MOLECULAR AND CELLULAR BIOLOGY, Sept. 1998, p. 5121–5127 Vol. 18, No. 9
0270-7306/98/$04.00+0

Targeted Recruitment of the Sin3-Rpd3 Histone Deacetylase Complex Generates a Highly Localized Domain of Repressed Chromatin In Vivo

DAVID KADOSH† AND KEVIN STRUHL*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received 15 May 1998/Returned for modification 3 June 1998/Accepted 16 June 1998

Eukaryotic organisms contain a multiprotein complex that includes Rpd3 histone deacetylase and the Sin3 corepressor. The Sin3-Rpd3 complex is recruited to promoters by specific DNA-binding proteins, whereupon it represses transcription. By directly analyzing the chromatin structure of a repressed promoter in yeast cells, we demonstrate that transcriptional repression is associated with localized histone deacetylation. Specifically, we observe decreased acetylation of histones H3 and H4 (preferentially lysines 5 and 12) that depends on the DNA-binding repressor (Ume6), Sin3, and Rpd3. Mapping experiments indicate that the domain of histone deacetylation is highly localized, occurring over a range of one to two nucleosomes. Taken together with previous observations, these results define a novel mechanism of transcriptional repression which involves targeted recruitment of a histone-modifying activity and localized perturbation of chromatin structure.

Although it has been known for more than 3 decades that histone acetylation is associated with transcriptional activity in eukaryotic cells (2, 27), the causal relationship and the underlying molecular mechanisms have been elusive. The recent identification of proteins with intrinsic histone acetylase and deacetylase activities has dramatically enhanced our understanding by providing a critical link between chromatin structure and transcriptional output (for recent reviews, see references 11, 26, 32, and 34). Some histone acetylases are intrinsic components of the basic RNA polymerase II (Pol II) machinery or are closely associated with this machinery. In essence, therefore, the transcription machinery (broadly defined) contains histone acetylase activity, which suggests a mechanism for the general correlation between histone acetylation and transcriptional activity. In this regard, *Saccharomyces cerevisiae* Gcn5 histone acetylase (8), the enzymatic component of the SAGA complex that functionally interacts with TBP (10), specifically acetylates histones in the vicinity of the promoter in vivo in a manner that is correlated with Gcn5-dependent transcriptional activity (20).

Some histone-modifying activities interact with DNA-binding activator or repressor proteins, suggesting that they modulate transcriptional activity of specific promoters by locally perturbing chromatin structure. For example, the p300/CBP histone acetylase (4, 25) interacts with numerous activator proteins (17), and the ACTR and SRC-1 histone acetylases associate with nuclear receptors in a hormone-dependent manner (9, 31). These proteins acetylate histones in vitro and function as transcriptional coactivators in vivo, but it is unknown whether histones are physiological substrates or whether the chromatin structure of the relevant target genes is locally affected. The Ada2 component(s) of Gcn5 histone acetylase complexes can interact with acidic activation domains in vitro (30), and this interaction might contribute to promoter-specific histone acetylation in vivo (20).

The yeast and mammalian Sin3-Rpd3 histone deacetylase complexes mediate transcriptional repression by interacting with specific DNA-binding proteins (e.g., Ume6, YY1, and Mad) or associated corepressors (NCoR, SMRT, and Rb) and being recruited to target promoters (1, 7, 13, 15, 18, 21–24, 35, 36). In yeast, the Sin3-Rpd3 complex is required for transcriptional repression by Ume6, a zinc finger protein that binds URS1 elements and regulates genes involved in meiosis and arginine catabolism (18). A short region of Ume6 interacts directly with the Sin3 corepressor, and this region is necessary and sufficient for recruitment of the complex to promoters and for transcriptional repression. The Sin3-Rpd3 complex is not required for the function of other transcriptional repressors (Tup1 and Acr1) under equivalent experimental conditions, indicating that repression by Sin3-Rpd3 requires recruitment to target promoters (18). Yeast Rpd3 can deacetylate histones H3 and H4 in vivo (28), and histone deacetylase activity is important for repression; Rpd3 mutants that are catalytically impaired in vitro but competent for Sin3-Rpd3 complex formation are severely or completely defective for transcriptional repression in vivo (19). These observations strongly suggest that transcriptional repression occurs by targeted histone deacetylation and the establishment of a locally repressive chromatin structure. However, little is known about the nature or extent of the locally perturbed chromatin domain in vivo.

