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

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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 *HM* 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

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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 (Carmen *et al.* 2001). It was recently shown that Sir2p was an 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 *Hin*dIII-*Bam*HI fragment of chromosome III (coordinates 14,838–16,263) inserted. The 1.1-kb *Bgl*II-*URA3*-*Bgl*II fragment of plasmid pFL44 (Chevallier *et al.* 1980) was inserted at the *Eco*RV site of pAR61 to make plasmid pMB22-a. A sequence containing a ColE1 operator (boldface type), TCTTACCTCGACTG**CTGTATATAAAACCAGTGGTTATAT GTACAG**TACGTCGAGGGATGATAATGC, was inserted at the *Sna*BI, *Ngo*MIV, and *Eag*I sites of pMB22-a to make plasmids pQY38, pQY39, and pQY37, respectively. The ColE1 operator contains two variants of the consensus sequence for LexA binding, CTGTATAT*NNANN*CAG, 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), GGGGTA CGTA**CTGTATGTACATACAG**GATATCGGGG, were inserted at the *Bam*HI site of pADH4.UCA4 (Sandell and Zakian 1993) to make pXB363. A sequence containing four tandem copies of the ColE1 operator [coordinates 271–450 of pSH18-34 (Gyuris *et al.* 1993)] was inserted at the *Eag*I site of pMB22-a to make plasmid pXB306. The same sequence was inserted at the *Bst*1107I site of pYXB61 (Bi and Broach 1999) to make plasmid pQY57. This sequence was also inserted at the *Spe*I 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 pBTM 116 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 pYC07 was made by replacing the *Xba*I-*Pvu*II fragment of pBTM116 with the *Xba*I-*LEU2*-*Pvu*II fragment of pRS425. The MCS sequence of pYC07 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 pKQL 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 *Bgl*I-*SIR3*-*Bgl*I fragment at the *Bgl*I site. Plasmids pRS425, pXB323, pXB301, pRQ12, pYC33, pYC34, and pYC35 were referred to as 1–7, respectively, for convenience (Figures 1–6). Plasmid pKQL was referred to as 4′.

Most yeast strains used in this study were derived from strain YXB76 [*MAT***a** *ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100* E-*HML*-(inverted I)] (Bi *et al.* 1999). Strain YXB85-new was made by transforming YXB76 to Ura^+ with the *Hin*dIII-*Bam*HI fragment containing the *URA3* gene from pMB22-a. Strains YQY10, YQY11, YQY09, and YXB227 were similarly constructed using plasmids pQY38, pQY39, pQY37, and pXB306, respectively. Strain YXB401 was made by transforming YXB76 to Ura^+ with the *Eco*RI-*Sal*I fragment from pXB363.

Strains YQY05 and YQY13 were made by transforming strain Y728 (Mahoney and Broach 1989) to Ura^+ with the *Eco*RI-*Pvu*II fragment of pQY58 and pQY57, respectively. Note that YXB76 is identical to Y728 except that the orientation of the *HML*-I silencer in it is inverted. Strain YQY54 was made by introducing the sequence $I_{inverted}$–*URA3*-ColE1-operator (same as in YQY11) into ResGen/Invitrogen strain 4282 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ada2Δ::kanMX*). Strain Y1838 was *MAT*α *ura3-52 leu2-3,112 his3*Δ *trp1-289* Δ(*hht1-hhf1*) Δ(*hht2-hhf2*) + plasmid pMS329 (*CEN-URA3-HHT1-HHF1*). YQY91 was made by introducing the construct $I_{inverted}$ –*URA3*-ColE1-operator (same as in YQY11) into Y1838 and replacing its pMS329 plasmid with pMP3-59b (*CEN-TRP1-HHT1-hhf2-H75Y*; Smith *et al.* 2002). Strain JJSy319 was made by replacing the *GCN5* gene in YXB85-new with the kanMX module. The relevant genotypes of these strains were confirmed by Southern blotting. Strain LPY3498 (*ESA1*$^+$) and its *esa1-L327S* derivative LPY2639 have been previously described (Clarke *et al.* 1999). For convenience, strains YXB85-new, YQY10 and YQY11, LPY3498, LPY3430, YXB401, YQY09, YXB227, YQY05, YQY13, YQY54, and YQY91 were denoted a–l, respectively (Figures 1–6). JJSy319 was denoted strain a′ (Figure 1E).

