A Targeted Histone Acetyltransferase Can Create a Sizable Region of Hyperacetylated Chromatin and Counteract the Propagation of Transcriptionally Silent Chromatin

Ya-Hui Chiu,1,2 Qun Yu,1,2 Joseph J. Sandmeier1,2 and Xin Bi2,3

Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588
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ABSTRACT

Transcriptionally silent chromatin is associated with reduced histone acetylation and its propagation depends on histone hypoacetylation promoted by histone deacetylases. We show that tethered histone acetyltransferase (HAT) Esa1p or Gcn5p creates a segment of hyperacetylated chromatin that is at least 2.6 kb in size and counteracts transcriptional silencing that emanates from a silencer in yeast. Esa1p and Gcn5p counteract URA3 silencing even when they are targeted 1.7 kb downstream of the promoter and >2.0 kb from the silencer. The anti-silencing effect of a targeted HAT is strengthened by increasing the number of targeting sites, but impaired by events that enhance silencing. A tethered HAT can also counteract telomeric silencing. The anti-silencing effect of Gcn5p is abolished by a mutation that eliminated its HAT activity or by deleting the ADA2 gene encoding a structural component of Gcn5p-containing HAT complexes. These results demonstrate that a tethered HAT complex can create a sizable region of histone hyperacetylation and serve as a barrier to encroaching repressive chromatin.

THE eukaryotic genome is packaged into chromatin via the formation of nucleosomes and higher-order structures. Histones form the core of the nucleosome around which DNA is wrapped. Besides being structural components of chromatin, histones play a pivotal role in the regulation of gene transcription (Jenuwein and Allis 2001). Acetylation of histones has long been linked to gene activation (Allfrey et al. 1964). Histone acetylation is carried out by histone acetyltransferases (HATs) and deacetylation is carried out by deacetylases (HDACs; Kuo and Allis 1998; Roth et al. 2001). Many HATs have been shown to be transcription co-activators or adaptors, reinforcing the link between histone acetylation and gene expression. The known HATs fall into distinct families with unique substrate preferences (Roth et al. 2001). For instance, yeast Esa1p belongs to the MYST HAT family that preferentially acetylates histone H4 whereas Gcn5p is a member of the GNAT family that has a preference for histone H3. In vivo, a HAT usually functions as part of a regulatory complex or the transcription machinery in which HAT activity can be appropriately directed to particular gene targets. In addition, other factors in the complex can help the HAT gain access to histones in nucleosomes (Roth et al. 2001). Esa1p is the catalytic subunit of the NuA4 complex while Gcn5p is a component of the SAGA or ADA complex (Grant et al. 1997; Allard et al. 1999). The NuA4 and SAGA complexes can be recruited to specific promoters by direct interactions with the acidic activation domains of certain activators (Utley et al. 1998; Bhaumik and Green 2001; Larschan and Winston 2001). It was shown that targeted NuA4 or SAGA complex led to a localized domain of histone acetylation. However, attempts to estimate the sizes of such domains have led to seemingly conflicting results ranging from 2 to 20 nucleosomes (Kuo et al. 1998; Krebs et al. 1999; Vignali et al. 2000). It is not clear if the discrepancy reflected distinct genomic contexts of targeted HATs and/or different sensitivities of the assays used in those experiments.

Consistent with its correlation with gene activation, histone acetylation is reduced in transcriptionally silent domains like the yeast HML and HMR loci (Braunstein et al. 1993, 1996). The silent HML loci are established and maintained through combined actions of cis-acting and trans-acting factors (Lustig 1998; Moazed 2001). The cis-acting elements are the silencers flanking each HM locus. The trans-acting proteins include histones, the Sir2p–Sir4p proteins, and silencer-binding proteins. It was proposed that silencer-binding proteins recruit a complex of Sir2p/Sir3p/Sir4p, which then propagates sequentially along neighboring nucleosomes to form a silent chromatin similar to metazoan heterochromatin that represses gene expression. In this mechanism, the SIR complex is an integral part of the silent chromatin, and interactions between Sir3p/Sir4p and histones H3/H4 are essential to the establishment and maintenance

1These authors contributed equally to this work.

2Present address: Department of Biology, University of Rochester, Rochester, NY 14627.

3Corresponding author: Department of Biology, University of Rochester, Rochester, NY 14627. E-mail: xinbi@mail.rochester.edu

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of silenced chromatin (Hecht et al. 1995). Sir3p and Sir4p interact with the N-terminal tails of histones H3 and H4, and there is evidence that Sir3p (hence the SIR complex) has much higher affinity to unacetylated histone H4 than to acetylated H4 (Carmes et al. 2001). It was recently shown that Sir2p was a NAD-dependent protein deacetylase that was likely involved in reducing the level of histone acetylation in silent chromatin (Imai et al. 2000). Taking these findings into consideration, a refined model of silencing featuring histone deacetylation by Sir2p can be proposed. In this model, Sir2p, when recruited to a silencer, deacetylates histones in an adjacent nucleosome, which then binds another SIR complex with high affinity. The nucleosome-bound SIR complex then deacetylates the neighboring nucleosome, which then binds a new SIR complex. In this manner, the SIR complex promotes its own sequential propagation along an array of nucleosomes.

Since histone deacetylation is essential to the establishment and maintenance of a silenced domain, a counteracting HAT may disrupt silencing if it is directed to the silenced domain. To test this hypothesis, we targeted a LexA-Esa1p or LexA-Gcn5p fusion protein to LexA-binding sites inserted near or within the silent HML locus or near a telomere. We showed that either fusion protein was able to create a region of histone hyperacetylation of at least 2.6 kb in size and counteract the propagation of silencing.

