Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity

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We investigated the influence of telomere proximity and composition on the expression of an *EGFP* reporter gene in human cells. In transient transfection assays, telomeric DNA does not repress *EGFP* but rather slightly increases its expression. In contrast, in stable cell lines, the same reporter construct is repressed when inserted at a subtelomeric location. The telomeric repression is transiently alleviated by increasing the dosage of the TTAGGG repeat factor 1 (TRF1). Upon a prolonged treatment with trichostatin A, the derepression of the subtelomeric reporter gene correlates with the delocalization of HP1 α and HP1 β . In contrast, treating the cells with 5 azacytidin, a demethylating agent, or with sirtinol, an inhibitor of the Sir2 family of deacetylase, has no apparent effect on telomeric repression. Overall, position effects at human chromosome ends are dependent on a specific higher-order organization of the telomeric chromatin. The possible involvement of HP1 isoforms is discussed.

INTRODUCTION

Telomeres have a structure that allows the cell's DNA repair machinery to distinguish natural chromosome ends from 'broken' DNA ends (Lundblad, 2000). They also provide a means for the complete replication of the chromosomal DNA (Blackburn, 2000). Furthermore, the structure and spatial localization of telomeric chromatin play an important role in the nuclear compartmentalization of gene expression and probably of other chromosomal transactions, such as replication initiation, condensation, segregation, recombination and repair (Gilson *et al.*, 1993). These properties are usually revealed by telomeric

position effect (TPE). For instance, telomere proximity induces gene silencing and late activation of replication origins (Gottschling *et al.*, 1990; Ferguson and Fangman, 1992; Nimmo *et al.*, 1994; Rudenko *et al.*, 1995; Cryderman *et al.*, 1999).

The heterochromatic nature of mammalian telomeres and their capacity to induce position effect have been controversial for many years (Bayne *et al.*, 1994; Sprung *et al.*, 1996; Ofir *et al.*, 1999; Smith and Higgs, 1999; Wright *et al.*, 1999; Baur *et al.*, 2001). Until now, the best evidence for human TPE (hTPE) consists in the delayed replication timing induced by a chromosome truncation (Ofir *et al.*, 1999) and in the telomere length dependency of the expression of a subtelomeric reporter gene (Baur *et al.*, 2001). In diseases involving telomeric rearrangements, a modulation of hTPE might lead to inappropriate gene expression, as in the myopathy FSHD (Gabellini *et al.*, 2002).

We investigated the influence of telomere proximity and composition on the expression of an *EGFP* reporter gene in human cells. Our findings demonstrate that TPE in human cells is dependent on a specific higher-order organization of the telomeric chromatin.

RESULTS AND DISCUSSION

The proximity of telomeric DNA activates gene expression in transient assays

We first asked whether a stretch of telomeric DNA could act as a *cis*-acting regulatory element in transient transfection assays. For this purpose, C33-A cells (a cervical undifferentiated carcinoma

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Fig. 1. Transient expression assays reveal a transactivation activity of telomeric repeats. (A) Schematic map of the two reporter plasmids used in this study. pCMVTelo contains a stretch of 1.6 kb TTAGGG in close proximity to the reporter *EGFP* gene driven by a CMV promoter. At 1.8 kb from the TTAGGG repeats, we introduced the fusion gene between hygromycin phosphotransferase and HSV1 thymidine kinase (*HyTK*) conferring the resistance to hygromycin as a selectable marker. The control vector lacking the telomeric 1.6 kb stretch was named pCMV. (B) Transient expression assays with various amount of pCMV or pCMVTelo circular DNA. A time course of the *EGFP* expression after transfection was determined. The percentage of *EGFP*-positive cells is corrected for transfection efficiency determined by CAT assay. The values correspond to the average of at least three independent experiments. We estimated the standard error to be <20%. (C) Ratio of the percentage of *EGFP*-positive cells in pCMVTelo transfection to that in pCMV transfections.

line) were transfected with increasing amounts of plasmid DNAs bearing an *EGFP* gene under the control of the CMV promoter with or without 1.6 kb of adjacent TTAGGG repeats (pCMVTelo and pCMV, respectively; Figure 1A). The molar concentration of the transfected CMV promoter DNA was

maintained constant by adding an appropriate amount of plasmid containing only the CMV promoter DNA. Putative variations in transfection efficiency were evaluated by co-transfecting with pBLCat DNA (Waltzer et al., 1996) and by normalizing the percentage of *EGFP*-expressing cells to the amount of CAT expression.