In this work, we utilize the technique of chromatin immunoprecipitation (3, 6, 14, 20) to analyze the chromatin structure of a repressed promoter in vivo. We demonstrate that transcriptional repression is associated with localized deacetylation of histones H3 and H4 (preferentially lysines 5 and 12) and that histone deacetylation occurs over a limited range of one to two nucleosomes. These findings are consistent with a recent report that appeared after the present work was initially submitted (29). Taken together with previous observations, these results define a novel mechanism of transcriptional repression which involves targeted recruitment of a histone-modifying activity and a localized domain of modified chromatin structure.

* Corresponding author. Mailing address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115. Phone: (617) 432-2104. Fax: (617) 432-2529. E-mail: kevin@hms.harvard.edu.

† Present address: Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

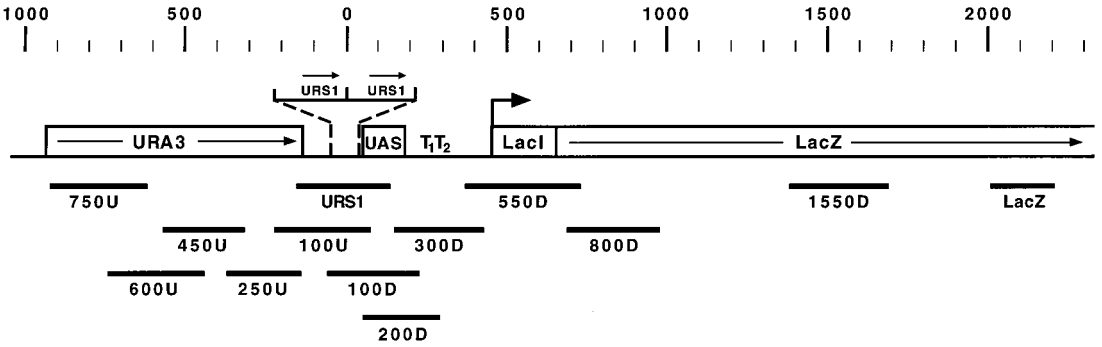

FIG. 1. Promoter structure. The promoter used in these experiments has two copies of a URS1-containing fragment from the *IME2* promoter upstream of the *CYC1* promoter (UAS, TATA elements T_1 and T_2, and mRNA initiation site [bent arrow] indicated), and it drives expression of a *LacI-LacZ* fusion gene. As described previously, *LacZ* expression is repressed in a manner dependent on the URS1 elements Ume6, Sin3, and Rpd3 (18, 19). The region upstream of this promoter contains sequences from the *URA3* gene, which serves as the plasmid marker. Shown below the promoter structure are the regions (typically 300 bp, with the upstream and downstream boundaries being defined by a pair of PCR primers) that are analyzed by the chromatin immunoprecipitation procedure. The regions labeled URS1 and *LacZ* are analyzed in Fig. 2 and 3, whereas the upstream (U) and downstream (D) regions analyzed in Fig. 4 are defined by the approximate number of base pairs from the center of the URS1 elements to the center of the indicated region.

MATERIALS AND METHODS

Yeast strains. The isogenic wild-type (FT5), Δ*rpd3::HIS3*, Δ*sin3::HIS3*, and Δ*ume6::LEU2* strains (18, 33) and the *LacZ* reporter plasmid, pLGΔ312S, containing two URS1 elements from the *IME2* promoter located upstream of the *CYC1* upstream activated sequence (UAS) and TATA region (12, 18) have been described previously.