MATERIALS AND METHODS

Plasmids and strains: Plasmid pAR61 was derived from pUC12 with the HindIII-BamHI fragment of chromosome III (coordinates 14,838–16,265) inserted. The 1.1-kb BglII-URA3-BglII fragment of plasmid pFL44 (Chevallier et al. 1980) was inserted at the EcoRI site of pAR61 to make plasmid pMB22a. A sequence containing a ColE1 operator (boldface type), TCTTACC-TGACTGT-GCTGTATAAAAAACCTAGGTGTATATGTACGTTAGCGAGGGGTATAAGGC, was inserted at the SmalI, NcoI, and EcoII sites of pMB22a to make plasmids pQY38, pQY39, and pQY37, respectively. The ColE1 operator contains two variants of the consensus sequence for LexA binding, CTGTATAANAAACAG, where N can be A, T, G, or C (Ebina et al. 1983). Three copies of a sequence containing a single synthetic LexA-binding site (boldface type), GGGTTA CGTACTGTATGTCATAACAGGTATCGGGG, were inserted at the BamHI site of pAD14/UC4 (Sandell and Zakian 1993) to make pXBY61. A sequence containing four tandem copies of the ColE1 operator [coordinates 271–450 of pSH18-34 (Gyuris et al. 1993)] was inserted at the EagI site of pMB22a to make plasmid pQY35. The same sequence was inserted at the BstI site of pXBY61 (Bi and Broach 1999) to make plasmid pQY37. This sequence was also inserted at the SpeI site of pQY57 to make pQY58.

Plasmid pRS425 is a 2-μm-based vector that has the LEU2 gene in it (Christianson et al. 1992). Plasmids carrying LexA fusion genes were derived from the two-hybrid vector pBTM116 containing the 2-μm origin and the ADH1 promoter-LexA-ADH1 terminator module. Note that in pBTM116, a sequence containing the multiple cloning sites (MCS) was fused to the LexA open reading frame (ORF). As a result, LexA encoded by pBTM116 has 25 extra amino acids at the carboxyl terminus. Plasmid pVC07 was made by replacing the XhoI-PvuII fragment of pBTM116 with the XbaI-LEU-PvuII fragment of pRS425. The MCS sequence of pVC07 was engineered to make pXB323, which encoded the wild-type LexA protein without the extra 25 residues. pXB301 was derived from pYC07 by fusing the ORF of ESA1 to the LexA gene. Plasmid pRQ12 was derived from pYC07 by fusing the ORF of the yeast GCN5 gene to the LexA gene. Plasmid pQKL was identical to pRQ12 except that the GCN5 sequence 376-AAGCAATTA-384 encoding amino acids Lys-Gln-Leu (KQL) was replaced by GCTGCAGCC encoding Ala-Ala-Ala (AAA). Plasmids pYC33-pYC35 were derived from pXB323, pXB301, and pRQ12, respectively, by inserting a BglII-SIR3-BglII fragment at the BglII site. Plasmids pRS425, pXB323, pXB301, pRQ12, pYC33, and pYC35 were referred to as pAR61-4, respectively, for convenience (Figures 1–6). Plasmid pQKL was referred to as pRS425-4.

Most yeast strains used in this study were derived from strain YBX76 [MATa ura3-52 leu2-3,112 ade2-1 his3-11,15 met15::kanMX4]. Strain YXB85-new was made by transforming YBX76 to Ura+ with the HindIII-BamHI fragment containing the URA3 gene from pMB22a. Strains YQY10, YQY11, YQY09, and YXB227 were similarly constructed using plasmids pQY38, pQY39, pQY37, and pXB306, respectively. Strain YXB401 was made by transforming YBX76 to Ura+ with the EcoRI-SalI fragment from pXB363.

Strains YQY05 and YQY13 were made by transforming strain Y728 (Mahoney and Broach 1989) to Ura+ with the EcoRI-PvuII fragment of pQY38 and pQY37, respectively. Note that YXB76 is identical to Y728 except that the orientation of the HML-I silencer is inverted. Strain YQYS1 was made by introducing the sequence Iinverted–URA3-ColE1-operator (same as in YQY11) into ResGen/Invitrogen strain 4282 (MATa his3A1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ::kanMX). Strain Y1838 was MATa his3A1 leu2-3,112 his3-11α tryp1-289 Δ(hht2-hhf1) Δ(hht2-hhf2) + plasmid pMS329 (CEN-URA3-HHT1-HHT1). YQY91 was made by introducing the construct Iinverted–URA3-ColE1-operator (same as in YQY11) into Y1838 and replacing its pMS329 plasmid with pMP3-59b (CEN-TRP1-HHT1-hhf2-HHT1) (Clarke et al. 1999). For convenience, strains YXB85-new, YQY10 and YQY11, LYP3498, LYP3430, YXB401, YQY09, YXB227, YQY05, YQY15, YQY34, and YQY91 were denoted a–l, respectively (Figures 1–6). JSW319 was denoted strain a’ (Figure 1E).