An enhanced expression of *EGFP* correlates with the dosage of the plasmid DNA and peaks 3 days after transfection (Figure 1B). The increase in the percentage of EGFP-positive cells is much more pronounced with pCMVTelo than with pCMV (Figure 1B and C). Therefore, TTAGGG repeats do not exhibit silencing properties in transient transfection assays. Thus, it appears unlikely that hTPE results simply from the binding of a transcriptional repressor to telomeric DNA.

Repressive effects of telomere proximity in stably transfected cells

In order to test whether the chromosomal context is important to uncover the repressive properties of telomeric DNA, we integrated the same *EGFP* reporter cassette at the immediate proximity of a telomere. Since cloned human telomeric DNA can seed the formation of new telomeres (Farr *et al.*, 1991; Hanish *et al.*, 1994), linearized pCMVTelo DNA was transfected into C33-A cells. Transfections with linearized pCMV DNA was used as a negative control. Figure 2 shows that, in three independent transfections (1, 2 and 3), within the first few days, the percentage of EGFP-positive cells is significantly higher among pCMVTelo than among pCMV transfectants. This effect is more pronounced for transfection 1, but it can clearly be seen in 2 and 3 (see the enlarged views in Figure 2). This transactivation of the telomeric DNA-linked reporter at an early stage of the transfection process is in agreement with the results obtained in transient transfection with circular DNA (Figure 1).

From the three transfections shown in Figure 2, several clones were established from hygromycin resistant cells. Successful telomere seeding in pCMVTelo clones was confirmed by detection of a diffuse hybridization signal using the EGFP probe (Hanish *et al.*, 1994). Sixteen out of 18 analyzed clones correspond to a telomeric fragmentation (data not shown). For three out of three randomly chosen telomeric clones, the presence of the *EGFP* DNA at one chromosome end was confirmed on metaphase spreads by fluorescence *in situ* hybridization (FISH), using a pCMV DNA probe (Figure 3A; data not shown). These data reveal a very high seeding efficiency for C33-A cells, indicating that the population of pCMVTelo-transfected cells is likely to contain a large majority of telomeric integration sites, probably at different chromosome ends.

After 10 days in hygromycin, the percentage of EGFP-positive cells among the population of transfected cells becomes higher with pCMV than with pCMVTelo, reversing the transactivation effect observed with pCMVTelo-transfected cells at the beginning of the kinetics (Figure 2). This striking change is likely to coincide with the integration of the plasmid DNA into chromosome and the seeding event in pCMVTelo-transfected cells. With time, the percentage of positive pCMV transfectants increases progressively, whereas the rate of positive cells after transfection with pCMVTelo plateaus at ~10%.

Starting from the cells transfected with pCMVTelo, sorted populations either enriched or depleted in EGFP-positive cells were cultured for ~20 mean population doublings (MPDs). The *EGFP* expression remained relatively stable, whether or not the medium contains hygromycin (data not shown). Thus, the percentage of EGFP-positive cells is a long-term characteristic of a population of cells. Furthermore, this indicates that the selective pressure exerted by hygromycin on the expression of

Fig. 2. Telomeric silencing in populations of transfected cells. The pCMV and pCMVTelo DNA were linearized with *NotI* (Figure 1A) and used to transform C33-A cells. By co-transfection with pBLCat, we estimated that the efficiencies were roughly similar in all situations (data not shown). The percentage of EGFP-positive cells was analyzed by Facscan. Three independent transfections are shown (1, 2 and 3). Three days after transfection, hygromycin (400 µg/ml) was added. The boxes show an enlarged view of the beginning of the kinetics for 2 and 3.

the *HyTK* gene does not greatly influence the expression of *EGFP*, at least for 20 MPDs. This uncoupling between the *HyTK* and the *EGFP* expression might be attributed to a putative boundary activity of the CMV promoter.