Chromatin preparation. Chromatin was prepared by a procedure similar to that described previously (5). Wild-type and deletion strains bearing the $URS1^{IME2}$ plasmid described above were grown overnight in 100 ml of glucose-minimal medium with Casamino Acids to an optical density at 600 nm of ~0.5. Formaldehyde was added to 1% final concentration, and the cells were incubated at room temperature, with gentle swirling, for 20 min. Cells were resuspended in 5 ml of 0.1 M Tris (pH 9.4)–10 mM dithiothreitol, placed on ice for 20 min, washed with 5 ml of 20 mM HEPES (pH 7.4)–1.2 M sorbitol, and resuspended in 5 ml of the same HEPES-sorbitol solution with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 40 μl of yeast-lytic enzyme (1 mg/ml). After incubation at 30°C for 30 min, 10 ml of 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8)–1 mM $MgCl_2$–1 mM sorbitol was added and the cells were immediately spun down. Spheroplasts were washed three times, sequentially, with 5 ml of ice-cold phosphate-buffered saline–0.5 mM PMSF, 5 ml of ice-cold 0.25% Triton X-100–10 mM EDTA–0.5 mM EGTA–10 mM HEPES (pH 6.5)–0.5 mM PMSF–pepstatin A (0.8 μg/ml), and 5 ml of ice-cold 200 mM NaCl–1 mM EDTA–0.5 mM EGTA–10 mM HEPES (pH 6.5)–0.5 mM PMSF, pepstatin A (0.8 μg/ml). Spheroplasts were resuspended in 1 ml of 1% sodium dodecyl sulfate (SDS)–10 mM EDTA–50 mM Tris (pH 8.1)–1 mM PMSF–pepstatin A (0.8 μg/ml) and sonicated eight times for 20 s (with 5 min on ice between sonications); fragment DNA sizes ranged from 180 to 550 bp, with the average size being approximately 350 bp. After microcentrifugation for 10 min at 15,000 rpm, the supernatant was diluted with 10 ml of IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8.1], 167 mM NaCl, 1 mM PMSF, pepstatin A [0.8 mg/ml]). This chromatin solution was used for subsequent immunoprecipitations.

Chromatin immunoprecipitation. Immunoprecipitations were carried out by a procedure similar to that described previously (5), with antibodies to specific acetylated lysine residues of histone H4 (Serotec) or antibodies to generally acetylated or unacetylated histone H3 (kindly provided by C. D. Allis). Chromatin solution (0.5 ml) was combined with the following volumes of antisera: 11.5 μl of acetylated H4 lysine 5, 7.5 μl of acetylated H4 lysine 8, 21 μl of acetylated H4 lysine 12, 23 μl of acetylated H4 lysine 16, 6.3 μl of acetylated histone H3, and 23 μl of unacetylated histone H3 antisera. Immunoprecipitations were carried out at 4°C overnight (with rotation), and immune complexes were harvested by the addition of 0.66 μg of sonicated bacteriophage λ DNA and 13.3 μl of protein A-Sepharose beads (50% slurry in Tris-EDTA [TE]–0.1% bovine serum albumin), followed by incubation at room temperature for 2 h. The beads were then washed, sequentially, with 0.33 ml of the following buffers: twice with TSE-150 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), once with 0.25 M LiCl, 1% Nonidet P-40), 1% deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8.1), and twice with TE. Immune complexes were eluted with 500 μl of 1% SDS–0.1 M $NaHCO_3$.