Western blotting: Yeast cells carrying a LexA fusion gene were grown in −Leu liquid medium at 30°C to late log phase. −Leu medium was synthetic complete medium lacking leucine. Protein extract was prepared from ~3 × 107 cells by glass-bead lysis and 20 μg of it was run on a 4–20% SDS ready gel (Bio-Rad, Hercules, CA). The gel was then blotted with nylon membrane, blocked in 3% BLOTTO solution (10 mM NaH2PO4, 140 mM NaCl, 0.05% Tween 20, 3% nonfat dry milk), washed, and then incubated with 1:5000 polyclonal α-LexA (Invitrogen, San Diego). The blot was washed, incubated with horseradish peroxidase-conjugated goat anti-rabbit antiserum (1:2000, Santa Cruz Biotechnology), washed, and processed for colorimetric HRP detection using the Oxy-4CN substrate kit (Bio-Rad).

Chromatin immunoprecipitations: The method for chromatin immunoprecipitations was identical to a previously described protocol (Sandmeier et al. 2002) except for a few minor modifications. SC-Leu cultures (50 ml) of strain carrying plasmid 2, 3, or 4 were grown to log phase (0.8–1.2 OD600) and then fixed for 1 hr at room temperature (RT) in 1%
formaldehyde. Cells were harvested and washed twice with dH2O and FA-lysis 140 buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholic acid, and Sigma P-8215 at 15 μl/ml). Cell pellets were resuspended in 400 μl FA-lysis 140 and acid-washed glass beads (425–600 μm) were added ~3:4 to the meniscus. A BioSpec Mini-Beadbeater was used to make the extracts (eight 20-sec cycles) that were then transferred to new Eppendorf tubes. With the use of a Branson Sonifier 450 the extracts were sonicated 12 times for six pulses each time at 90% duty cycle and output setting 4. The lysate was then clarified by centrifugation at 14,000 rpm for 20 min. According to the A260, 120 units of whole-cell extract was added to each immunoprecipitation (IP) for a final volume of 240 μl in FA-lysis 140. Serum antibodies against acetyl-H3 (K9/K14) and acetyl-H4 (Penta; kindly provided by Dr. David Allis) were added at 1 μl/IP. Incubation of IP reactions was done at 4°C overnight.

Bound chromatin was precipitated with 20 μl of Protein A Sepharose beads (50% slurry in 1× TE/0.1% BSA/0.1% Na Azide) for 2 hr at 4°C. The beads were washed extensively and the immune complexes were eluted twice with 200 μl 1% SDS/0.1 M NaHCO3 at RT. The cross-links were then reversed for 5 hr in the presence of NaCl and ethanol precipitated overnight at −20°C. The recovered material was RNase A and Proteinase K treated and phenol:chloroform extracted. Purified DNA was resuspended in 150 μl 1× TE. Six microliters of each sample was used in 50-μl PCR reactions where the Tc was 50°C for 28 cycles. In PCR reactions the proper amount of input and IPed chromatin DNA used was predetermined to be in the linear range by serial dilutions. Input chromatin was added to PCR reactions as a 1:10 dilution. PCR products were separated on a 1.2% agarose gel. Images were captured with VisionWorks 32 software from UVG (San Gabriel, CA) and bands were quantified using Quantity One from Bio-Rad.

RESULTS

Targeted LexA-Esa1p or LexA-Gcn5p can counteract transcriptional silencing: The URA3 gene has been frequently used as a reporter in studies of transcriptional silencing. Its expression can be assessed by cell viability on medium containing 5-fluoroorotic acid (5-FOA). Ura3p, the protein encoded by URA3, converts 5-FOA to a toxic metabolite, so that cells with basal-level URA3 expression are sensitive to 5-FOA (Boeke et al. 1987). When URA3 is inserted at HML, its basal expression is silenced by the HML silencers such that cells are able to grow on medium containing 5-FOA. By examining URA3 silencing at HML, we have previously demonstrated that the HML1 silencer defined the right (centromere-proximal) boundary of the silent HML domain by initiating silencing in only one direction (toward the HML2 genes; Bt et al. 1999). When we inverted the direction of the HML1 silencer, however, silencing could spread ~1.4 kb to the right of HML1 (Bt et al. 1999). On the basis of these observations we designed a silencer-blocking assay to test if a sequence has the ability to prevent the spread of silencing. URA3 was inserted 0.6 kb to the right of the inverted HML1 silencer (Figure 1A, strain a), where its basal expression was silenced as reflected by cell growth on 5-FOA medium (Figure 1A, strain a, SC + FOA plot). A sequence to be tested would be inserted between the HML-I silencer and URA3 (e.g., Figure 1A, strain b). Elimination of silencing of URA3 would indicate that the inserted sequence has anti-silencing activity. The sequence can also be inserted downstream of URA3 (e.g., Figure 1A, strain c) to test if it has any effect on URA3 silencing. When a ColE1 operator consisting of two LexA-binding sites was tested in such a silencer-blocking assay, no effect was detected on URA3 silencing (Figure 1A, compare b and c to a on SC + FOA medium). Similar results were obtained with other sequences containing LexA-binding sites used in this study (data not shown). Therefore, LexA-binding sites per se do not affect HML silencing in yeast and thus can be used to test LexA-fusion proteins in a silencer-blocking assay.

