Chromatin Domain Boundaries Delimited by a Histone-binding Protein in Yeast*§

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Received for publication, September 9, 2004
Published, JBC Papers in Press, October 7, 2004, DOI 10.1074/jbc.M410346200

When located next to chromosomal elements such as telomeres, genes can be subjected to epigenetic silencing. In yeast, this is mediated by the propagation of the SIR proteins from telomeres toward more centromeric regions. Particular transcription factors can protect downstream genes from silencing when tethered between the gene and the telomere, and they may thus act as chromatin domain boundaries. Here we have studied one such transcription factor, CTF-1, that binds directly to histone H3. A deletion mutagenesis localized the barrier activity to the CTF-1 histone-binding domain. A saturating point mutagenesis of this domain identified several amino acid substitutions that similarly inhibited the boundary and histone binding activities. Chromatin immunoprecipitation experiments indicated that the barrier protein efficiently prevents the spreading of SIR proteins, and that it separates domains of hypoacetylated and hyperacetylated histones. Together, these results suggest a mechanism by which proteins such as CTF-1 may interact directly with histone H3 to prevent the propagation of a silent chromatin structure, thereby defining boundaries of permissive and silent chromatin domains.

The expression state of a eukaryotic gene depends in part on its location in the chromosome. This position effect results from the organization of eukaryotic genomes into discrete functional domains, defined by local differences in chromatin structure. The expression of genes within each domain appears to be defined and maintained by the concerted action of regulatory elements such as promoters, enhancers, silencers, and locus control regions. Individual domains may be bordered by boundary elements that separate regions of permissive and silent chromatin. Experimentally, boundary elements have been defined functionally by their ability to protect against position effect when flanking the assayed gene. Examples of boundary elements in metazoans include the subtelomeric anti-silencing regions at yeast telomeres, the scs and scs' elements flanking the 87A1 hsp70 locus in Drosophila, and the chicken β-globin boundary elements (1–4). Several proteins have been associated with barrier activities in yeast, Drosophila, and mammalian cells (5).

Transcriptional repression of the yeast Saccharomyces cerevisiae subtelomeric regions is one of the best-studied examples of position-dependent gene expression. This phenomenon is referred to as telomere position effect and is similar to the position effect variegation (PEV) initially described in Drosophila (6). Transcriptional silencing at yeast telomeres is associated with a heterochromatin-like structure (7, 8). DNA wrapped in transcriptionally silent chromatin replicates late in S phase and is refractory to some modifying agents. In addition, nucleosomes have reduced acetylation compared with nucleosomes from active region of the genome (9, 10).

Transcriptional silencing at subtelomeric regions is mediated by a multiprotein nucleosome binding complex called the SIR complex, composed of the Sir2, Sir3, and Sir4 proteins (11, 12). This complex is recruited to DNA by the telomere-binding protein Rap1 (13). A series of physical and genetic arguments imply that this SIR complex may nucleate from the telomere and spread along the chromosome to coat more centromeric regions and thereby silence intervening genes (14, 15). Silencing in natural subtelomeric regions further involves auxiliary recruitment at protosilencer relay elements found in the middle-repetitive X and Y' subtelomeric elements (1, 8, 16).

The proteins involved in silencing form extensive homotypic and heterotypic interactions. Both Sir3 and Sir4 can homodimerize and heterodimerize. They also bind to Sir2, Rap1 (17), and histones H3 and H4 (18, 19). In addition Sir3 interacts with histones H2A and H2B (20). Sir4 can interact with Rap1 at telomere proximal positions in the absence of Sir2 and Sir3 proteins, but Sir4 binding at telomere-distal heterochromatin is strongly dependent on other Sir proteins (21). These interactions and the discovery of the NAD-dependent histone deacetylase activity of Sir2 (22–24) has led to the current model for the establishment of telomeric silencing. In this model, Sir4 is the first of the SIR proteins that is recruited by Rap-1 and helps determine the subsequent association of heterochromatin proteins. The Sir4-mediated recruitment of Sir2 to the DNA is followed by deacetylation of the tails of histones H3 and H4. This then allows the binding of the Sir3 and Sir4 proteins to deacetylated histone tails and the recruitment of a new Sir2-Sir4 complex. Repetition of this binding/modification cycle would result in spreading of the SIR complex outward from the nucleation site (25, 26).

How boundary proteins protect against the encroachment of repressive chromatin complexes remains unclear. However, the activity of boundary elements is usually conserved when transposed into other organisms, indicating that they share an evolutionary conserved mechanism (27, 28). Two different models for boundary function have been discussed: the “nuclear orga-
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nization” and the “chromatin modifying” models (29). The nuclear organization model suggests that boundary elements congregate to create a subnuclear compartment or are tethered to a nuclear structure, which then forms a physical block against the spreading of heterochromatin (30–32). The chromatin modifying model suggests that boundary proteins mediate an active process by recruiting a multiprotein complex, bearing acetyltransferase and/or nucleosome remodelling activities, which precludes nucleosome assembly, thereby disrupting a contiguous array of hypoacetylated nucleosomes required for the spread of silent chromatin (27, 33, 34).

In yeast, transcriptional activation domains of various species were shown to efficiently protect reporter genes against telomeric silencing (35). These were shown to prevent the silencing of telomere-proximal genes, when interposed via a heterologous GAL4 DNA-binding domain between the gene and the VII-L telomere, and to generate an apparent discontinuity in silent chromatin propagation (35). Transcriptional activity per se appears not to be involved in these effects. One likely interpretation for these findings is that the transcription factors somehow prevent the propagation of a silent chromatin structure from the telomere. However, a direct implication of chromatin structure in this effect is currently lacking, and other interpretations are possible, including mechanisms involving changes in subnuclear localization.

