Transcription Factor CTF1 Acts as a Chromatin Domain Boundary That Shields Human Telomeric Genes from Silencing

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Received 15 May 2008/Returned for modification 10 June 2008/Accepted 5 February 2009

Telomeres are associated with chromatin-mediated silencing of genes in their vicinity. However, how epigenetic markers mediate mammalian telomeric silencing and whether specific proteins may counteract this effect are not known. We evaluated the ability of CTF1, a DNA- and histone-binding transcription factor, to prevent transgene silencing at human telomeres. CTF1 was found to protect a gene from silencing when its DNA-binding sites were interposed between the gene and the telomeric extremity, while it did not affect a gene adjacent to the telomere. Protein fusions containing the CTF1 histone-binding domain displayed similar interactions rather than silencing at human telomeres. Overall, these results indicate that transcription factors can act to delimit chromatin domain boundaries at mammalian telomeres, thereby blocking the propagation of a silent chromatin structure.

In eukaryotes, regulation of gene expression is believed to rely largely on modifications of the structural organization of chromatin, which may include the relative positioning of chromosomal domains in the cell nucleus, nucleosome localization on regulatory sequences, and covalent modifications of histones and DNA or the incorporation of histone variants. For instance, the heterochromatin structure frequently associated with gene silencing has been associated with low levels of histone acetylation and with a variety of other epigenetic markers, such as changes in the methylation status of histones and of the DNA. Silent heterochromatin portions of the chromatin are interspersed with euchromatic structures that are more permissive for gene expression, and boundaries between the two types of chromatin structures have been found to be enriched with specific epigenetic markers, such as incorporation of the H2A.Z histone variant (25, 28, 46).

Constitutive heterochromatin, as found at telomeres or centromeres, has been associated with the silencing of adjacent genes. In Saccharomyces cerevisiae, the telomere position effect (TPE) has been well studied, and it is attributed to the spreading of the SIR complex from the telomere along the chromosome. Proteins of the SIR complex associate with deacetylated nucleosomes, where their histone deacetylase activity may modify adjacent histones, allowing autopropagation of the hypoaecetylated structure along the chromosome (18). TPE-associated gene silencing has also been observed in human and mouse cells (3, 34), but a potential role of SIR-like proteins and/or of another mechanism that propagates a telomeric heterochromatich structure remains to be identified in mammals.

The mammalian telosome is composed of a multiprotein complex that binds to repetitive telomeric DNA sequences. This complex, named Shelterin, may shield the telomere from being recognized as a double-strand break through the formation of the T loop (6, 9, 17) while ensuring the maintenance of a correct length of telomeric repeats by interacting with the telomerase (21, 45). Chromatin features present at telomeres and nearby subtelomeric sequences include histone modifications, such as the trimethylation of H3 on lysine 9 (H3K9Me3) and of H4 on lysine 20 (H4K20Me3) (5, 36, 39). These modifications have been associated with constitutive heterochromatin, as exemplified by the interaction of H3K9Me3 with heterochromatin protein 1 (HP1) (8, 35, 39), although other studies have indicated that H3K9Me3 and H4K20Me3 modifications may also occur transiently upon transcriptional activation (41). Trimethylation of H3K9 and H4K20 are catalyzed by the Suv39H1/2 and Suvar4-20H1/2 histone methyltransferases, which are specifically recruited to the telomeres (5, 15, 23). The relatively low activities of the cellular demethylases capable of removing methylation residues at the telomeric locus are consistent with the persistence of these modifications in the constitutive heterochromatin (7, 22, 27, 40).