We intended to examine if targeted LexA-Esa1p or LexA-Gcn5p fusion protein could counteract transcriptional silencing. To this end, we constructed 2-μm-based plasmids that carried a LEU2 marker gene and the LexA-Esa1p and LexA-Gcn5p fusion genes, respectively (Figure 1B, plasmids 3 and 4). Plasmids pRS425 (plasmid 1) and pXB323 carrying the LexA gene (plasmid 2) were used as controls. Expression of the fusion proteins from these plasmids was analyzed by Western blotting (Figure 1C). The cellular levels of LexA-Esa1p and LexA-Gcn5p proteins were comparable to that of LexA (Figure 1C, lanes 2–4). We noted that the level of LexA-Gcn5p was always approximately two- to threefold higher than that of LexA-Esa1p in all the strains tested (Figure 1C, compare lanes 3 to 4, and data not shown). ESA1 is essential for cell growth and esal temperature-sensitive (ts) alleles have been created (Clarke et al. 1999). Strain e carrying the esal-L327S mutation grew normally at 30°C but was inviable at 37°C (Figure 1D, top, strain e, compare 30°C and 37°C growth). When LexA-ESA1 was introduced into strain e, cells grew normally at 37°C, just like the ESA1+ strain d (Figure 1D, bottom). Therefore, LexA-ESA1 is able to complement the loss of Esa1p activity, indicating it is functional in vivo. Unlike ESA1, GCN5 is not essential for cell growth. Deletion of GCN5 leads only to a slight slow-growth phenotype (Figure 1E, compare strains e’ and a bearing plasmid 1). LexA-GCN5 was able to complement this defect (Figure 1E, strain a’, compare 4 and 1), indicating that LexA-Gcn5p is also functional in vivo.

When introduced into strain a in which there was no LexA-binding sequence in the genome, LexA-ESA1 and LexA-GCN5 had no effect on cell growth (Figure 1B, –Leu plot) or URA3 silencing at HML (Figure 1B, –Leu + FOA plot), indicating that an untargeted LexA-HAT does not counteract silencing. However, when a ColE1 operator consisting of two LexA-binding sites was integrated between the inverted HML-I silencer and URA3 (Figure 2, strains b), expression of LexA-Esa1p or LexA-Gcn5p led to the elimination of cell viability on 5-FOA medium (Figure 2A, compare rows 3 and 4 to 1 and 2, –Leu + FOA plot). This indicates that
tethered LexA-Esa1p or LexA-Gcn5p prevents HML-I from silencing URA3. This is in accord with similar observations that tethered Gal4p-Gcn5p or Gal4p-Sas2p [Sas2p is a HAT (Sutton et al. 2003)] decreases transcriptional silencing (Donze and Kamakaka 2001; Ishii and Laemmli 2003), although the anti-silencing effect of LexA-Gcn5p or LexA-Esa1p that we observed here is much stronger than that of Gal4p-Gcn5p reported earlier. Therefore, a HAT, regardless of its substrate specificity, can counteract transcriptional silencing when tethered near a silencer. LexA-Esa1p or LexA-Gcn5p can also counteract telomeric silencing of URA3 inserted near the left telomere of chromosome VII (Figure 2B, significant effect on URA3 tethered near a silencer. LexA-Esa1p or LexA-Gcn5p can also counteract transcriptional silencing (Figure 3A), LexA-Gcn5p still completely abolished silencing at a distance:

In the above experiments and earlier. Therefore, a HAT, regardless of its substrate over, even when the two LexA-binding sites were inserted 1.7 kb downstream from the URA3 promoter as in strain g (Figure 3A), LexA-Gcn5p still completely abolished silencing whereas LexA-Esa1p had a reduced but still significant effect on URA3 silencing (Figure 3A, strain g, compare rows 3 and 4 to 2). The stronger anti-silencing effect of LexA-Gcn5p as compared to that of LexA-Esa1p may reflect the two- to threefold higher expression of LexA-Gcn5p (Figure 1C). The above results demonstrated that LexA-Esa1p or LexA-Gcn5p eliminated URA3 silencing when tethered up to 1.7 kb downstream from the URA3 promoter. This argues against the possibility that LexA-Esa1p or LexA-Gcn5p directly activates URA3 expression. Consistent with this conclusion, Ishii and Laemmli (2003) showed that tethered Gcn5p did not activate a lacZ reporter gene. We speculate that a targeted HAT can create a sizable domain of ~2–4 kb in size that is resistant to transcriptional silencing.

![Figure 1](image-url) — Assay for testing the effect of a targeted HAT on transcriptional silencing. (A) Insertion of LexA-binding sites near HML did not affect cell growth or HML silencing. In strain a (YXB85-new), the direction of the HML-A silencer was flipped and the URA3 gene was inserted to the right of HML. Strains b (YQY10) and c (YQY11) were derived from a by inserting a ColE1 operator upstream or downstream of URA3, respectively. Construction of these strains was detailed in MATERIALS AND METHODS. The two open bars denote the two LexA-binding sites in the ColE1 operator. The growth phenotypes of strains a, b, and c were shown on the right. Cells of each strain were grown to late log phase and serial dilutions (10-fold) were spotted on test plates and allowed to grow for 3 days at 30°C. SC, synthetic complete medium; FOA, 5-fluoroorotic acid. (B) Expression of untargeted LexA-HAT fusion proteins did not affect cell growth or HML silencing. Left, plasmids. Plasmids 1–4 were described in MATERIALS AND METHODS. Right, growth phenotypes of strain a with plasmid 1, 2, 3, or 4 in it. –Leu, SC medium lacking leucine. (C) Expression of LexA-HAT fusion proteins. Twenty micrograms of total protein from cells of strain a bearing plasmids 1–4 (B), respectively, was analyzed by SDS-PAGE followed by Western blotting and probed with an anti-LexA antibody. Bands corresponding to LexA, LexA-Esa1p, and LexA-Gcn5p were indicated. (D) LexA-Esa1 complemented the ts growth phenotype of esa1-L327S. Top, growth phenotypes of strains d (LPY3498) and e (LPY3430) on SC medium at 30°C and 37°C, respectively. Bottom, growth phenotypes of strains d and e bearing plasmid 3 (LexA-Esa1) on –Leu medium at 30°C and 37°C, respectively. (E) LexA-Gcn5 complemented the slow-growth phenotype of gcn5Δ. Growth phenotypes of strain a' (JJSy319) bearing plasmids 1 and 4, respectively, were shown. Also shown were growth phenotypes of strain a bearing plasmids 1 and 4, respectively. Medium used was –Leu.
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LexA-Esa1p in strain h now totally abolished silencing of URA3 (Figure 3A, plasmid 3). As for LexA-Gcn5p, the two LexA-binding sites in strain g were sufficient to abolish URA3 silencing (Figure 3A); thus, as predicted, the eight LexA sites in strain h also completely eliminated URA3 silencing (Figure 3A, strain h with plasmid 4).