Western blotting: Yeast cells carrying a LexA fusion gene were grown in −Leu liquid medium at 30° to late log phase. −Leu medium was synthetic complete medium lacking leucine. Protein extract was prepared from $\sim 3 \times 10^8$ 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 NaH_2PO_4, 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 Opti-4CN substrate kit (Bio-Rad).

Chromatin immunoprecipitations: The method for chromatin immunoprecipitation was identical to a previously described protocol (Sandmeier *et al.* 2002) except for a few minor modifications. SC-Leu cultures (50 ml) of strain c bearing plasmid 2, 3, or 4 were grown to log phase (0.8–1.2 OD_{600}) and then fixed for 1 hr at room temperature (RT) in 1%

formaldehyde. Cells were harvested and washed twice with dH_2O 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 A_{260}, 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° 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°. The beads were washed extensively and the immune complexes were eluted twice with 200 μl 1% SDS/0.1 M $NaHCO_3$ at RT. The cross-links were then reversed at 65° for 5 hr in the presence of NaCl and ethanol precipitated overnight at −20°. 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 T_A was 50° 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 UVP (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 *HML*-I silencer defined the right (centromere-proximal) boundary of the silent *HML* domain by initiating silencing in only one direction (toward the *HMLα* genes; Bi *et al.* 1999). When we inverted the direction of the *HML*-I silencer, however, silencing could spread ~1.4 kb to the right of *HML*-I (Bi *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 *HML*-I 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-*ESA1* and LexA-*GCN5* 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 *esa1* temperature-sensitive (ts) alleles have been created (Clarke *et al.* 1999). Strain e carrying the *esa1-L327S* mutation grew normally at 30° but was inviable at 37° (Figure 1D, top, strain e, compare 30° and 37° growth). When LexA-*ESA1* was introduced into strain e, cells grew normally at 37°, 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 a′ 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, −Leu + FOA plot, compare 3 and 4 to 2).

Targeted LexA-Esa1p or LexA-Gcn5p can counteract silencing at a distance: In the above experiments and other similar studies on the anti-silencing effect of a tethered HAT, each HAT was targeted close to the silencer, close to the promoter of the reporter gene, or both. Therefore, it is not clear if the anti-silencing effect of tethered HAT is a localized effect on the silencer or the promoter. To address this question, we tested if LexA-Esa1p and LexA-Gcn5p could counteract silencing when targeted to sites that are far from the silencer and the promoter of *URA3*. In strain c, two LexA-binding sites were inserted 1.2 kb downstream of the promoter of *URA3* and 2 kb from the *HML*-I silencer (Figure 3A). In this strain, it was obvious that LexA-Esa1p and LexA-Gcn5p completely eliminated *URA3* silencing (Figure 3A, strain c, compare rows 3 and 4 to 2). Moreover, 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.