Insulators and boundaries are DNA elements that may alter gene expression by preventing activation or inhibitory effects that stem from their chromosomal environment (4, 43). Insulators are often defined as DNA elements that can prevent the action of an enhancer or silencer on a promoter when inter-
posed between the promoter and the regulatory sequence. Chromatin domain boundaries are defined as elements that prevent the propagation of chromatin features, such as heterochromatin, and they may thereby demarcate chromosomal domains that possess distinct chromatin features and gene expression status. Nonetheless, while insulator and boundary elements may be distinguished experimentally, the frontier between these two types of epigenetic regulators is not entirely clear, as expected from the fact that enhancers’ or silencers’ function may include the regulation of chromatin structure. For instance, the chicken HS4 enhancer-blocking insulator has also been found to protect transgenes from TPE (37). In yeast, boundary activities have been observed for proteins such as DNA-binding transcription factors (14, 19). Notably, the activation domains of the human transcription factors, such as CTF1, have been found to block SIR-mediated silencing when recruited to the yeast telomere (12). However, mammalian and yeast cells have distinct chromatin structures, and whether silent chromatin may propagate from mammalian telomeres as it does in yeast is unclear because an equivalent of the yeast SIR protein complex has not been found in mammalian cells. In addition, whether DNA-binding proteins such as transcription factors may insulate genes from telomeric silencing effects remains to be established.

CTF1 is a member of the CTF/NFI family of mammalian transcriptional regulators that may activate or repress the expression of cellular and viral genes and whose presence is required for proper mouse development. CTF1 was proposed to interact with the histone H3.3 variant and thereby to mediate transcriptional regulation in response to growth factors (1). CTF1 derivatives were also shown to prevent the silencing of transgenes upon chromosomal integration (33). However, the precise regulatory mechanism and potential action on chromatin structure by this class of transcription factor have remained unclear.

In this study, we have evaluated whether proteins such as CTF1 may prevent the silencing of telomeric transgenes in mammalian cells and whether this may result from the interruption of the propagation of a specific chromatin structure from the telomere. To do so, we generated a dual reporter fusion protein expression. Fluorescence in situ hybridization (FISH) was performed 48 h later. Stable populations expressing Gal-DVD/Gal-Pro were obtained by cotransfecting the Gal fusion expression plasmid, a BFP-encoding plasmid, and a phleomycin resistance plasmid at a 45:45:10 weight ratio. Cell culture, transfection, and in situ hybridization. HeLa cells were harvested at a confluence of 90% and cross-linked with 1% formaldehyde for 4 min. After lysis of the nuclei, chromatin was sonicated to 200 bp and digested with BamHI. The chromatin solution was diluted to a volume of 300 μl in a buffer containing 200 mM HEPES, 2 M NaCl, 20 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, and 1 mg/ml bovine serum albumin. Chromatin fragments were precleared for 30 min with 10 μl Protein A Sepharose (Amersham Bioscience), and immunoprecipitation were incubated at 4°C overnight with 5 μl of antibody. Immunoprecipitated complexes were incubated with 10 μl Protein A Sepharose, and pellets were washed three times with immunoprecipitation buffer (20 mM HEPES, 0.2 M NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100). Immunoprecipitated complexes were eluted in 100 mM Tris-Cl/1% sodium dodecyl sulfate, and cross-links were reversed at 65°C for 1 h. Precipitated DNAs were eluted in 50 μl Tris-EDTA. Quantitative PCR. Quantitative PCR was performed using a 7900 sequence detector (Applied Biosystems) using the SYBR green reagent (Eurogentec). Chromatin immunoprecipitation samples and chromatin input were diluted 10-fold before analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was performed using primers 5'-GGCCTCGTTTCTATAA-3' and 5'-AGAGCTGCAACAGGAGGCAGCAG-3', that for EGFP using primers 5'-ACCAAAGCCACCCAGAAGG-3' and 5'-GGCCGCGTCACGCAAGA-3', that for DsRed using 5'-TTCCGACTCCGGTCCAAAGT-3' and 5'-GGAGAAGACAGCTTCTCTTGTAGTCG-3'. The relative positions of these amplitons are shown in Fig. 1A. EGFP and DsRed values were normalized to that of GAPDH.