Interestingly, one of the strongest boundary activities detected in this organism consisted of the transcriptional activation domain of CTF-1, a member of the CTF/NF-I family of mammalian transcription and replication factors previously shown to interact directly with histone H3 (35, 36). Furthermore, CTF-1 was shown to remodel reconstituted chromatin in vitro (36) and native chromatin at yeast origins of replication in vivo (37, 38). Here, we have evaluated whether CTF-1 H3 binding activity is required for the boundary activity, whether it can modify the structure of chromatin in vitro in subtelomeric regions, and whether this may mediate the boundary effect. Saturation mutagenesis and in vivo chromatin immunoprecipitation assays indicate that the CTF-1 histone binding activity is required for boundary activity, and that the histone-binding domain can act as a barrier against the propagation of a SIR-containing, histone-hypocetylated, silent chromatin structure from telomeres. This contrasts the activity of the VP16 transcriptional activator, which induces local histone hyperacetylation bidirectionally and prevents the propagation of SIR proteins, thus acting as a bidirectional anti-silencer. These results indicate that the boundary activity of transcription factors involves specific actions beyond DNA binding and that interaction with histones is one of the mechanisms by which the propagation of silent chromatin can be blocked.

MATERIALS AND METHODS

Mammalian Cells Expression Vectors—Mammalian expression vectors for fusion polypeptides consisting of the histone H3 mutants or derivatives linked to the VP16 TAD at the N terminus are based on pMLV-VP (39) and fusions were generated as for the parental plasmid pMLV-VP-H3 (36). For VP-yH3-1 and VP-mH3.2, the full-length yeast S. cerevisiae pMLV-VP-H3 (36). For VP-yH3-1 and VP-mH3.2, the full-length yeast pMLV-VP-H3 (36). For VP-yH3-1 and VP-mH3.2, the full-length yeast pMLV-VP-H3 (36). For VP-yH3-1 and VP-mH3.2, the full-length yeast

The transforming growth factor-β responsive domain of CTF-1 (TRD), encoding amino acids 486–499, was extensively mutagenized and introduced in-frame into the pRSV GAL4 expression vector (41) coding for the first 147 amino acids of GAL4 DNA-binding domain. The pRSV GaTRD mutated fusion proteins were constructed by replacing the Oct2 (mammalian octamer factor 2) activation domain in pRSV GalOct2 (41), by double-stranded oligos using the XhoI and XbaI restriction sites. Except for amino acid at position 496, where Asp(GAC) was mutated to Lys(AAG) (D486K), all amino acids were mutated to an asparagine, drastically changing both charge and polarity of the asparagine as compared with that of the wild type protein. The TRD mutations fused to the pRSV GAL4 DNA-binding domain are: P487D, A488D, G489D, I490D, Y491D, Q492D, A493D, Q494D, S495D, W496D, Q497D, Y498D, L499D, and Q510D.

Yeast Cell Expression Vectors—All mutated sequences of CTF-1 TRD were inserted in-frame at the C-terminal coding extremity of the GAL4 DNA-binding domain sequence (amino acids 1–93, abbreviated Gal). pHAC GaTRD and all TRD point mutants were constructed by inserting double-stranded oligos containing XhoI/XbaI sites into the corresponding sites of the yeast pHAC GAL4 vector (pHAC-GalPRO, containing the ARS1 replication origin, the CEN4 and HIS3 genes; a gift from W. Schaffner), producing: pHAC Gal TRD, pHAC Gal D486K, pHAC Gal P487D, pHAC Gal A488D, pHAC Gal G489D, pHAC Gal I490D, pHAC Gal Y491D, pHAC Gal Q492D, pHAC Gal A493D, pHAC Gal Q494D, pHAC Gal Q510D, pHAC Gal S495D, pHAC Gal W496D, pHAC Gal Y497D, pHAC Gal L499D, and pHAC Gal Q510D.

Mammalian Cell Culture and Transfection—Mouse NIH3T3 cells were grown and transfected by electroporation as described previously (36). Cells destined for both CAT (chloramphenicol acetyltransferase) and β-galactosidase assays were harvested and lysed for 10 min at room temperature in 100 µl of 1× Reporter Lysis Buffer (Promega). CAT activities were determined using standard procedures and normalized to the amount of protein loaded onto the internal control CMV-β-galactosidase (Clontech) that gives an indication on the relative efficiency of transfection. Transcriptional activation assay in a chromatin context were performed in NIH3T3 cells stably transfected with the G5BCAT reporter construct as described earlier (36). Western Blot Analysis—Transfected NIH3T3 cells were grown at 37 °C for 90 h and lysed in 100 µl of 2× loading buffer (Promega), and cell lysates were recovered by centrifugation. Equal amounts of proteins, corresponding to roughly 10% of the total transfected cells, were loaded on a 12% SDS-polyacrylamide gel and electroblotted for 1 h at 70 V onto a nitrocellulose membrane. Immunoblot detection was performed using standard procedures using the rabbit LA-2 polyclonal anti-GAL4 (1–147)-VP16 antibody (a gift from S. Triezenberg) diluted 5000-fold. The rabbit antibodies were detected using horseradish peroxidase- conjugated anti-rabbit IgG (Sigma) and the ECL kit (Amersham Biosciences) as recommended by the manufacturers. Molecular weights were estimated by comparison with the relative migration of a low range prestained SDS-PAGE marker (Bio-Rad).