Formaldehyde cross-links were reversed by the addition of 20 μl (for immunoprecipitates) or 2.5 μl (for 0.3 ml of total chromatin solution) of 5 M NaCl and incubation at 65°C for 5 h. DNA was ethanol precipitated overnight, resuspended in 100 μl of TE, and treated with 1.5 μl of proteinase K (18.6 mg/ml) (42°C, 2 h). Following extraction with phenol-chloroform-isoamyl alcohol and chloroform, DNA was ethanol precipitated overnight in the presence of 5 μg of glycogen. DNA recovered from the immunoprecipitates was resuspended in 50 μl of TE. Total chromatin was resuspended in 300 μl of TE.

Quantitation of immunoprecipitated DNA. Amounts of DNA present in the immunoprecipitates and total chromatin were determined by quantitative PCR. Each PCR mixture contained two primer sets: one corresponding to the *LacZ* internal control (located in the *LacZ* coding sequence, 2.1 kb downstream from the URS1 sites) and a second corresponding to a test region. The *LacZ* internal control primers generated a fragment of 206 bp, whereas the test primer sets yielded PCR products with lengths ranging between 260 and 310 bp. PCRs were first performed with decreasing concentrations of template to determine the linear range for each combination of primer sets and DNA (typically 1 μl of a 1/3 or 1/9 dilution of the immunoprecipitated DNA or 1 μl of a 1/27 dilution of total chromatin was in the linear range). All subsequent reactions were carried out with templates prediluted to the linear range. Following 26 cycles of PCR, fragments were resolved on a 2.5% agarose gel stained with ethidium bromide. Photographs of the stained gels were scanned directly into Canvas 5.0, and bands were quantitated by using Image Gauge (version 3.0). Values were calibrated to standards containing known quantities of DNA.

For all experiments the ratio of test PCR product to *LacZ* internal control PCR product was determined (note that this ratio can vary depending on the particular primer set combinations used). These ratios are normalized, as described in the captions to Tables 1 and 2, to allow for a comparison of the amount of immunoprecipitated DNA from wild-type and deletion strains. The absolute values of the band intensities reflect the amounts of input DNA in each PCR mixture and hence are irrelevant to the analysis.

RESULTS

Experimental strategy. In a previous work (18), we characterized a promoter in which two copies of the URS1 element from the *IME2* promoter were located upstream of the intact *CYC1* promoter and *LacZ* structural gene (Fig. 1). In wild-type strains, the URS1 elements repress transcription from this promoter by a factor of 13; repression is virtually abolished in *ume6* and *sin3* deletion strains and significantly reduced in an *rpd3* deletion strain. Because this promoter is well defined and has served as part of the basis for elucidating the repression mechanism involving Ume6 recruitment of the Sin3-Rpd3 histone deacetylase complex, we directly analyzed its chromatin structure in yeast cells.

The histone acetylation status of this promoter was analyzed by a chromatin immunoprecipitation procedure (3, 6, 14, 20). Isogenic wild-type, *ume6*, *sin3*, and *rpd3* strains containing this promoter were treated with formaldehyde to cross-link proteins to DNA. Following fragmentation of the DNA to an average length of 350 bp, protein-DNA complexes were immunoprecipitated with appropriate antibodies, and the resulting DNA was analyzed by quantitative PCR. Each PCR mixture contained two probe pairs; one of these corresponded to a region of the promoter, whereas the other corresponded to a