RESULTS

Design of telomeric gene silencing quantitative assay. In order to analyze both telomere-insulated and noninsulated genes cointegrated at the same telomeric locus, we generated the reporter plasmids shown in Fig. 1A. Reporter vectors con-
sist of the green fluorescent protein (GFP) and DsRed coding sequences placed on either side of four DNA binding sites for the yeast GAL4 protein. Each reporter gene was placed under the control of a minimal cytomegalovirus promoter in an orientation mediating either a convergent or divergent direction of transcription to control that transcriptional orientation relative to the telomere or the distance between the insulator element and reporter gene promoters would not affect silencing or insulation. An antibiotic resistance gene was placed adjacent to DsRed, while telomeric (TTAGGG)n repeats were placed next to the GFP expression cassette. Previous studies have demonstrated that stable transfections of telomeric repeat-containing plasmids yield mostly single-copy integration at a telomeric position, possibly because integration of the telomeric repeats induces a chromosomal break and the formation of a new telomere (3, 34, 37). These constructs were transfected, and antibiotic-resistant cells having stably integrated the transgenes in their genome were selected and sorted.
into monoclonal populations. Clones showing the following properties were discarded: (i) heterogeneous or disproportionately DsRed and GFP fluorescence, probably because of multiple insertions and/or a nonclonal nature, (ii) no activation of DsRed and/or GFP upon transfection of a Gal-VP16 expression vector, which often coincided with the lack of reporter genes as based on PCR amplification assays (data not shown), and (iii) high basal expression of GFP and DsRed, which may result from nontelomeric integrations. FISH analysis indicated a telomeric or subtelomeric transgene position for all retained clones (see Fig. S1 in the supplemental material). Monoclonal populations were also generated from the transfection of reporter plasmids deleted of the telomeric repeats to obtain integration at nontelomeric loci, and comparably silent cell clones were selected according to the above criteria.

This yielded clonal populations generated from telomeric repeat-containing plasmids with either divergent or convergent transcription. Clones displaying a telomeric transgene location had essentially undetectable or low transgene expression (see Fig. S1, S2A, and S2B in the supplemental material). These results are consistent with previous reports of the low expression of telomeric transgenes in mammalian cells (3, 34, 37). Clones generated using the constructs devoid of telomeric repeats displayed random chromosomal integration sites and more-variable levels of expression. Clones displaying clear internal chromosome integration and low or essentially silent expression levels were kept as controls (see Fig. S2C and S3 in the supplemental material).

**CTF1 protects telomeric transgenes from TPE.** The proline-rich domain of CTF1 has been shown to interact with histone H3.3 and to activate gene transcription in response to growth factors in mammalian cells (1). To specifically assess CTF1 activity at mammalian telomeres and to exclude possible interference from other members of the HeLa cell CTF/NFI family (38), the CTF1 proline-rich domain was transiently expressed as a fusion to the DNA binding domain of the yeast GAL4 protein (Gal-Pro). Expression vectors encoding either the unfused GAL4 DNA-binding domain (Gal-DBD) or a fusion with the strong herpes simplex virus VP16 activator (Gal-VP16) were used as controls. These plasmids were cotransfected with a BFP expression vector as a transfection marker, and transiently transfected BFP-expressing cells were analyzed for GFP and DsRed fluorescence. Gal-Pro expression resulted in an increase of DsRed fluorescence without an increase of GFP fluorescence in the telomeric clones (compare Fig. 1B and E with C and F, respectively). Moreover, the use of plasmid construct derivatives carrying DsRed and GFP in a reversed configuration yielded a preferential expression of the telomere-distal GFP reporter (see Fig. S4 in the supplemental material). This indicates that the expression of the telomere-distal gene in clones expressing Gal-Pro is not gene specific but that it is dependent on its position relative to the telomere and to the GAL-Pro binding sites. In contrast, the Gal-VP16 fusion did not significantly activate the transgenes when transcribed in a convergent fashion (Fig. 1B and D; see Fig. S4B in the supplemental material), while it activated DsRed and GFP divergent transcription to a similar extent (Fig. 1E and G; see Fig. S4D in the supplemental material). The lesser activation of the convergent construct is explained by the more distant location of the Gal-VP16 binding sites in relation to the promoters driving the reporter genes (Fig. 1A, top drawing). Assays of GAL4 fusions to other proteins that bind insulator and/or boundary elements, such as CTCF or USF1 (3, 34, 37), failed to affect DsRed or GFP expression (data not shown).