Gel Mobility Shift Analysis—For gel mobility shift analysis, the GalTRD mutant fusion proteins were transiently transfected into NIH3T3 cells. At 24 h post-transfection the cells were lysed twice with phosphate-buffered saline, before lysis in 80 µl of extraction buffer (20 mM Tris, pH 7.5, 20% glycerol, 500 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) by repeated freeze-thaw cycles (42). Alternatively, yeast cell extracts were prepared in lysis buffer (50 mM Tris, pH 7.5, 10 mM MgSO4, 1 mM EDTA, 10 mM potassium acetate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) by mechanical breakage using a bead beater (Biospec).

14 µg of protein extract was incubated on ice in 15 µl of reaction mixture (0.01 mM ZnCl2, 4.5 µl of poly(dI-dC), 4.7 µl of sonicated salmon sperm DNA, 20 µl Hepes, pH 7.9, 7.5% glycerol, 5 mM MgCl2, and 0.01% Nonidet P-40) with a 2P end-labeled double-stranded DNA probe containing the 17-bp GAL4-binding site (43). After incubation at room temperature for 10 min, the protein-DNA complexes were separated from the free probe on a native 5% polyacrylamide gel as performed by Armentero et al. (44) and revealed by autoradiography and phosphoimaging.

Yeast Strains and Media—All yeast media were used as described by Adams et al. (45). Yeast cells were always grown in selective minimal media (Strategene Inc.) at 30 °C. The boundary assay of telomeric genes is usually performed using yeast strains W303-1a (MATa, ade2-1, ura3-1, his3-11, 15 leu2-3, 112 trp-1, con-1 100 containing the TRP1 GAL4AUS URA3 inserted at telomere VII-L) for the boundary assay and with Y187 (MATα, TRP1-901, leu2-3, ade2-101, lacZ, URA3-52, His3, gal4, gal80, URA3::GAL1-lacZ) to assess transcriptional activity of Gal-TRD fusions.

Analysis of URA3 Expression—The variegated expression of URA3 was monitored by scoring the fraction of yeast cells from a culture
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pre-grown in non-selective conditions either expressing or not the URA3 gene. Cells with a repressed URA3 gene were identified as those able to form colonies in the presence of 5-FOA, a compound toxic for cells expressing a functional URA3 gene product (46). After overnight growth to saturation, 10-μl drops of serial dilutions were plated onto SC and SC + 5-FOA (1 g/liter), as well as onto SC-Trp and SC-Trp + 5-FOA for a strain carrying both URA3 and TRP1 reporter genes.

Chromatin Immunoprecipitation—Yeast cells containing the URA3 gene and the TRP1 gene deleted of its promoter were grown as above. 50 ml of cells (2.0 × 10⁷ cells/ml) were cross-linked with 1% formaldehyde for 15 min at room temperature. Glycine was added to a final concentration of 125 mM and the incubation continued for 5 min. The cross-linked cells were washed twice with digestion buffer (1 M sorbitol, 1× XbaI buffer, 0.05 M phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml antipain, 1 μg/ml leupeptine, and 1 μg/ml pepstatin) and sonicated to obtain chromatin fragments of ~500 bp. Chromatin was then digested by adding 50 units of XbaI for 45 min at 37°C to cleave the DNA between the URA3 and the TRP1 genes. Immunoprecipitation was performed with precoupled Dynabeads overnight at 4°C and using specific antibodies against tetra-acetylated histone H3 (K9, 14), diacylated histone H3 (K9, 14) (Upstate), or against SIR2, SIR3, and SIR4 proteins (Santa Cruz). An aliquot of each sample was not immunoprecipitated (Input). Input and immunoprecipitated samples were incubated 6 h at 65°C to revert the formaldehyde cross-linking, prior to DNA precipitation and quantitative PCR analysis.

Quantitative PCR Analysis of Immunoprecipitated DNA—Quantitative PCR was performed using the qPCR Core Kit for Sybr Green I from Eurogentec and the ABI Prism 7700 sequence detector. PCR were carried out in a 25-μl volume with 1/1000 of the immunoprecipitated material and 1/10,000 of the input material. Enrichment ratios were measured using the absolute quantification method, as described by Aras et al. (47).

Primers used are specific for the reporter URA3 gene (5'-GGTTTCCTTCCGCAACAGT-3' and 5'-GTTCTGATGTGGAGACAAAT-3'), producing a 179-bp fragment, and for the reporter TRP1 gene (5'-GGGAGGTTGGAGACAAAT-3' and 5'-GACAGTGCCCTGCAGGATGCT-3'), producing a 142-bp fragment. Both include specific junctions generated in the cloning process. Additional PCRs were performed on the telomere distal ADH4 gene (5'-GCAACAAAGGCCCTTCATGTTTC-3' and 5'-GTCGATGTGGAGACAAAT-3'), producing a 1112-bp fragment.

Control PCRs of each immunoprecipitated DNA were performed in the MATA locus (5'-GAATTTGGATACGACGGAGGAGGA-3' and 5'-AAGTCTGTAGCCAGCT-3'), producing a 103-bp fragment, and in the HMR locus (5'-CCTCGTGGGATACGACGGAGGAGGAGGA-3' and 5'-CCTCGTGGGATACGACGGAGGAGGAGGA-3'), producing a 140-bp fragment.

RESULTS

Chromatin Boundary Activity of the CTF-1 Transcriptional Activation Domain—The CTF-1 transcription activation domain was previously shown to display strong boundary activity in yeast (Fig. 1A and Ref. 35). To investigate the possibility that CTF-1 histone interaction might be involved in its boundary activity, we first evaluated whether the histone-binding portion of CTF-1 might bear the boundary activity.

