cells were stably co-transfected with the tetracyclin-controlled transactivator (tTA) expression vector pUHD15-1 (ref. 16) and plasmid pBPGKHyg containing the hygromycin-resistance gene expressed from the PGK (phosphoglycerol kinase) promoter. About 50 hygromycin-resistant clones were expanded and tested for expression of transiently transfected luciferase reporter plasmid pUHC13-1 (ref. 16) in the absence or presence of doxycyclin (100 ng ml^{-1}). Clone HTC75 showed a ~100-fold increase in luciferase expression upon withdrawal of doxycyclin. Next, HTC75 cells were stably co-transfected with the neomycin-resistance plasmid pSXneo[11] and TRF1, Flag-TRF1 or $TRF1^{66-385}$ cloned into the tTA-regulated expression vector pUHD10-3 (ref. 16). For each construct, about 25 G418-resistant clones were expanded in the presence of doxycyclin and tested for inducible expression of the appropriate protein by immunofluorescence microscopy and western blotting using anti-Flag antibody M2 (Eastman–Kodak) and gel-shift analysis of whole-cell extracts using a telomeric-repeat probe[12].

Long-term cell culture. Cells were grown in DMEM supplemented with 10% bovine calf serum (Irving Science). Clones indicated in the text were passaged 1:16 when ~80% confluent (typically every 3 days). All clones were grown in parallel with or without doxycyclin (Sigma; 100 ng ml^{-1}). Addition of hygromycin (90 μg ml^{-1}) or G418 (150 μg ml^{-1}) to culture media was alternated every two weeks.

Antibodies. Antibody 371C2 against the acidic domain of TRF1 was affinity-purified in two steps from a rabbit polyclonal serum raised against baculovirus-expressed TRF1 protein (bacTRF1)[17]. The antiserum was purified against bacTRF1 coupled to CNBr-activated agarose and subsequently purified against a bacterially expressed fusion protein consisting of glutathione-S-transferase and residues 19–71 of TRF1. Antibody 371C2 did not crossreact with other parts of TRF1 (M. Schäfer, B.v.S. and T.d.L., unpublished observations). The following secondary antibodies were used in the experiments shown in Fig. 2: TRF1 detection with 371C2 was achieved with FITC-conjugated donkey anti-rabbit antibody (Fig. 2c), or with a Cy3-conjugated donkey anti-rabbit antibody (Fig. 2g). For FISH, digoxigenin-labelled RNA[8] was detected with a sheep anti-digoxigenin antibody (Boehringer) and a TRITC-conjugated donkey anti-sheep IgG. The Flag-tagged protein was detected with a monoclonal M2 (Kodak), followed by FITC-labelled donkey anti-mouse. All fluorochrome-conjugated antibodies (Jackson ImmunoResearch Labs) were multilabelling grade. Control experiments indicated that there was no cross-reactivity between antibodies.

Whole-cell extracts. Cells grown to ~80% confluence in 10-cm dishes were collected by scraping in PBS, then pelleted and incubated for 30 min at 4 °C in 250 μl buffer C[8] with 0.2% Nonidet P-40. After centrifugation (10 min at 14,000*g*) the supernatant was dialysed against buffer D[8] and stored at − 70 °C until use in gel-shift assays, western blotting and TRAP assays. Protein was determined by the Bradford assay (BioRad).

Genomic blotting and telomere-length estimation. Genomic DNA was isolated from cells at the indicated population doublings (PDs), digested to the completion with *Hin*fI and *Rsa*I and quantified by fluorometry using Hoechst 33258 dye. Genomic blots were prepared as described[13]. The median telomeric restriction-fragment length was determined by PhosphorImager analysis using ImageQuant software and not corrected for the dependence of signal strength on telomere length. The length of the subtelomeric DNA on the *Hin*fI/*Rsa*I fragments was estimated[3] to be ~1.5 kb.

Received 4 December; accepted 31 December 1996.

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Acknowledgements. We thank M. Schäfer for isolation and characterization of the 371C2 TRF1 antibody, H. Bujard for tetracyclin system plasmids, and A. Lustig, R. Lang and members of our laboratory for criticism of this manuscript. This work was supported by grants from the NIH, the Rita Allen Foundation, and the Irma T. Hirschl Foundation (to T.d.L.). B.v.S. is the recipient of a Human Frontier Science Program postdoctoral fellowship.

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