Quantification of the Gal-Pro effect indicated that it occurs in independent clones that have telomeric transgenes integrated in various chromosomes (Fig. 2A and B; see Fig. S2 in the supplemental material). In contrast, Gal-VP16 activated the expression of the reporter genes to various extents but without a marked preference for the activation of DsRed over GFP. Gal-Pro had variable but generally small effects on the expression of silent transgenes integrated at nontelomeric po-
sitions, where it could activate DsRed and also GFP expression (Fig. 2C).

We previously showed that the proline-rich activation domain of CTF1 possesses two regions that cooperate to bind histone H3 and that this domain may reposition nucleosomes close to its binding site (12, 32, 33). Thus, we assessed whether the H3 interaction domains may mediate the boundary activity. Gal fusions previously characterized by their ability to bind H3 were expressed in the telomeric clones B09 and D17, where Gal-Pro shows strong boundary effects. In both cases, deletions in the H3 interaction domains were associated with a reduction of DsRed expression (see Fig. S5 in the supplemental material), matching previously reported results on the interaction of these mutants with histone H3.3 as probed by two-hybrid assays (1). Similarly, single proline-rich domain point mutations known to decrease or abolish interaction with H3 consistently reduced the boundary effect (see Fig. S6 in the supplemental material). Taken together, these data are consistent with an involvement of H3 interaction activity in the boundary effect.

To assess if the boundary effect can also be observed from the expression of native transcription factors such as CTF1, we analyzed clones generated with reporter constructs carrying seven CTF/NFI binding sites inserted between the two reporter genes instead of GAL4 sites. Since various members of the family of CTF/NFI proteins are expressed in HeLa cells (38), we sought to identify clones in which the additional expression of CTF1 may mediate a boundary effect. Clones having integrated the reporter genes in telomeric or internal chromosomal positions were thus isolated and analyzed after the transient expression of CTF1. The boundary effect was observed upon CTF1 expression in cells with telomeric transgenes (Fig. 3A and B), while a commensurate activation occurred for both reporter genes inserted at an internal location on the chromosome (Fig. 3C and D). The boundary effect at telomeric loci was observed in three independent clones with telomeric transgenes, but the boundary effect observed upon CTF1 overexpression was smaller overall than that obtained with the GAL4 fusion protein (data not shown). This may stem from the background of CTF/NFI proteins, since they may already mediate some boundary effects on the reporter constructs containing CTF/NFI binding sites and/or from the strong interaction of GAL4 with heterochromatic DNA compared to that of CTF1 (33).

Taken together, these results indicate that CTF1 and its fusion derivatives act specifically to prevent silencing of the telomere-distal gene but not of the telomere-proximal gene, implying that they may prevent the propagation of a silencing signal from the telomere toward more centromeric sequences. Thus, these results suggested that this protein may act as a
boundary or barrier element that blocks the spreading of a repressive chromatin structure from the telomere.

Chromatin landscape at mammalian telomeric loci. Chemical agents that affect histone acetylation or DNA methylation were used to assess whether telomeric transgenes are subjected to chromatin-mediated silencing effects. TSA, a broad-specificity inhibitor of class I and II histone deacetylase (HDAC), was found to strongly increase transgene expression at various telomeric positions in independent cell lines (see Fig. S7 in the supplemental material). In contrast, NaB, a more specific inhibitor of the HDAC I and IIa classes, mediated lower unsilencing effects in some clones, suggesting an involvement of the HDAC IIb class in gene silencing at some but not all telomeres. Thus, several HDAC activities may be involved in telomeric gene silencing. HDAC inhibitor treatment of telomeric clones with lower transgene expression generally resulted in greater enhancement of gene expression, as would be expected from a chromatin-mediated silencing process (compare Fig. S1A and S1B with S7A and S7B in the supplemental material).

Treatment of telomeric clones with the 5azadC DNA methylation inhibitor had little effect on transgene expression (see Fig. S6 in the supplemental material). Thus, DNA methylation is unlikely to be the primary determinant of telomeric silencing in this cellular model. Several studies have shown that BrdU can abolish expression variegation, namely, the cycling between semistable expressing and nonexpressing states. Its mode of action remains unclear, but it may act by decreasing histone mobility (26). Treatment of telomeric clones with BrdU was associated with an increase in expression of the reporter genes but to a lesser extent than that noted with TSA.