Silencing within the HML locus is stronger than that in its surrounding regions due to the concerted actions of both the E and I silencers flanking HML (Bt et al. 1999; Bi 2002). We tested if targeting a HAT could also overcome the strong silencing within HML. In strain i, the URA3 gene was bracketed by two copies of a sequence containing eight LexA-binding sites within the HML locus (Figure 3B). When only LexA was expressed, URA3 was strongly silenced as evidenced by the robust growth of cells on 5-FOA medium (Figure 3B, strain i bearing plasmid 2). However, expressing LexA-Esa1p or LexA-Gcn5p completely eliminated URA3 silencing (Figure 3B, growth phenotype of strain i bearing plasmid 3 or 4). Similar results were obtained when only eight LexA sites were inserted at HML (Figure 3B, growth phenotype of strain j bearing plasmids 3 or 4). These data indicate that a targeted HAT is able to overcome the strong silencing within HML. They also imply that the LexA-binding sites embedded in silent chromatin were accessible to the LexA-HAT fusion proteins.

The HAT activity of Gcn5p is required for its anti-silencing function. We wanted to test if increasing the amount of targeted LexA-Esa1p molecules could restore the strong anti-silencing effect of LexA-Esa1p. To this end, we inserted four ColE1 operators containing a total of eight LexA-binding sites at a position 1.7 kb downstream of the URA3 promoter (Figure 3A, strain h). Expression of

Increasing the number of binding sites for LexA-Esa1p enhances its anti-silencing effect: As evident from Figure 3A, the ability of LexA-Esa1p to counteract silencing was weakened when the two LexA-binding sites were 1.7 kb downstream of the URA3 promoter (strain g). We wanted to test if increasing the amount of targeted LexA-Esa1p molecules could restore the strong anti-silencing effect of LexA-Esa1p. To this end, we inserted four ColE1 operators containing a total of eight LexA-binding sites at a position 1.7 kb downstream of the URA3 promoter (Figure 3A, strain h). Expression of

Figure 2.—Targeted LexA-Esa1p or LexA-Gcn5p could counteract transcriptional silencing. (A) Growth phenotypes of strain b bearing plasmids 1–4, respectively, on –Leu medium (left) and –Leu + FOA medium (right) were shown. (B) Growth phenotypes of strain f (YXB401) bearing plasmids 2–4, respectively.

Figure 3.—Targeted LexA-Esa1p or LexA-Gcn5p can counteract silencing at a distance. (A) Growth phenotypes of strains c, g (YQY09), and h (YXB227) bearing plasmids 2–4, respectively. In strains c and g, two LexA sites (open bars) were inserted 1.2 and 1.7 kb downstream of the URA3 promoter, respectively. In strain h, eight LexA sites (shown as a shaded box) were inserted 1.7 kb downstream of the URA3 promoter. (B) Growth phenotypes of strains i (YQY05) and j (YQY13) bearing plasmids 2–4, respectively. In strain i, eight LexA sites (shaded box) were inserted upstream and another eight downstream of URA3 that was integrated at HML. Strain j was identical to i except that eight LexA sites were inserted only between URA3 and HML.
LexA-gcn5-KQL had the amino acids 126–128 (KQL) of Gcn5p replaced by three alanines (AAA). This substitution mutation was previously shown to eliminate the HAT activity of Gcn5p (Wang et al. 1998). As demonstrated in Figure 4A, LexA-gcn5-KQL expressed in strain c had no effect on URA3 silencing (top, compare rows 4’ to 2). This was in contrast to the robust anti-silencing activity of LexA-GCN5 (Figure 4A, compare rows 4 and 4’). Note that the levels of LexA-gcn5-KQL and LexA-GCN5 proteins were comparable in the cell (Figure 4A, bottom, compare lanes 4 and 4’). These results indicate that the HAT activity of Gcn5p is required for its anti-silencing function.

The ability of LexA-Gcn5p to counteract silencing is dependent on ADA2. Although the Gcn5p or Esa1p HAT can catalyze histone acetylation in vitro by itself, it has to work as a component of a HAT complex to perform its proper functions in vivo (Roth et al. 2001). Gcn5p alone cannot be recruited to specific sites. Since LexA-Gcn5p can be directly targeted to LexA-binding sites, it is reasonable to think that LexA-Gcn5p may bypass the need for the Tra1p or SAGA complex for HAT function. Candau et al. (1997) have addressed this issue by testing if LexA-Gcn5p still required Ada2p to activate gene expression. They demonstrated that deleting ADA2 abolished the activating function of LexA-Gcn5p. Is ADA2, or an intact SAGA complex, also required for LexA-Gcn5p’s function in anti-silencing? The answer is yes as evidenced by the lack of anti-silencing activity of LexA-Gcn5p in an ADA2 deletion strain (Figure 4B, growth phenotype of strain k with plasmid 4 on −Leu + FOA medium). As a control, we demonstrated that the anti-silencing activity of LexA-Esa1p was not affected by ADA2 deletion (Figure 4B, growth phenotypes of strain k with plasmid 3). This is expected since Ada2p is not related to Esa1p function. Therefore, LexA-Gcn5p does not act alone but rather, most likely, works as part of the SAGA complex. This is in accord with our observation that LexA-GCN5 can complement the growth defect caused by the deletion of GCN5 as described earlier.