Only clones containing a single copy of the *EGFP* gene were studied further. Single-copy insertions were revealed as an equal signal intensity between a pCMV plasmid and a β -actin probe by Southern blotting (data not shown). By these criteria, all 16 telomeric clones and 10 out of 20 analyzed pCMV clones exhibit a single integrated copy of the plasmid DNA. Both the pCMV and the pCMVTelo clones are mosaics of EGFP-positive and -negative cells (Figure 3B). For all these clones, a population deprived of EGFP-positive cells was sorted and cultured for 3 months without any significant change in *EGFP* expression (data not shown). This indicates that the repression of *EGFP* is not spontaneously reversible within the time limits of this experiment. For pCMV clones, the rate of positive cells within a positive clone ranges from 0.02 to 70%. In contrast, the pCMVTelo clones have an expression efficiency ranging from 0.1 to 3% in positive clones (Figure 3B). The fact that the range of *EGFP*-expressing cells is more constrained when the transgene is juxtaposed to a

Fig. 3. Telomeric silencing in clones. (A) Localization of the *EGFP* gene at 16p by chromosome 16 painting (image a) and FISH with an *EGFP* probe (image b); the position of 16p is marked by arrows. (B) The percentage of *EGFP*-positive cells in clones presenting a single insertion of the reporter gene. These clones were obtained from three independent transfections with either pCMV or pCMVTelo. (C) The percentage of *EGFP*-positive cells plotted versus the length of the *EGFP*-linked telomere [eTRF in kb of (TTAGGG)_n]. The eTRF value was determined by Southern blotting after probing *Hind*III-cut genomic DNA with an *EGFP* probe.

telomere than when it is internally inserted indicates that telomere proximity confers a characteristic position effect.

TPE correlates with telomeric protein composition

We plotted the percentage of *EGFP*-positive cells corresponding to the different pCMVTelo clones as a function of the median length of the associated telomeric DNA (*EGFP* telomeric restriction fragment, eTRF; Figure 3C). There is no obvious correlation

between the two values (Figure 3B), in agreement with previous studies (Sprung *et al.*, 1996). This seemingly contrasts with the fact that a telomere overelongation of 9 kb correlates with an increased TPE (Baur *et al.*, 2001). This discrepancy can be explained by cell type differences and/or the range of telomere length variation. Alternatively, what controls hTPE might not be the telomere length *per se* but accompanied changes in chromatin structure.

Therefore, we modified the dosage of a major component of the telomeric chromatin, the TTAGGG repeat factor 1 (TRF1) protein (Chong *et al.*, 1995; Billaud *et al.*, 1996). We constructed a TRF1 expression vector (pTRF1) where a FLAG-tagged *TERF1* gene is co-transcribed with the neomycin phosphotransferase gene. The two open reading frames are co-translated due to the presence of an internal ribosome entry site sequence (IRES). The pattern of TRF1-FLAG expression was roughly similar after transfection of two pCMVTelo clones, named 4C4 and 4C11, or of two pCMV clones, named 2A3 or 3B10. By immunoblotting using anti-FLAG antibodies, TRF1-FLAG reaches a plateau after ~11 MPDs after selection and then remains relatively constant for at least 17 MPDs (Figure 4A and B for 4C11 and 2A3, respectively; data not shown for 4C4 and 3B10). Importantly, by immunoblotting with anti-TRF1 antibodies, it appears that the total amount of TRF1 protein, i.e. the endogenous one and TRF1-FLAG, is significantly enhanced, compared to pIRES-transfected cells, after 11 MPD days and then remains constant (Figures 4A and B; data not shown). This is in agreement with the kinetics of TRF1-FLAG expression and indicates that the level reached by TRF1-FLAG at the plateau is higher than that of the endogenous TRF1. Although it was previously reported that an increased TRF1 or FLAG-TRF1 dosage decreases telomere length (van Steensel and de Lange, 1997), no apparent change of telomere length was observed for 20 MPDs, both for the *EGFP*-linked telomere and for the bulk of telomeres (data not shown). More cell divisions might be required to detect a change in telomere length dynamics. Indeed, overexpression of full-length TRF1 leads to a relatively slow rate of shortening, ranging between 3 and 10 nt/MPD (van Steensel and de Lange, 1997). Alternatively, a small subset of cells could express TRF1-FLAG at a very high level, leading to a rapid telomere shortening in only a minority of cells. However, this possibility appears highly unlikely, since immunofluorescence studies with FLAG antibodies revealed that >90% of them expressed TRF1-FLAG at a high level during the time course of the experiments (see Supplementary figure 1 available at *EMBO reports* Online). Importantly, we have been unable to detect a small fraction of cells expressing a significantly higher amount of TRF1