The GAL4 DNA-binding domain, either alone or tethered to various portions of the CTF-1 activation domain was expressed in yeast cells containing an URA3 gene inserted in a telomeric position (35). As shown in Fig. 1B, the GalPRO fusion protein containing the complete transcriptional activation domain of CTF-1 prevented URA3 silencing when interposed between the telomere and the reporter gene, but not when located in a telomere-distal position. Similarly, when placed between the telomere TRP1 and URA3 genes, it selectively prevents silencing of the telomere distal TRP1 gene but not that of the telomere-proximal URA3 gene (Fig. 1C), in agreement with Fournel et al. (35). Thus, the CTF-1 transcriptional activation domain functions as a boundary element that prevents the silencing of telomere distal genes, irrespective of the gene and promoter type but in a position dependent fashion. Thus it acts effectively as a boundary element between active and inactive chromosomal domains. In contrast, the VP16 transcriptional activator induced the expression of both the TRP1 and URA3 genes, thus acting as a bidirectional anti-silencer (Fig. 1C and Ref. 35).

Another fusion of the GAL4 DNA-binding domain to the C-terminal 14 amino acids of CTF-1, known to contain the minimal interaction domain for histone H3 (36, 41), was similarly able to prevent silencing of the URA3 gene when recruited to telomere-proximal UAS elements and it similarly acted as a boundary element (Fig. 1, B and C). A point mutation in this subdomain, previously shown to decrease interaction with histone H3 (GalTRD Y497D (36)), significantly impaired its ability to counteract silencing. These data taken together gave a first indication that the histone interaction ability of CTF-1 may be linked to its barrier properties.

Specificity of the Histone Binding Activity of CTF-1—The possibility that the CTF-1 boundary activity may be mediated by its histone H3 binding activity was further addressed by evaluating if CTF-1 can indeed interact with the yeast histone H3. Previous studies had demonstrated that the CTF-1 transcriptional activation domain selectively interacts with the mammalian histone H3.3 variant but not with H1, H2A, H2B, and H4, both in vivo using two-hybrid assays, and in vitro in co-precipitation studies (36). However, whether it can also interact with the yeast histone H3, although probably given the extremely high degree of conservation of histone sequences across species, has not been reported as yet. This was tested in vivo using a two-hybrid assay where the GAL4 CTF-1 hybrid protein was co-expressed in mammalian cells together with either a fusion of histone H3 and the strong activation domain of the viral VP16 activator, or with the unfused VP16 moiety as a control. In this assay, interaction of GalPRO with the VP16-H3 fusion leads to increased transcriptional activation, as mediated by the recruitment of the strong viral VP16 activator, in comparison to the activation mediated by GalPRO alone, when expressed with unfused VP16 (36). Expression of GalPRO together with a fusion of VP16 to the mammalian H3.3 protein induces a strong increase in the activity of the reporter promoter (Fig. 2A), which was not observed with the GAL4 DNA-binding domain alone (Ref. 36, see also Fig. 3A, left panel), conveying specific interaction between the CTF-1 activation domain and histone H3.3. Mammalian cells contain several other forms of histone H3, termed H3.2 and H3.1. These did not interact significantly with GalPRO in this assay, although they were expressed at similar levels as the H3.3 fusion protein (Fig. 2B), demonstrating the high specificity of the interaction. Yeast cells possess only one H3 variant, which was found to interact well with GalPRO (Fig. 2A). This correlates well with the fact that yeast H3 is most related to the mammalian H3.3 variant. Similar results were obtained with two-hybrid assays performed in yeast cells (data not shown). Altogether, these data indicate that the CTF-1 activation domain is capable of interacting specifically with yeast histone H3.

Correlation of the Histone Binding and Boundary Activities of GalPRO—Having determined that the activation domain of CTF-1 interacts with the yeast histone H3, and that it displays boundary activity, we set up to assess whether the two effects may be linked. Because the N-terminal extremity of the CTF-1 activation domain contains a weaker histone H3 interaction domain (41) and displays moderate boundary activity (35), we rather focused on the shorter and more potent C-terminal TRD domain. We approached this by performing a saturation mutagenesis of the TRD domain, and by probing its histone binding and boundary activities. Each of the 14 amino acids of the minimal histone-binding TRD domain was mutated independently. To ascertain strong phenotypic effects, negatively charged amino acids were introduced, except at position 486.
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where the negatively charged aspartic acid was replaced by a lysine. These mutants were first assayed in the mammalian cell two-hybrid assay by transient transfections as above. Mutations in two regions of the TRD affected most significantly H3 binding, namely the regions encompassing the D486K and Y496D/L497D mutations, whereas mutation of residues I490D and Y491D had a milder but significant effect (Fig. 3A, compare light and dark gray columns of the left panel). These effects are specific and cannot be ascribed to lower expression levels and/or to lower DNA binding activities of the mutated proteins, as each mutant displays similar or higher activities than the wild type TRD parental construct in mobility shift assays (Fig. 3B). Overall, lack of interaction of these mutants with VP16-H3 cannot be ascribed either to a generally lower transcriptional activation potential, as determined in transient transfection assays (Fig. 3A, light gray columns of the left panel). In contrast, the L498D mutant is more difficult to interpret. It allows a significant increase of the reporter gene expression in the presence of the VP16-H3 fusion as compared with the control with unfused VP16, suggesting efficient interaction with H3. The L498D mutant does not display intrinsic transcriptional activity in the absence of VP16-H3, which can account for the overall low expression in presence of VP16-H3 (Fig. 3A, left panel, light and dark gray columns, respectively), as the two types of domains cannot synergize to activate transcription. However, weaker interaction with H3 is an alternative interpretation that cannot be formally excluded, and the two interpretations are not mutually exclusive.