Targeted LexA-Gcn5p or LexA-Esa1p creates a sizable segment of hyperacetylated chromatin: It could be inferred by data described so far in this report as well as results from other studies that histone acetylation underlying the anti-silencing activity of targeted HATs. However, this has not been experimentally tested. We decided to directly address this issue by examining the acetylation levels of histones around the target site of a HAT using a chromatin immunoprecipitation (ChIP) assay. Since in general Gcn5p has specificity for histone H3, an antibody against H3 with K9/K14 acetylation (designated α-H3-Ac) was used to examine the function of LexA-Gcn5p. PCR primers were designed to detect DNA fragments 1–9 (200–450 bp in length) on both sides of the LexA sites in strain c that were precipitated by α-H3-Ac in ChIP (Figure 5A). PCR product corresponding to each fragment was examined by agarose gel electrophoresis (Figure 5A, left). The intensity of each fragment was quantified and normalized against input control. Strain c expressing LexA was used as a control for background in the ChIP assay. LexA-Gcn5p-induced H3 acetylation in a particular sequence (1–9) was estimated as the ratio of the intensity of the corresponding fragment in LexA-Gcn5p-expressing cells over that in LexA-expressing cells (Figure 5A, right). The ChIP assay was repeated at least three times and a representative gel picture was presented (Figure 5A, left). For each DNA segment, the mean of data from all the repeats (together with standard deviation) was graphed in Figure 5A, right (1–9). Also included as a control (designated 0) was the result for the TDH3 locus at which histone acetylation was not affected by Gcn5p (Kuo et al. 2000). It was clear that tethered LexA-Gcn5p...
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Figure 5.—A targeted HAT created a sizable domain of histone hyperacetylation. (A) Domain of histone H3 acetylation generated by LexA-Gcn5p. Left, chromatin-immunoprecipitation from strain c bearing plasmids 2 (LexA) and 4 (LexA-GCN5), respectively. The antibody used, designated α-H3-Ac, was specific for histone H3 with K9/K14 acetylation. DNA sequences tested (1–9) by PCR were illustrated on the right. Sequence 0 was within the upstream regulating sequence of the TDH3 gene. It was used as a background control. Right, histone H3 acetylation generated by LexA-Gcn5p around the LexA-binding sites in strain c. Sequences to the right of the HML locus (roughly proportional) in strain c were illustrated. The relative positions of the DNA segments 1–9 were shown. Each bar represents the fold increase in acetylation in strain c bearing LexA-GCN5 compared to strain c bearing LexA, which was calculated as the IP/input ratio for LexA-GCN5 divided by the IP/input ratio for LexA. Standard deviation from at least three independent experiments was shown (error bars). The URA3 gene was shown as a solid box with arrow. The 5’ and 3’ flanks of URA3 were shown as hatched boxes. Note that strain c has an endogenous ura3-52 allele, which resulted from a Ty insertion at an RsaI site within the URA3 ORF (Rose and Winston 1984). We have designed PCR primers to specifically amplify sequences within the URA3 allele near HML but not sequences at ura3-52. Detailed information about the primers is available upon request. (B) Domain of histone H4 acetylation generated by LexA-Esa1p. Left, chromatin-IP from strain c bearing plasmids 2 (LexA) and 3 (LexA-ESA1), respectively. The antibody used, designated α-H4-Ac, was specific for multiple acetyl-H4 isoforms. DNA sequences tested (1–9) by PCR were the same as those in A. Sequence 0 was within the upstream regulating sequence of the ACT1 gene. It was used as a background control. Right, histone H3 acetylation generated by LexA-Esa1p around the LexA-binding sites in strain c. Each bar represents the fold increase in acetylation in strain c bearing LexA-ESA1 compared to strain c bearing LexA.

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increased H3 acetylation to a great extent (up to ninefold) near the LexA sites (Figure 5A, 5 and 6). In a region of at least 1 kb in size to the right of the LexA sites, H3 acetylation was significantly elevated (approximately fourfold; Figure 5A, 7–9). Remarkably, H3 acetylation was also increased (two- to sixfold) in sequences encompassing 1.6 kb to the left of the LexA sites. Note that this region was under the influence of the HML-I silencer. These results demonstrated that targeted LexA-Gcn5p created a sizable zone (>2.6 kb) of histone H3 hyperacetylation in which transcriptional silencing was abolished (Figure 3A, strain c with plasmid 4).

Using the ChIP assay, we also demonstrated that LexA-Esa1p created a sizable zone of histone H4 acetylation (Figure 5B). LexA-Esa1p induced a great increase in H4 acetylation (approximately sevenfold) at/near the LexA sites in strain c (Figure 5B, 5 and 6), as well as significant acetylation (three- to fivefold) in an ~1.4-kb region to the left (Figure 5B, 2–4) and an at least 1-kb region to the right of the LexA sites (Figure 5B, 7–9). In summary, tethered Gcn5p or Esa1p can create a sizable (>2 kb) region of histone H3 or H4 acetylation that is centered at/near the targeting site. Notably, for both LexA-Gcn5p- and LexA-Esa1p-induced histone acetylation, the level is the highest immediately to the left of the targeting site but not at the targeting site. This may be due to the limited resolution of the ChIP assay. Alternatively, this may indicate that histones in sequence 5 are better substrates for the targeted HAT.