Nevertheless, the expression of TRF1-FLAG at 7 MPDs after selection is sufficient to lead to a large increase in the percentage of *EGFP*-positive 4C4 and 4C11 cells when normalized to the expression of *EGFP* in cells transfected with the parental DNA (pIRES) (Figure 4C). The level of *EGFP*-positive cells remains elevated until 20 MPDs, when it returns to base line (Figure 4C). The increase is more pronounced with the 4C11 clone (culminating at ~8-fold) than with the 4C4 clone (culminating at 3-fold) (Figure 4C). No variation in *EGFP* expression was monitored when TRF1-FLAG was expressed in 3B10 or 2A3 cells (Figure 4C), indicating that the TRF1 effect is specific for the

Fig. 4. Overexpression of TRF1 antagonizes telomeric silencing. The pITRF1 or pIRES plasmid DNAs were transfected in the 3B10 and 2A3 pCMV clones and the 4C4 and 4C11 pCMVTelo clones. Four days after transfection, a neomycin selection was applied (t0) and cells were cultured for >30 MPDs. (A) TRF1 expression was determined in 4C11 cells transfected with pIRES or pITRF1 by immunoblotting using either anti-FLAG antibodies (a monoclonal anti-M2 antibody; Sigma) or anti-TRF1 antibodies. Anti-tubulin antibodies were used to evaluate the loading variations. (B) The same type of immunoblotting experiments as in (A) but with 2A3 transfected cells. (C) The entire population of neomycin resistant clones was analyzed by Facscan for *EGFP* expression.

EGFP gene adjoining a telomere. We conclude that TRF1-FLAG expression correlates with the derepression of a subtelomeric *EGFP* gene. This establishes that TRF1 is an hTPE-modifier in two different cell lines. However, this does not imply that the effect of TRF1 on hTPE is direct. For instance, a modified dosage of TRF1 might perturb the integrity of telomeric chromatin, altering the binding or titrating out some key TPE players. Finally, it seems unlikely that the re-establishment of TPE corresponds to a counterselection of TRF1-expressing cells because the TRF1 dosage remains unaltered (Figure 4A). Therefore, we favor the idea that the transient derepression reveals an adaptation process of the telomeric chromatin to the new concentration of TRF1, allowing it to regain at least part of its properties.

These results show that hTPE occurs at a seeded telomere in human C33-A cells. Studies on yeast TPE indicate that the repressive properties of such a newly formed telomere are shared by native telomeres, except for chromosome-specific modulations exerted by the natural subtelomeric regions (Fourel *et al.*, 1999; Pryde and Louis, 1999). Therefore, hTPE is highly likely to be a general property of human telomeres.

A specific chromatin structure determines hTPE

Trichostatin A (TSA) inhibits the HDAC families of histone deacetylases and leads to the loss of structure of the heterochromatin compartment (Yoshida *et al.*, 1990; Taddei *et al.*, 2001). An increase of *EGFP* expression rapidly occurs in a population of 4C11 cells after a first treatment with a subtoxic dose of TSA (Figure 5). Impressively, the derepression induced by TSA is exacerbated upon a second treatment (Figure 5), indicating a potentiation of the effect. No such phenomenon was detected in populations of 2A3 or 3B10 cells (Figure 5). This demonstrates that it is not an intrinsic property of the *EGFP* reporter cassette to be highly sensitive to TSA and indicates that the TSA effect in 4C11 cells is dependent on the telomeric location of the *EGFP* gene. A similar conclusion was reported in HeLa cells (Baur *et al.*, 2001). In contrast, no apparent change in *EGFP* expression was observed when cells were treated by two or more successive doses of 5 azacytidin (5 azaC), which reduces cytosine methylation (Harrison *et al.*, 1983), and of sirtinol, a drug recently identified as a specific inhibitor of the sir2/sirtuin family of NAD-dependant deacetylases (Grozinger *et al.*, 2001).