The transcriptional activation property of these mutants in a chromatin context was determined in parallel using a reporter gene stably integrated in a long-term transfection experiment. Transcriptional activities in this assay correlated well with histone interaction properties, with mutations around positions 486, 491, and 496–498 displaying the most prominent effects (Fig. 3). The transcriptional activity of the TRD mutants correlates well with their interaction with H3 in stable but not in transient expression levels and/or to lower DNA binding activities of the mutated proteins, as each mutant displays similar or higher activities than the wild type TRD parental construct in mobility shift assays (Fig. 3B).

FIG. 1. Anti-silencing and boundary activities of CTF-1 fusion proteins at telomere. A, maps of the GAL4-CTF-1 fusions proteins used in this study. The GAL4 93 N-terminal amino acids comprising the DNA-binding domain were expressed alone (GalDBD), or as fusions to CTF-1 complete proline-rich activation domain (PRO) in the GalPRO protein, or fused to the minimal activation domain (TRD) in GalTRD. Positions of the main histone binding determinant (TRD) and of the accessory histone H3-binding domain, as well as of the GalTRD-Y497D point mutation that inhibits binding to H3 are as indicated (38, 41). B, anti-silencing activities of GAL4-CTF-1 fusion proteins. Yeast strain W303-1a containing the URA3 gene integrated at the VII-L telomeric locus, in the presence or absence of a GAL4 DNA binding array containing four copies of GAL4 UAS sequences, were transformed with plasmids coding for GalDBD, GalPRO, GalTRD, and GalTRD-Y497D as shown in A. The transformants were assayed for URA3 expression by plating serial dilutions of cells on 5-FOA containing solid media as outlined under “Materials and Methods.” The ratio of the population that is repressed for URA3 gene transcription and forms colonies in the presence of 5-FOA is represented by a histogram bar as a fraction of the total yeast cell number. Thus a ratio of 1 on the logarithmic scale indicates that 100% of the cells have a silenced URA3 gene. C, boundary activities of GAL4-CTF-1 fusion proteins. Yeast W303-1a pER5 cells, containing the TRP1 and URA3 genes inserted at telomere VII-L in opposite orientation and separated by four copies of GAL4 UAS sequences, were transformed with expression vectors for the indicated fusion proteins. The ratio of cells that are both silent for the URA3 gene and active for TRP1 expression were counted after growth on 5-FOA containing but tryptophan-deprived solid media, and are expressed as a ratio to the total number of cells that grew on non-selective media.
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assays. Moreover, this shows that the observed effects are specific, as for instance, mutant D486K is affected for histone H3 binding and transcriptional activation in a proper chromatin context, whereas it retains significant transcriptional activity in yeast, such P487D, I490D, and Y491D, also display correspondingly decreased boundary activity, implying that interaction with histone H3 contributes to the boundary activity of the TRD. However, an implication of other TRD functions in the boundary activities cannot be excluded, as the correlation is not always absolute. For instance, mutant G499D has normal anti-silencing and activation functions in a chromatin context and it interacts efficiently with H3. Nevertheless, this mutant displays a reduced boundary activity. Thus, the histone H3-binding function of the TRD domain appears to be required for the boundary effect, but other as yet unidentified TRD functions may also contribute to the full boundary activity.

Chromatin Remodeling Activities of GAL4 Fusion Proteins—The possibility that CTF-1 boundary activity is mediated by its histone H3 binding and chromatin remodeling activities was evaluated by directly probing the chromatin structure of the URA3 and TRP1 telomeric reporter genes in vivo. First, spheroplasts generated from yeast cells expressing the various GAL4 fusion proteins were treated with micrococcal nuclease I, and genomic DNA was extracted and probed to determine nucleosomal positioning. Expression of GAL VP16 and GalPRO mediated a general increase in micrococcal nuclease I accessibility over the TRP1 promoter and TRP1 gene, consistent with the increase of the accessibility of telomere-distal nucleosomal DNA next to the GAL DNA binding sites (see Supplemental Materials). However, nucleosomes appeared to be poorly positioned overall on the silent or active TRP1 gene locus.

To further characterize the mechanism by which CTF-1 prevents telomeric repression, we set up to determine the structure and composition of chromatin on each side of the GAL4 fusion proteins. Given that yeast repressive chromatin is characterized by histone hypoacetylation and that acetylated histone tails inhibit SIR complex binding (9, 50), we examined the histone H3 and H4 acetylation levels by chromatin immunoprecipitation assays using specific antibodies against tetra-acetylated H4 (H4 Lys-5, Lys-8, Lys-12, and Lys-16) and di-acetylated H3 (H3 K9, 14). Yeast cells containing telomeric URA3 and TRP1 genes, separated by four GAL4-binding sites as before, were assayed, except that the promoter of TRP1 gene
Histone-binding Protein Sets Chromatin Domains