In summary, tethered Gcn5p or Esa1p can create a sizable (>2 kb) region of histone H3 or H4 acetylation that is centered at/near the targeting site. Notably, for both LexA-Gcn5p- and LexA-Esa1p-induced histone acetylation, the level is the highest immediately to the left of the targeting site but not at the targeting site. This may be due to the limited resolution of the ChIP assay. Alternatively, this may indicate that histones in sequence 5 are better substrates for the targeted HAT.

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Enhanced silent chromatin can overcome the anti-silencing effect of a HAT: The above results indicate that a targeted HAT in the path of silent chromatin in effect serve as a barrier to its propagation. This barrier functions by acetylating histones to counteract histone deacetylation essential to the spread of silent chromatin. Consequently, this barrier is not a passive physical roadblock but rather an active far-reaching anti-silencing center. The strength of this barrier should depend on the balance of the silencing function of the silencing machinery and the anti-silencing activity of a HAT. We tested if enhancing silencing could overcome such a barrier.
Sir3p has been shown to be a limiting component in silent chromatin; hence overexpressing Sir3p enables silencing to propagate farther (RENAULD et al. 1993; HECHT et al. 1996). We introduced an ectopic copy of the SIR3 gene on a 2-μm plasmid in strain b (Figure 6A). Although LexA-Esa1p or LexA-Gcn5p still significantly decreased URA3 silencing in this strain (Figure 6A, compare rows 6 and 7 to 5), it was no longer able to completely eliminate silencing as in strain b in the absence of 2-μm SIR3 (Figure 2A). This indicates that overexpression of SIR3 decreases the barrier function of a targeted HAT.

SMITH et al. (2002) have identified histone H3 and H4 mutations that increased telomeric silencing. We have shown that one of their H4 mutations, hhfH75Y, also enhanced HML silencing (X. Bi and J. R. BROACH, unpublished results). As evident in Figure 6B, this mutation also significantly impaired the anti-silencing effect of LexA-Esa1p and LexA-Gcn5p (compare strain l with plasmids 3 or 4 in Figure 6B to strain c with plasmids 3 or 4 in Figure 3A). Taken together, the above results indicate that enhanced silent chromatin can overcome a HAT barrier.

**DISCUSSION**

Reversible acetylation of the N-terminal tails of histones plays a crucial role in the fine regulation of gene expression in eukaryotes. Acetylation and deacetylation of histones are carried out by HATs and HDACs that are targeted to specific promoters by transcriptional activators and repressors, respectively (Kuo et al. 1998; ROTH et al. 2001). For example, the NuA4 and SAGA HAT complexes are targeted to certain promoters by transcriptional activators bearing acidic activation domains (UTLEY et al. 1998; KUO et al. 2000; BHAUMIK and GREEN 2001; LARSCHEEN and WINSTON 2001), and the Sin3p-Rpd3p HDAC complex is targeted to promoters by the Ume6p repressor (KADOISH and STRUHL 1997).

Although it has been generally thought that a targeted HAT or HDAC creates a localized domain of histone hyper- or hypoacetylation in vivo, the underlying mechanism is not well understood. In this report we demonstrate that a targeted histone acetyltransferase can create a segment of hyperacetylated chromatin of at least 2.6 kb, indicating that a HAT tethered to DNA is able to reach and acetylate up to eight nucleosomes on each side. These results provide strong support for the notion that one mechanism for chromatin boundary or insulator function is the recruitment of HAT activity that counteracts encroaching histone deacetylation that is a hallmark of heterochromatin (LITT et al. 2001a; DONZE and KAMAKARA 2002).

**A targeted HAT can overcome transcriptional silencing:** Transcriptional silencing at the HM loci in yeast is maintained by a special silent chromatin similar to metazoan heterochromatin in many aspects (MOAZED 2001). Histones H3 and H4 in silent chromatin have reduced acetylation compared to those in active chromatin (BRAUNSTEIN et al. 1993, 1996). This may be the result of the protein deacetylase activity of Sir2p, a component of the silent chromatin. The hypoacetylated silent chromatin prevents RNA polymerase machinery from transcribing genes embedded in it. The fact that tethering the HAT Esa1p or Gcn5p to the HML locus results in the abolishment of silencing reinforces the notion that hypoacetylation is key to silencing. Assuming that Esa1p fused to LexA still retains its substrate specificity (for histone H4) and LexA-Gcn5p is still specific to H3, this result would imply that elevating the acetylation level of either histone H4 or H3 is sufficient to disrupt the silent chromatin and allow activation of transcription. Thus it may be a net level of hypoacetylation of histones H3 and H4 that is required for maintaining silencing. Conversely, a net level of acetylation of histones H3 and H4 may be required for transcriptional activation. Consistent with this notion, VIGNALI et al. (2000) showed that acetylation of either H4 by targeted NuA4 or H3 by targeted SAGA was sufficient for transcriptional stimulation of a gene in a nucleosomal template in vitro. Also in accordance with the notion was the finding that opposing Esa1p- or Gcn5p-dependent acetylation and Hda1p- or Rpd3p-dependent deacetylation determined global acetylation of histones H3 and H4, thereby regulating gene activity (VOGELAUER et al. 2000).