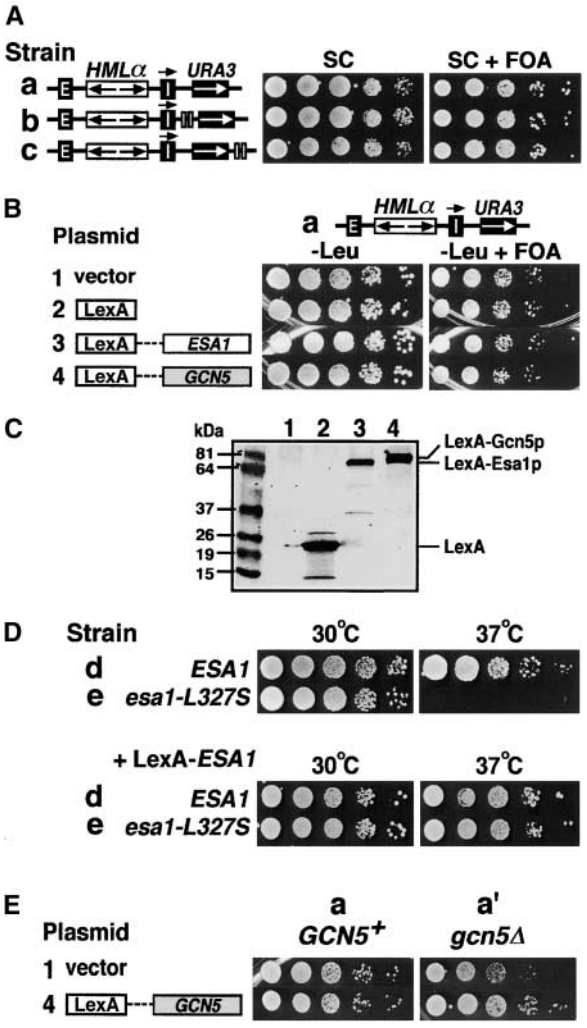

Figure 1.—Assay for testing the effect of a targeted HAT on transcriptional silencing. (A) Insertion of LexA-binding sites near *HML* did not affect cell growth or *HML* silencing. In strain a (YXB85-new), the direction of the *HML*-I silencer was flipped and the *URA3* gene was inserted to the right 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°. 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° and 37°, respectively. Bottom, growth phenotypes of strains d and e bearing plasmid 3 (LexA-*ESA1*) on −Leu medium at 30° and 37°, respectively. (E) LexA-*GCN5* complemented the slow-growth phenotype of *gcn5*Δ. Growth phenotypes of stain a′ (JJSy319) bearing plasmids 1 and 4, respectively, were shown. Also shown were growth phenotypes of stain a bearing plasmids 1 and 4, respectively. Medium used was −Leu.

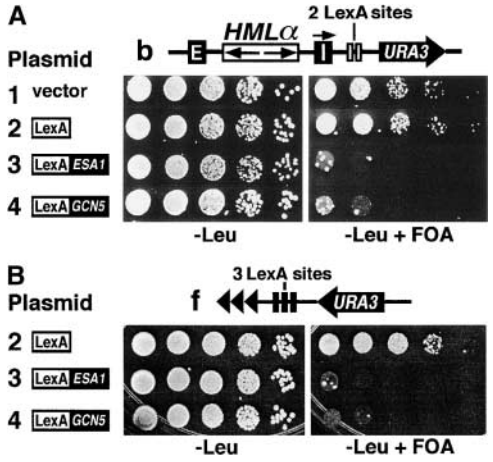

FIGURE 2.—Targeted LexA-Esa1p or LexA-Gcn5p could counteract transcriptional silencing. (A) Growth phenotypes of strain b bearing plasmids 1–4, respectively, on −Leu medium (left) and −Leu + FOA medium (right) were shown. (B) Growth phenotypes of strain f (YXB401) bearing plasmids 2–4, respectively.

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 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* (BI *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 know if the enzymatic activity of a HAT was necessary for its anti-silencing function. To answer this question, we mutated *GCN5* in the LexA-*GCN5* fusion gene so that its product no longer possessed HAT activity. Specifically, the mutagenized