A

FIG. 3. Scanning of CTF-1 TRD domain by point mutagenesis and analysis of the mutants histone H3 binding and transcriptional activities. A, the left-hand panel shows a two-hybrid assay of the interaction of GalPRO and GalTRD wild type and mutant derivatives with a VP16-H3 fusion. NIH3T3 cells were transiently transfected with the VP16 activation domain, either alone (light gray bars) or fused to mouse histone H3 variant (dark gray bars), together with the indicated GalTRD derivatives, as described in the legend to Fig. 2. The light gray columns illustrate the intrinsic transcriptional activities of the GalTRD derivatives in transient transfection experiments. The dark gray columns show the additional activation of the reporter gene resulting from the interaction of the TRD with the VP16 histone H3 fusion. In the right-hand panel, the indicated GalCTF-1 fusion proteins were expressed in a polyclonal NIH3T3 cell population containing the chromosomally integrated G5BCAT reporter construct (36). Expression of this reporter is thus indicative the transcriptional activity of the different GalCTF-1 fusion proteins in a native chromatin. Mean ± S.D. values of normalized CAT activities are shown in arbitrary units relative to that GalTRD, which was assigned a value of 100. B, NIH3T3 cells transfected transiently with GalPRO or GalTRD wild type and mutant expression constructs as in A, and total cell extracts were probed by gel mobility shift assays using a probe containing a 17-mer GAL4 DNA binding sequence. The position of the GalPRO and GalTRD complexes are as indicated. N.S. indicates a nonspecific complex also observed with extracts of untransfected cells (not shown).

was deleted to avoid any changes in the chromatin structure that may result from transcription rather than from the boundary effect. Chromatin was subjected to cross-linking in vivo, followed by sonication and digestion with the restriction enzyme XbaI to dissociate the URA3 and the TRP1 reporter genes, as well as the telomere-distal ADH4 gene (Fig 5A).
**Fig. 4.** Scanning of the CTF-1 minimal activation domain point mutant for transcriptional, anti-silencing, and boundary activities in yeast cells. 

A, yeast strain Y187 (light gray columns) and W303-1a pER5 (dark gray columns) were transformed with expression vectors for the indicated Gal-CTF1 fusion proteins. The intrinsic transcriptional activity of GAL fusions was estimated with the Y187 strain that contains the β-galactosidase gene driven by the UAS containing GAL1 promoter, inserted within the chromosomal URA3 gene at its native position. Cells were grown in non-selective conditions and β-galactosidase activities quantified as described under “Experimental Procedures,” are shown as arbitrary units. Anti-silencing and boundary activities of Gal fusions were estimated, using the W303-1a strain that contains the TRP1 and URA3 genes separated by four tandem UAS inserted at the telomere VII-L, as represented in Fig. 1C. Anti-silencing activities were determined by plating cells on tryptophan-depleted medium to probe for TRP1-expressing cells over the total cell population (dark gray columns of left hand panel). Whereas cells were plated on tryptophan-depleted medium containing 5-FOA to determine the proportion of cells being silenced for the telomere proximal URA3 gene but expressing the telomere distal TRP1 gene, as shown in Fig. 1C, reflects the boundary effect (right hand panel). 

B, the expression levels of various fusion proteins in yeast strains, used in A, in a gel mobility shift assay were estimated using equivalent protein amounts of total yeast cell extracts and a GAL4 DNA probe. Reactions performed with extracts of cells expressing wild type GalTRD fusions (TRD), the indicated TRD mutants, or untransformed cells (minus sign) were loaded as indicated.
Complete digestion was confirmed in each assay by quantitative PCR with primers flanking the XbaI sites. In each experiment, immunoprecipitations were also performed on the silent HMRa locus and on the active MATa locus as controls for immunoprecipitation and quantitative PCR efficiencies.

As expected, histones H3 and H4 were hyperacetylated in the MATa locus, and hypoacetylated at the repressed HMRa locus, and this was not affected by the expression of the GAL4 fusion proteins (Fig. 5, B and C). In cells expressing the unfused GAL4 DNA-binding domain, histones H3 and H4 were hypoacetylated over both the URA3 and TRP1 genes, respectively, as schematically represented in A. Immunoprecipitations were performed using specific antibodies against diacetylated histone H3 (H3 K9, 14) (B), against tetra-acetylated histone H4 (H4 K5, 8, 12, and 16) (C), or against Sir2 protein (D), Sir3 protein (E), and Sir4 protein (F). Precipitated DNA levels obtained with histone H3 and H4 antibodies were arbitrarily set to 100 at the MATa locus (B and C), and a value of 100 was also ascribed to Sir levels at the HMRa locus (D–F).

The extent of acetylation seen on the TRP1 and ADH4 genes in the presence of GalPRO is weaker than that obtained at the MATa locus. This difference might be because of the absence of transcription of both genes compared with the fully transcribed MATa locus. Alternatively, the degree of acetylation might vary between a telomeric region and a more centromeric locus. To determine whether the chromatin centromeric to GalPRO is fully acetylated, we used the GalVP16 fusion protein, as it is known to recruit histone acetyltransferases and thereby induces high histone acetylation levels. A 7-fold increase in acetylation over the URA3 and TRP1 genes was obtained in the presence of GalVP16, indicating that it induces histone acetylation on both sides of its binding site independently of gene expression. Interestingly, GalPRO and GalVP16 induced a similar increase in histone acetylation on the TRP1 and ADH4 genes. Thus, the chromatin protected from telomeric silencing by GalPRO is strongly hyperacetylated. Furthermore, acetylation levels observed on the TRP1 gene in the presence of GalPRO and GalVP16 were similar to those obtained with SIR4-deficient cells, thus reflecting the maximal acetylation level of this non-transcribed gene in the absence of telomeric repression (data not shown). These results indicate that GalPRO can maintain the chromatin acetylation levels of the protected telomeric-distal region in the absence of transcription and thereby further define CTF-1 as an efficient boundary element.
GalPRO Establishes a Barrier to SIR Complex Propagation—One model of the boundary action of transcription factors is that they may protect telomere-distal genes from repressive chromatin by impairing SIR complex propagation. We addressed this by performing chromatin immunoprecipitation assays using specific antibodies against Sir2, Sir3, and Sir4 proteins. Chromatin immunoprecipitations were performed as before, using the silent HMRa and active MATa loci as controls. As expected, the Sir proteins were present on the HMRa locus, but absent on the MATa locus (Fig. 5, D–F).