In order to monitor the impact of the TSA treatments on the spatial organization of the centromeres and telomeres in our cellular system, FISH experiments with alphoid DNA and telomeric PNA probes were performed. Remarkably, each of the three types of treatments correlates with distinct patterns of heterochromatin destructure (see Supplementary figure 2A). These changes occur at the first TSA treatment and do not seem to be exacerbated at the second treatment. The spotty pattern of telomeric DNA appears unchanged upon the two successive TSA treatments (see Supplementary figure 2B). These results prompted us to determine the localization of the three mammalian isoforms of the heterochromatin protein 1 (HP1 α , β and γ). HP1 α and HP1 β localize predominantly to centromeric heterochromatin, and HP1 γ localizes to both heterochromatin and euchromatin (Minc *et al.*, 1999; Aagard *et al.*, 2000). Importantly, they bind heterochromatin in a TSA-sensitive way (Taddei *et al.*, 2001). In interphase nuclei, the three HP1 isoforms exhibit a punctated pattern, whereas, in metaphase chromosomes, HP1 α and HP1 β show a predominant centromeric staining (see Supplementary figure 2C). As previously reported (Minc *et al.*, 1999; Aagard *et al.*, 2000), we also noted infrequent signals at chromosome tips. This observation indicates that HP1 α and HP1 β play a role at telomeres.

In contrast to alphoid DNA, the first treatment with TSA does not seem to greatly alter the HP1 pattern in interphase nuclei. However, the second TSA treatment clearly leads to a release of HP1 α and HP1 β but not of HP1 γ (see Supplementary figure 2C). We conclude that the first treatment of TSA is sufficient to perturb the organization of centromeric DNA but is insufficient to massively dissociate HP1 α and HP1 β from centromeres. Interestingly, the correlation between hTPE potentiation and HP1 α / β release at the time of the second TSA treatment indicates an involvement of HP1 isoforms in hTPE.

Finally, we investigated whether hTPE can be related to a preferential association of the reporter gene with the centromeric heterochromatin compartment. The proportion of subtelomeric *EGFP* genes associated with various types of centromeric regions

Fig. 5. TSA alleviates telomeric silencing. Populations of 4C11, 2A3 or 3B10 cells sorted for a deprivation of EGFP-positive cells were incubated for 3 days with a culture medium containing the indicated drug at T1; then the medium was changed with one of identical composition (T2).

remains essentially unchanged in EGFP-positive and -negative cells or when cells are treated with TSA (see Supplementary figure 3). Thus, there is no apparent correlation between the silencing state of the subtelomeric gene and its association with centromeric heterochromatin. This does not favor a model where hTPE would result from the recruitment of chromosome ends at or near centromeres (Brown *et al.*, 1997). However, it is plausible that the silent telomere localizes to alternative areas of heterochromatin. In particular, the telomere itself might recruit one or several repressive factors in a TSA-sensitive way. The correlation between the HP1 delocalization and hTPE alleviation, together with the occasional staining of HP1 isoforms to telomeres, indicate that one of these factors could be HP1.

METHODS

Plasmids and molecular biology. The pCMV and pCMVTelo plasmids derived from pEGFP1 (Clontech) (Figure 1A) carry the *HyTK* gene excised from pNYH3 (Lupton *et al.*, 1991) (obtained through the courtesy of T. de Lange). The pITRF1 results from the cloning of a FLAG version of the *TERF1* gene (Vassetzky *et al.*, 1999) into pIRES1neo (Clontech). Details concerning the exact cloning procedure will be given upon request. Cellular DNA was prepared by using Nucleon Bacc2 kits (Amersham). FISH experiments were performed with biotin or digoxigenin labeled pCMV plasmids or with a chromosome-specific set of probes (Vysis).

Cells methods. The human cell line C33-A (carcinoma, cervix) was grown in DMEM with bicarbonate supplemented with 10% fetal calf serum, 1% L- glutamine and antibiotics. Transfections were performed by using a modified calcium phosphate method (Chen and Okayama, 1987). The transfection efficiency of C33-A cells was estimated by transfecting 2 μg of a *LacZ*-expressing vector and was found to vary between 30 and 40% in independent experiments. The CAT expression was determined by ELISA (Boehringer). The percentage of EGFP-positive cells was monitored by Facscan. Populations of cells either enriched or deprived in EGFP-positive cells were sorted through Facstar +.

To determine the highest subtoxic concentration of each drug (TSA, 5-azaC and sirtinol) that was non-toxic and did not affect cell growth, a C33-A cell line was cultured in various concentrations of the drug for 1 week. The highest concentrations that did not cause a decrease in cell growth rate were chosen.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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