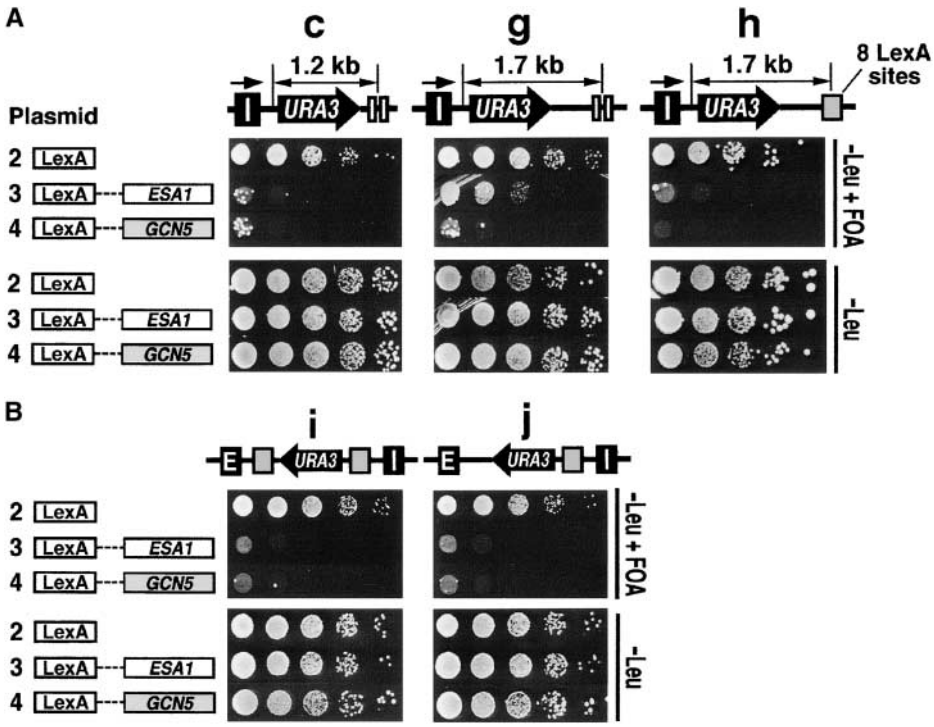

FIGURE 3.—Targeted LexA-Esa1p or LexA-Gcn5p can counteract silencing at a distance. (A) Growth phenotypes of strains c, g (YQY09), and h (YXB227) bearing plasmids 2–4, respectively. In strains c and g, two LexA sites (open bars) were inserted 1.2 and 1.7 kb downstream of the *URA3* promoter, respectively. 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*-I.

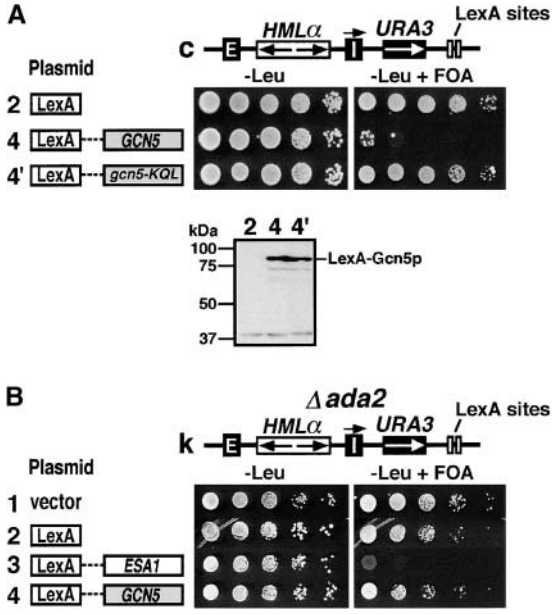

FIGURE 4.—The anti-silencing effect of tethered Gcn5p was abolished by the *gcn5-KQL* mutation or deletion of *ADA2* encoding a component of Gcn5p-containing HAT complexes. (A) Top, growth phenotypes of strain c bearing plasmids 2, 4, and 4′, respectively. Plasmid 4′ (pKQL) bore a LexA-*gcn5-KQL* fusion gene. Bottom, Western-blotting analysis of LexA-fusion proteins encoded by LexA-*GCN5* and LexA-*gcn5-KQL*. Twenty micrograms of total protein from cells of strain c bearing plasmids 2, 4, and 4′, respectively, was analyzed by SDS-PAGE followed by Western blotting and probed with an anti-LexA antibody. Bands corresponding to LexA-Gcn5p were indicated. (B) Growth phenotypes of strain k (YQY54) bearing plasmids 1–4, respectively, were shown. Note that strain k had its *ADA2* gene replaced by kanMX.