In cells expressing the unfused GAL4 DNA-binding domain, Sir2, Sir3, and Sir4 proteins were detected over both the URA3 and TRP1 genes, confirming that SIR complexes repress both genes. Little Sir binding was observed on the ADH4 gene, thus ADH4 defines the limit of SIR complex propagation. Interestingly, in cells expressing the unfused GalDBD, there was similar binding of Sir4 on the URA3 gene as on the MATa locus, but strikingly, twice as much Sir4 proteins were bound to the TRP1 gene. In contrast, amounts of Sir2 and Sir3 proteins were not similarly increased over the TRP1 gene. This difference is not because of a bias in PCR amplification, because quantitative PCR performed on plasmid DNA containing both the URA3 and TRP1 genes gave similar amounts of amplified product (data not shown). Thus, materials immunoprecipitated by anti-Sir4 antibodies accurately reflect higher Sir4 abundance near the TRP1 gene. This finding may be explained by a telomere-looping mechanism allowing Rap1-bound Sir4 to interact with Sir complexes bound on the TRP1 gene (1, 17, 51). Following formaldehyde cross-linking, DNA fragments containing the TRP1 gene would be immunoprecipitated through both Rap1-bound Sir4 and TRP1-bound Sir4, thereby more of the TRP1 fragment would be obtained as compared with URA3 (Fig. 6B). Alternatively, a proto-silencer present over the TRP1 gene might recruit additional Sir4 proteins over this gene (8).

In yeast cells expressing the GalVP16 fusion protein, little Sir2 and Sir3 proteins were detected over both the URA3 and TRP1 genes, confirming its bidirectional anti-silencing activity. In contrast, results obtained with Sir4 were unexpected. Little Sir4 binding was observed on the URA3 gene, consistently with the expression of this gene, but Sir4 protein co-precipitation with the TRP1 gene still occurred to a significant extent (Fig. 5F). Cross-reactivity between the anti-Sir4 antibody and GalVP16 cannot account for this result, because the Sir4 protein level is not similarly affected over the URA3 gene. One possible interpretation for this finding is that Sir4 immunoprecipitation might result from the interaction of the TRP1 gene with Rap1-bound Sir4 proteins. For instance, GalVP16-recruited histone acetylases and ATP-dependent chromatin remodeling complexes would induce the formation of a relatively flexible chromatin structure upon the URA3 gene, allowing fortuitous interactions of Rap1-bound Sir4 with the TRP1 gene, or because of residual interactions of the telomer DNA structure with the TRP1 gene (Fig. 6D). The GalVP16-mediated remodeling of the TRP1 chromatin structure is nevertheless consistent with the expression of this gene, because the amount of TRP1-bound Sir4 was reduced when compared with GalDBD alone, and Sir2 and Sir3 binding was nearly abolished.

In the presence of GalPRO, SIR complexes were still bound to the URA3 gene, but disappeared almost completely on the TRP1 gene. These results imply that the activation domain of CTF-1 can efficiently block the propagation of SIR complexes and alter the chromatin structure/composition of the telomere-distal gene (summarized in Fig. 6A). Moreover, GalPRO may induce a telomere unfolding, because no Sir4 proteins were detected over the TRP1 gene unlike GalDBD and GalVP16. Therefore, CTF-1 mediates a chromatin domain boundary by separating the chromatin organization of the telomere-distal gene that is similar to permissive chromatin, even in the absence of transcription, whereas the telomere-proximal gene retains all properties of silent chromatin (Fig. 6, B and C). Overall, the chromatin effects observed in the presence of GalPRO clearly contrast those obtained with GalVP16 and may
discriminate the effects of boundary elements such as CTF-1 from those mediated by more general anti-silencers like VP16.

DISCUSSION

The eukaryotic genome is divided in regions of permissive and silent chromatin. Permissive chromatin is associated with an enrichment of particular histone variants such as H3.3 and H2AZ. How this occurs remains unclear, but it may conceivably result from the interaction of histones with DNA binding regulatory proteins (52–54). The finding that CTF-1 interacts preferably with the H3.3 variant associated with active chromatin is consistent with its transcriptional activation function, and this may provide a mechanisms by which promoter and enhancer regions are enriched in such histone variants.

Individual chromatin domains are thought to be separated by boundary elements that insulate domains from the influence of adjacent regions. Experimentally, boundary elements have been defined functionally by their ability to protect against position effect. In yeast, transcriptional activation domains were shown to act as efficient barriers against telomeric silencing (35). This boundary function does not involve direct activation of reporter genes and the transcriptional activation potential of these domains does not always correlate with their anti-silencing or boundary activities (35). Whether these boundary elements function by altering the chromatin structure or by affecting other chromatin properties such as subnuclear location remains unclear. Results presented here argue in favor of the first model, as they show that the activation domain of CTF-1 acts to partition adjacent chromosomal sequences into active or inactive chromatin structures at yeast telomeres, and because they further involve the histone binding activity of the short TRD motif in the boundary activity.