**A targeted HAT can generate a sizable domain of nucleosome hyperacetylation:** An important question con-
cerning the function of a HAT in vivo is if, and how, it carries out localized or long-range acetylation of histones along the chromatin. There is evidence that HAT complexes recruited by DNA-binding activators acetylate histones in localized regions. However, the estimated range of distribution of acetylated histones varied from experiment to experiment. Kuo et al. (1998) obtained evidence indicating that histone H3 acetylation induced by Gcn5p at a promoter spanned only ~2-3 nucleosomes. Parekh and Maniatis (1999) showed that in mammalian cells virus infection induced a p300-dependent domain of histone H3 and H4 hyperacetylation consisting of also 2-3 nucleosomes. However, Krebs et al. (1999) demonstrated that a 6- to 7-nucleosome domain in the yeast HO promoter could be acetylated by Gcn5p. In an in vitro experiment using a nucleosomal template, Vignali et al. (2000) showed that targeting the NuA4 complex by a transcriptional activator led to a large domain of H4 acetylation of >3 kb (~20 nucleosomes), whereas targeting SAGA resulted in a smaller region of H3 acetylation. The discrepancy in the estimated sizes of domain of acetylation from the above experiments may reflect different resolutions of the assays and/or the existence of locus-specific factors that regulate the actions of HAT complexes.

In this report we have shown that targeted LexA-Gcn5p or LexA-Esa1p generates a hyperacetylated chromosomal domain of at least 2.6 kb in size. Therefore, LexA-Gcn5p and LexA-Esa1p are able to reach at least 8-10 nucleosomes on each side. In vivo, Gcn5p is in either the SAGA or the ADA complex whereas Esa1p is incorporated into the NuA4 complex. The fact that LexA-ESA1 and LexA-GCN5 can complement the growth phenotypes of inactivation/deletion of ESA1 and GCN5, respectively, and that the anti-silencing function of LexA-Gcn5p is dependent on ADA2 indicate that LexA-Esa1p and LexA-Gcn5p are also incorporated into HAT complexes to carry out their proper functions. How a targeted HAT complex reaches and acetylates histones several nucleosomes away is not clear. The bromodomain in certain HATs, a protein motif capable of mediating protein-protein interactions with factors bound to DNA, is involved in protein-protein interactions with factors bound at the promoter thereby “reaching” the promoter. In the second model (the tracking model), a HAT complex recruited to a site was hypothesized to track along the DNA via interaction with acetylated nucleosomes, creating a hyperacetylated region. The third model (the spreading model) proposes that local acetylation carried out by a targeted HAT serves to recruit additional HATs, thereby initiating the spread of HAT complexes along the DNA. In the looping model nucleosomes within the loop are not acetylated by the HAT whereas the tracking and spreading models predict a continuous region of hyperacetylation. The tracking/spreading model is consistent with the possibility that the bromodomain of Gcn5p could mediate interactions between the SAGA complex and acetylated nucleosomes (Hassan et al. 2002). The tracking or spreading models could readily explain the acetylation pattern of LexA-Gcn5p (Figure 5A). However, since Esa1p lacks a bromodomain and the NuA4 complex cannot be anchored to acetylated nucleosomes (Hassan et al. 2002), the tracking/spreading model does not explain how LexA-Esa1p can induce an acetylation pattern similar to that of LexA-Gcn5p (Figure 5B). Further investigations are underway to gain more information on the mechanisms of long-range actions of LexA-Gcn5p and LexA-Esa1p.

**Targeted HATs can act as barriers to the spread of silent chromatin:** The existence of distinct active and silent chromosomal domains in the eukaryotic genome poses the question of how each domain is confined to a limited region. Chromatin boundary or insulator elements have been found to demarcate some well-defined domains of gene regulation (West et al. 2002). These elements, by definition, can block the spread of heterochromatin into euchromatin and/or the activation of a promoter by an upstream enhancer. Increasing evidence indicates that DNA-binding proteins and their associated factors are involved in the function of chromatin boundary elements. One of the best-analyzed boundary/insulator elements is the chicken HS4 insulator at the β-globin locus. This insulator can block nearby condensed chromatin from invading into the β-globin locus. Recent studies showed that histones surrounding the HS insulator were hyperacetylated, indicating that HAT activity was targeted to the insulator (Litt et al. 2001a,b). Since the propagation of silent chromatin in chicken involves a chain of events of histone H3 deacetylation → H3 methylation → binding of methylated H3 by the HP1 protein, it was proposed that a targeted HAT counteracted histone deacetylation and therefore
block the spread of silent chromatin although the identity of the HAT and how it is targeted to the insulator are not known (Litt et al. 2001a). Our demonstration that a targeted HAT can create a sizable domain of histone acetylation and counteract transcriptional silencing supports the HAT model for insulator function. The yeast version of this model is illustrated in Figure 7. In accordance with this model it was recently demonstrated that the HAT Sas2p was required for restricting the spread of telomeric silent chromatin in yeast (Kimura et al. 2002; Suka et al. 2002). This was achieved by Sas2p’s positive regulation of histone H4-K16 acetylation, which counteracted Sir2p’s negative regulation of H4-K16 acetylation, although how Sas2p was targeted to sequences near the telomeres to carry out its boundary function remained to be determined. Over the past few years, various sequences with the ability to block the spread of silencing have been identified in yeast (Bi and Broach 2001; Donze and Kamakaka 2002; Yu et al. 2003). It will be interesting to determine if any of these sequences function as a barrier to silencing by attracting HAT complexes.

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