The loss of silencing observed upon treatment with inhibitors of histone deacetylation implies a chromatin-mediated silencing of the telomeric genes. However, independent clones showed distinct sensitivities to the various chemical HDAC inhibitors, suggesting that different molecular mechanisms may lead to telomeric silencing. This was addressed using chromatin immunoprecipitation assays performed with two clones that showed strong telomeric silencing but that displayed distinct responses to the chemical agents. These two clonal populations were compared to that of a control clone having internally located transgenes but that also show low levels of transgene expression (Fig. 4). This revealed low levels of H3 acetylation on the telomere-proximal GFP genes, while relatively higher levels of acetylated H3 were found on the transgene integrated at an internal locus in the cD06 cells (Fig. 4A). However, differences were less prominent when the internal DsRed sequence were compared with their counterparts in clones D17 and B09. This correlates well with the fact that in the latter clones, the DsRed sequences are more distantly located in

![FIG. 4. Telomeric histones H3 and H4 are hypoacetylated. Chromatin immunoprecipitation was performed on two telomeric clones (B09 and D17) and one clone with nontelomeric integration (cD06). Chromatin fragments were immunoprecipitated using antibodies specific for acetylated H3 and H4 (A and B), trimethylated H3K9 (C), or the histone variant H2A.Z (D), and the precipitated DsRed and GFP genomic sequences were assayed by real-time PCR and normalized to values obtained by amplifying the GAPDH gene. Means and standard errors of the means of three independent experiments with at least two independent chromatin preparations are indicated.](http://mcb.asm.org/medias/journals/McB/2005/23/5/FIG.4.png)
relation to the telomeric repeats than the GFP genes (Fig. 1A). Significant hypoacetylation of histone H4 was observed only when the extremity of the GFP sequence adjacent to the telomeric repeats of clone D17 was assayed, not when the orientation of the gene was reversed and the probed sequence was more distant from the telomere, as in clone B09 (Fig. 1A and 4B). This observation further argues for a correlation between telomere proximity and histone hypoacetylation. These findings are consistent with the spread of a silencing signal from the telomeric repeats, and they are reminiscent of the distance-related silencing effects associated with the propagation of a silent chromatin structure from yeast telomeres (24).

The trimethylation of histone H3 on lysine 9 (H3K9Me3) has been associated with heterochromatin-mediated gene silencing (5, 36, 39). H3K9Me3 levels were similar when the chromatin structures of telomeric and nearly silent internal transgenes were compared (Fig. 4C). Similar observations were made when analyzing other histone modifications, such as H4K20Me3, H3K27Me3, or H3K79Me2 (data not shown). Thus, these modifications may be generally associated with silent or low-expression genes, but they are not specifically enriched at telomeric loci. The histone variant H2A.Z has often been located at the boundaries of silent and permissive chromatin domains (10, 28). Its relatively low levels at the telomeric reporter genes of clones B09 and D17 indicate that it may be excluded from these telomeric loci (Fig. 4D). Overall, these results may link telomeric gene silencing to histone hypoacetylation, and they suggest that a short-ranging gradient of such modifications may stem from the telomere.

**CTF1 fusion protein delimits distinct chromatin domains at telomeric boundaries.** Given the observation that telomeric transgene silencing may involve histone modifications, such as acetylation, we next assessed if Gal-Pro expression may selectively oppose these changes over the DsRed-coding sequence. Clones B09 and D17 were stably transfected with the Gal-DBD or Gal-Pro expression vector to ensure stable expression of the GAL4 fusion in a significant proportion of the cell population. Expression of these GAL4 fusions was assessed indirectly by measuring the fluorescence of the BFP expressed from a cotransfected vector.

Gal-Pro expression was associated with an increase of H4 acetylation on the DsRed sequence of clone B09. However, Gal-Pro expression did not affect histone acetylation on the GFP sequence, indicating that Gal-Pro mediated the formation of two chromatin domains of distinct acetylation status but that it did not act by recruiting histone acetyltransferases that would acetylate bidirectionally the GFP and DsRed genes. Gal-Pro expression also strongly increased H3K9Me3 on DsRed but not GFP at the B09 telomere. The trimethylation of H3K27 and H4K20, which are modifications generally associated with gene silencing, was similarly increased on the expressed DsRed sequence in the presence of Gal-Pro (data not shown). The HDAC inhibitor TSA yielded an increase of the acetylation of H3 and of the trimethylation of H3K9, suggesting that the latter modification may be a consequence of the increased histone acetylation on the silent telomeric genes.