Our results support the proposal that activation domains such as the one of CTF-1 have two independent modes of action: one that may directly result in the activation of transcription, whereas another would regulate chromatin structure. Activation domains thus would possess an intrinsic transcriptional activation function most apparent in transient transfection assays, which may directly influence the recruitment of the basal transcription machinery to the promoter, as the latter would not be in an inaccessible chromatin structure. The proposed interaction of the CTF-1 PRO domain with TFII-B and TBP may support this type of activity (55). Alternatively, a variety of transcription activators have been further shown to alter chromatin structure through the recruitment of chromatin remodeling/modifying complexes (56, 57). CTF-1 rather possesses a histone binding activity, which has been implicated in CTF-1 anti-silencing activity in mammalian cells (58). The GAL4 fusion proteins studied here support this conclusion, as the histone H3 binding activities of mutant proteins correlate well with transcriptional activation in a chromatin context, as illustrated by the boundary effect, but not with the transcriptional activation potential detected in the absence of native chromatin structure in transient assays.

Telomeric gene silencing in yeast results from the interaction of a complex formed by the Sir2, Sir3, and Sir4 proteins with telomere-binding proteins and further along the chromosome, silencing intervening genes (15). The interaction of Sir3 and Sir4 proteins with themselves and with the protruding N-terminal tails from histones H3 and H4 may form the basis of regular nucleosomal arrays, and any perturbation of this array might block the propagation of the silent chromatin structure. For instance, it has been proposed that any DNA-binding protein that would induce a nucleosomal gap might block the spreading of silent chromatin (59). This may occur passively by the formation of stable protein complexes that would act as physical barriers. However, results presented here clearly show that the GAL4 DNA-binding domain, alone or fused to many of the TRD point mutants, does not display anti-silencing or boundary effects. Thus, the DNA binding activity of these proteins does not account for their boundary activity. In contrast, gene regulation in a silent chromatin environment correlates more closely with histone H3 interaction, implying that the GalICTF-1 barrier protein must directly interact with histones in addition to its DNA binding ability. These results would support the hypothesis that CTF-1 prevents chromatin silencing by directly contacting histone H3, thereby altering the propagation of a silent chromatin structure.

It is now well established that Sir2 possesses NAD-dependent histone deacetylase activity (26). In yeast, the Sir complex spreads from nucleating sites to silence a large region of characteristically hypoacetylated chromatin (10, 15, 60). Sir2 mutants that are devoid of NAD-dependent histone deacetylase activity abrogate both rDNA and telomeric silencing (61). These data support a model in which histone deacetylation by Sir2 is required to propagate the spread of Sir1 silencing complexes. Moreover, direct targeting of acetyltransferases to the silenced domain is sufficient to create a region of histone hyperacetylation and to disrupt Sir1-mediated silencing (62, 63). Consistent with this model, heterochromatin barriers, such as HMR rRNA^Th and the H4S element of the chicken β-globin locus, are likely to block silent chromatin by providing a center of histone acetylation (27, 64).

Results presented here indicate that the high levels of histone acetylation mediated by VP16, and its ability to recruit ATP-dependent chromatin remodeling complexes and histone acetyltransferases (65–67), are sufficient to fully abrogate the spreading of Sir1 complexes. In this assay, VP16 acts to increase histone acetylation on both sides of its binding site, thereby activating nearby genes irrespective of their location relative to the telomere, as expected from a local increase of HAT concentration.

Fragments of the activation domain of VP16 have been shown to display some boundary activity, in addition to moderate anti-silencing of telomere proximal genes, and to disrupt silencing by a transcription independent mechanism (35). This raises the possibility that CTF-1 might act in a similar fashion as VP16, by displaying similar but weaker transcriptional activation properties. Thus, CTF-1 would appear to have a polar effect because it would successfully compete with silent chromatin on the telomere-distal genes, but not on the telomeric locus, whereas VP16 would be potent enough to activate both loci. If so, CTF-1 and VP16 would be expected to act similarly, but to a different extent. However, this possibility is not supported by the finding that CTF-1 displays stronger effects than VP16 on the chromatin structure, because it can fully abrogate telomere looping in vivo, even in the absence of a functional promoter sequence. Although the activation domain of CTF-1 was not found to bind chromatin-modifying enzymes, it thus allows the maintenance of high histone acetylation levels and efficiently blocks Sir1-mediated silencing. Thus, the barrier protein CTF-1 appears not to function like VP16 by inducing enzymatic modifications of histones, but rather by excluding the spreading of histone deacetylases like Sir2 and Sir complex binding (Fig. 6C). CTF-1 may prevent the propagation of the silent chromatin structure by perturbing local chromatin structure, for instance by altering nucleosomal position and/or spacing. Alternatively, it may directly interfere with the association of the Sir proteins and in particular the Sir2 histone deacetylase with nucleosomes, thus acting as a competitive inhibitor. Histone hyperacetylation in downstream regions would arise in this case as a secondary, by default modification of an open chromatin domain (68).
The boundary and anti-silencing activities of CTF-1 are unlikely to be limited to yeast cells, because the PRO domain is also able to modify a mammalian silenced locus to enhance chromatin accessibility to other factors. Moreover, mutations of the TRD domain that inhibit H3 interaction correspondingly decrease the ability of CTF-1 to achieve chromatin opening in mammalian cells (58). Irrespective of the precise molecular mechanism involved, these data give new insight into how the anti-silencing and boundary activities of CTF-1 may participate to its transcriptional activation function. CTF-1 may act as a “pioneer” transcription factor, by initiating chromatin opening through its direct interaction with histone H3, thus allowing other factors to bind and initiate transcription. A similar mechanism of chromatin remodeling was recently proposed for the histone-binding HNF-3 transcription factor (69).

Acknowledgments—We thank Drs. S. M. Gasser, W. Schaffner, D. Schumperli, and S. Triezenberg for the generous gift of plasmids and antibodies, and Drs. S. M. Gasser and D. Shore for helpful suggestions. We acknowledge Drs. A. McNair and D. Shore for comments on the manuscript.

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