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 functions as part of the SAGA or ADA complex, each containing Ada2p as a structural component that is required for the function of the complex. The SAGA complex, as a transcriptional co-activator, can be directed to specific loci in the genome via direct interactions between DNA-bound transcriptional activators and Tra1p, another component of the SAGA complex (BROWN *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 underlies 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

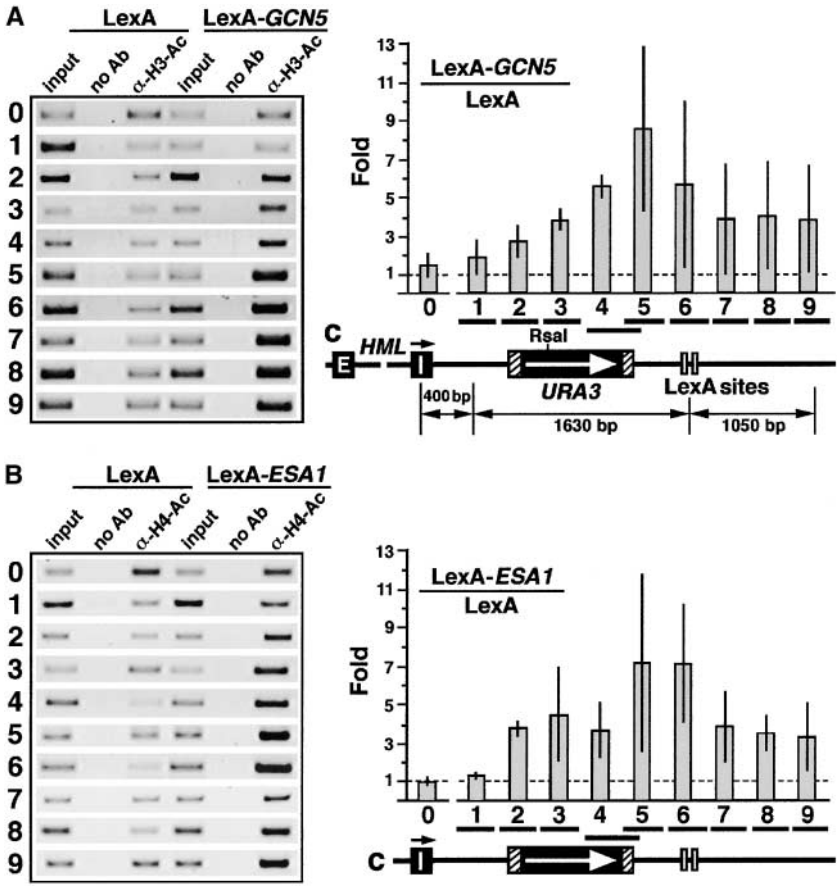

FIGURE 5.—A targeted HAT created a sizable domain of histone hyperacetylation. (A) Domain of histone H3 acetylation generated by LexA-Gcn5p. Left, chromatin-immunoprecipitation from strain c bearing plasmids 2 (LexA) and 4 (LexA-*GCN5*), respectively. The antibody used, designated α-H3-Ac, was specific 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.

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 for unknown reasons.

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 can 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.