To determine if histone acetylation changes are always involved in the boundary effect, clone D17 was similarly tested, since Gal-Pro had a strong boundary activity with this clone while the HDAC inhibitor NaB has little effect on telomeric gene expression (Fig. 2A; see Fig. S7 in the supplemental material). Expression of Gal-Pro was not associated with an increase of H3 and H4 acetylation compared to that with the unfused DNA binding domain nor with modifications such as H3K9Me3, H3K27Me3, or H4K20Me3 (Fig. 5 and data not shown). However, the occurrence of H2A.Z on DsRed was significantly increased upon the expression of Gal-Pro in clone D17, while it remained unaffected on GFP. These results strengthen the conclusion that Gal-Pro may act to separate chromosomal domains of distinct chromatin structures. In addition, comparison of the chromatin modifications elicited by Gal-Pro in the B09 and D17 clones indicates that several types of chromatin structures may be associated with the telomeric silencing and insulation effects, as suggested by the distinct sensitivities of these two clones to the NaB HDAC inhibitor (Fig. 5; see Fig. S7 in the supplemental material).

**DISCUSSION**

The eukaryotic genome is thought to be partitioned into euchromatic or heterochromatic domains in which chromatin may be either permissive for gene expression or, rather, silent. How the boundaries separating these chromatin domains are established and how they may influence gene expression remain poorly understood. In this work, we show that two genes colocalized at a telomeric locus can be partitioned into active and inactive chromatin structures by the CTF1 protein or fusions derived thereof. This mode of action is distinct from that of the VP16 transcriptional activator, which induces bidirectionally the expression of telomere-proximal and telomere-distal genes but only over a short distance. The latter effect most likely results from the ability of VP16 to recruit histone acetyltransferase and components of the basal transcription machinery to the promoter (20). In contrast, CTF1 derivatives protect the telomere-distal gene from silencing effects without significantly affecting the expression of the telomere-proximal gene and irrespective of the reporter gene identity or of its transcriptional orientation or distance from the promoter. This implies that CTF1 does not act as a classical transcriptional activator but rather that it mediates the establishment of a barrier that blocks the propagation of a silent chromatin structure from the telomere, thereby forming a boundary between expressed and silent genes. The CTF1 boundary effect is mediated by its histone-binding domain, and mutations that inhibit interactions with the histone also inhibit the boundary effect. Taken together with previous observations that CTF1 binds preferentially to H3.3 and that this histone variant is enriched at chromatin boundaries (12, 31), these findings imply a mechanism whereby the interaction of CTF1 with nucleosomes may establish a chromosomal structure that blocks the autopropagation of silencing signals from the telomere. These findings also provide a mechanistic explanation for the previous observations that CTF1 may contribute to reversing chromatin-mediated gene silencing but that it is unable to activate transcription alone (33).

In budding yeast, TFE is mediated by the spreading of the SIR protein complex from the telomere over subtelomeric regions, which results in histone deacetylation and gene silencing. However, a similar mechanism involving the propagation of SIR proteins has not been found in mammalian cells.
Rather, the establishment of a repressive telomeric structure has been associated with increased H3K9Me3 modifications at telomeres (5, 35). H3K9Me3 is known to bind HP1, which may in turn recruit the Suv39 histone methyltransferase to mediate further H3K9 methylation. Here we found that histone deacetylation is linked to silencing at several of the analyzed telomeric loci and that broad-range HDAC inhibitors, such as TSA, mediate an increase not only of histone acetylation but also of other types of modification, such as H3K9 trimethylation. This implies a causal effect of hypoacetylation on histone methylation levels and silencing effects in mammalian cells. This conclusion is further supported by the previous demonstration that H3K9Me3 modifications may occur as a result of gene transcription (41) and by the occurrence of H3K9Me3 on a transgene protected from a chicken telomere by the cHS4 betaglobin insulator (34, 37).