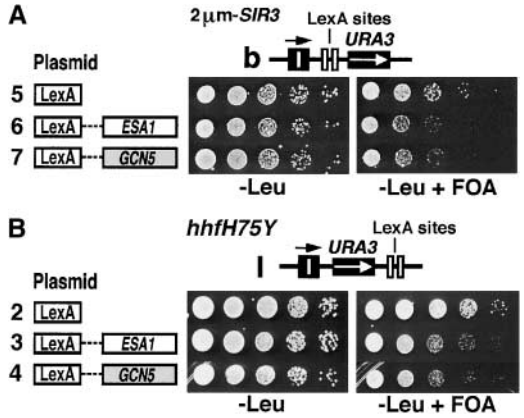

FIGURE 6.—Enhanced silent chromatin could overcome the anti-silencing effect of a HAT. (A) Effect of overexpressing *SIR3* on the anti-silencing effect of LexA-HAT. Plasmids 5 (pYC33), 6 (pYC34), and 7 (pYC35) were identical to plasmids 2–4 (2-μm based), respectively, except for bearing an additional copy of the *SIR3* gene. Therefore, strain b with plasmid 5, 6, or 7 in it carried an additional ectopic 2-μm *SIR3*. Its growth phenotypes were shown. Note that 2-μm *SIR3* exhibited some toxicity to cell growth (HOLMES *et al.* 1997). (B) Effect of the *hhfH75Y* mutation on the anti-silencing effect of LexA-HAT. Strain l (YQY91) was similar to strain c but had the *hhfH75Y* gene as the sole copy of the histone H4 gene. Growth phenotypes of strain l carrying plasmid 2, 3, or 4 were shown.

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, *hhf-H75Y*, 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; LARSCHAN and WINSTON 2001), and the Sin3p-Rpd3p HDAC complex is targeted to promoters by the Ume6p repressor (KADOSH 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 KAMAKAKA 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-chromatin interactions, is thought to help them contact nucleosome substrates (Roth *et al.* 2001). In fact, Hassan *et al.* (2002) has recently shown that the SAGA complex was retained on both H3- and H4-acetylated nucleosome arrays after the removal of the activator that first recruited it to DNA. The bromodomain of Gcn5p was required for this retention. In contrast, the NuA4 complex, which lacks a bromodomain, was not retained following activator removal. On the basis of these results, it was suggested that the SAGA complex binds to acetylated nucleosomes through the bromodomain of Gcn5p, thus providing a self-perpetuating epigenetic mark tethered to a small chromatin domain (Turner 2002), similar to the proposed self-perpetuated spread of silencing complexes through histone methylation or deacetylation (Moazed 2001; Czermin *et al.* 2002; Müller *et al.* 2002).

Three different models for how a targeted HAT establishes broad acetylation patterns have been proposed (Forsberg and Bresnick 2001). The first model (referred to here as the looping model) suggests that a HAT complex recruited to a regulatory element engages 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

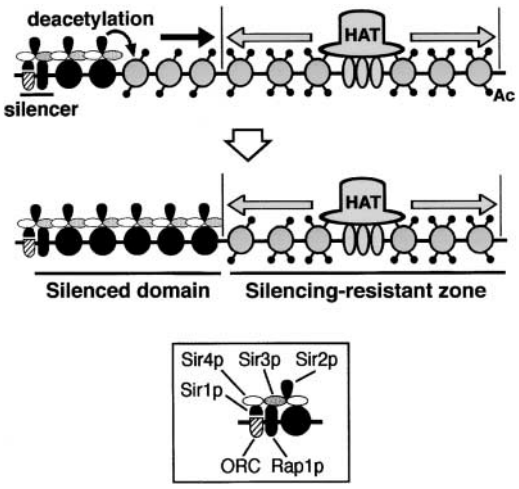

FIGURE 7.—Targeted HAT can create a hyperacetylated zone and counteract the spread of silent chromatin. Initiation of the formation of silent chromatin involves the binding of silencer-binding proteins (Rap1p and ORC complex) to a silencer and subsequent recruitment of Sir1p and the SIR complex (consisting of Sir2p, Sir3p, and Sir4p). Sir2p at the silencer deacetylates a nearby nucleosome. The hypoacetylated nucleosome (solid circle) then attracts a second SIR complex. Repetition of this process leads to the spread of silent chromatin (indicated by the solid arrow). A tethered HAT generates a zone of histone hyperacetylation on both sides (shaded arrows) and blocks the spread of transcriptional silencing. Shaded oval, DNA-binding protein that can recruit a HAT; shaded circles, acetylated nucleosomes. The inset illustrates various interactions among silencer-binding proteins, the SIR proteins, and the nucleosome.

blocked 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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