Although we observed various degrees of histone hypoacetylation when comparing different silent integration loci, the extent of histone deacetylation correlated with telomere proximity, since it was significantly lower over the telomere-distal gene. This finding suggests a short-ranging spread of a hypoacetylation signal from the telomere. This contrasts with the long-ranging histone hypoacetylation and silencing that stem from yeast telomeres, and it may explain why telomeric gene silencing has been more difficult to detect in mammalian cells. In human cells, we find that expression of Gal-Pro results in the recovery of higher levels of histone acetylation on the telomere-distal but not on the proximal gene on one of the telomeric clones, further supporting the notion that it can act to block the self-propagation of a deacetylated histone structure. This interpretation is consistent with the recent implication of the mammalian SIRT6 homolog of the yeast Sir2 HDAC in mammalian TPE and with its H3K9 deacetylase activity (29). Thus, these results suggest a mechanism by which SIRT6 and possibly other proteins may propagate along the mammalian chromosome to silence subtelomeric regions. However, whether this structure may propagate at distances sufficient to inhibit the expression of genes at their natural location remains to be ascertained.

Interestingly, our results imply that various chromatin structures and/or mechanisms may be implicated in the telomeric silencing and boundary effects. For instance, distinct telomeric clones display different responses to treatment with agents that affect chromatin-modifying activities, such as NaB, an inhibitor of the HDAC types I and IIa. Furthermore, the boundary effect elicited by the CTF1 fusion protein was not associated

![Graph image](http://mcb.asm.org/)

**FIG. 5.** Effect of the Gal-Pro boundary on telomeric chromatin structure. Chromatin immunoprecipitations were performed on telomeric clones B09 and D17 stably expressing Gal-DBD or Gal-Pro or treated with the HDAC inhibitor TSA. Antibodies were specific for acetylated H3 and H4 (A and B), trimethylated H3K9 (C), or the histone variant H2A.Z (D), and precipitated sequences were processed as for Fig. 4. *, P < 0.05; **, P < 0.01 (Student’s t test).
with major changes in histone acetylation in the clone whose silencing was not inhibited by NaB, but it was rather associated with the incorporation of the histone H2A.Z variant in the insulated gene. Thus, telomeric silencing and the boundary activity of the CTF1 derivative may involve distinct chromatin markers and telomeric contexts. The implication of H2A.Z in the boundary activity is reminiscent of the previous demonstration that the yeast homolog of H2A.Z is capable of synergizing with boundary elements and that it preferentially locates on insulated telomeric genes (25, 28, 46). Thus, in contrast to the view that mammalian H2A.Z may have the distinct function of mediating a silent heterochromatin structure (11, 25, 28, 46), our results rather indicate that it can be associated with gene expression at human telomeres.

What distinguishes telomeric loci where the boundary effect may be associated with histone acetylation or with H2A.Z enrichment is unclear at present, but it may stem from different chromosomal contexts and/or differences in the structures of distinct telomeres. It has been found that telomeric silencing is often counteracted by HDAC inhibitors in tumor cell lines but not in normal cells (3, 34, 37). While our results are consistent with these observations, they also raise the possibility that distinct mechanisms may operate at distinct chromosomal loci and that seemingly discrepant results may reflect distinct telomeric structures. This interpretation is supported by the inspection of histone modifications and variants in the genome of human or murine cells (2, 42), which reveal distinct structures when the telomeric and subtelomeric loci of different chromosomes are compared. For instance, the telomeres of the p arms of human chromosomes 1 and 6 may be relatively devoid of H3K9Me3, H3K27Me3, H3K14Ac, and H4K20Me3 and of H2A.Z, while these modifications and variant are more prominent at the telomeres of chromosomes 2 and 4. Similar differences can be noted when comparing the telomeric structures of different murine chromosomes in differentiated or embryonic stem cells, although heterochromatic markers were generally more prominent at the telomeres of the latter cells (30). Different telomeric structures may thus explain why several types of chromatin modifications can be associated with the boundary effect of CTF1 derivatives, depending on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed. This may also provide a rationale for the different effects of agents such 5azadC or TSA on the telomeric locus assayed.