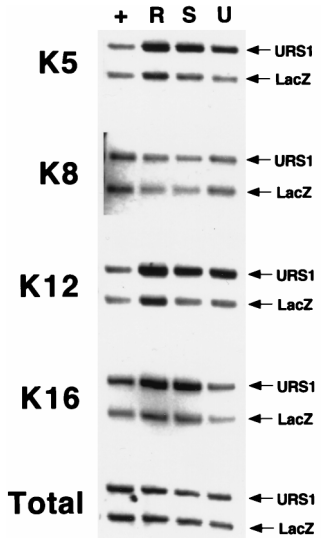

FIG. 2. Acetylation status of individual lysines of histone H4. Cross-linked and fragmented chromatin preparations from wild-type (+), *rpd3* (R), *sin3* (S), and *ume6* (U) strains were immunoprecipitated with the antibodies to acetylated histone H4 isoforms of lysines (K) 5, 8, 12, and 16 or were analyzed prior to immunoprecipitation (Total). Recovered DNA was analyzed by quantitative PCR; for each determination, the reaction mixture contained primers both for the region corresponding to URS1 and for the region corresponding to the *LacZ* structural gene (Fig. 1). Because individual PCRs are internally controlled, the relative level of histone acetylation in the URS1 is defined with respect to the level of histone acetylation within the *LacZ* region. These data are quantitated in Table 1 and expressed as the URS1/*LacZ* ratio of band intensities of the PCR fragments; the absolute level of band intensities reflects the amount of input DNA in each reaction mixture and is irrelevant to the analysis.

region of the *LacZ* structural gene located approximately 2 kb downstream. For each case, titration experiments were performed to ensure that reactions were in the linear range, i.e., the amounts of the two PCR products were proportional to the amount of input DNA. In this way, PCRs were internally controlled and the determinations were quantitatively reliable and unaffected by variations in plasmid copy number. The relative level of histone acetylation at the promoter is defined by the ratio of the amount of the promoter fragment to that of the *LacZ* fragment produced in the same PCR.

Transcriptional repression is associated with promoter-specific deacetylation of histone H4. The amino-terminal tail of histone H4 has four lysines (residues 5, 8, 12, and 16) that are potential substrates for acetylation. Analysis of bulk chromatin in wild-type and *rpd3* deletion strains indicates that Rpd3 deacetylates histone H4 with some specificity for the individual lysines; the effect of Rpd3 is strongest at lysines 5 and 12, moderate at lysine 16, and minimal at lysine 8 (28). In the initial experiments, cross-linked and fragmented chromatin was immunoprecipitated with antibodies recognizing acetylated forms of histone H4 that are specific for individual lysines (Fig. 2; data quantitated in Table 1). The promoter probe is approximately 300 bp long, with the URS1 elements being centrally located.

Analyses with antibodies to acetylated lysines 5 and 12 reveal a notable difference between wild-type and mutant strains at the promoter. Specifically, when normalized to the *LacZ* internal control fragment, the amount of promoter fragment from wild-type strains is twofold lower than the amounts in *ume6*, *sin3*, and *rpd3* strains. Thus, the promoter region is relatively deacetylated at lysines 5 and 12 of histone H4 in wild-type strains. Similar results are observed for lysine 16, although the difference between wild-type and mutant strains (1.6-fold) is less pronounced. In contrast, analysis with antibodies to acetylated lysine 8 indicated that the four strains behaved similarly. Indeed, the ratios of promoter to *LacZ* fragment in this case are comparable to that observed with total chromatin prior to immunoprecipitation.

TABLE 1. Relative histone acetylation of the promoter[a]

Antibody	Yeast strain			
	Wild type	*rpd3*	*sin3*	*ume6*
None	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
H4 lysine 5	1.0 ± 0.1	2.2 ± 0.5	2.3 ± 0.1	2.1 ± 0.3
H4 lysine 8	0.8 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
H4 lysine 12	1.5 ± 0.1	2.7 ± 0.1	2.9 ± 0.3	3.0 ± 0.2
H4 lysine 16	1.5 ± 0.3	2.4 ± 0.1	2.3 ± 0.4	2.3 ± 0.3
H3 acetylated	0.9 ± 0.1	1.9 ± 0.3	2.4 ± 0.3	2.0 ± 0.1
H3 unacetylated	3.6 ± 0.5	2.7 ± 0.2	2.3 ± 0.1	1.7 ± 0.2

[a] Each entry represents analysis of cross-linked and fragmented chromatin from the indicated yeast strain that was immunoprecipitated with the antibodies to unacetylated or acetylated isoforms (individual lysines indicated) of histone H4 or histone H3; "None" indicates total chromatin. Each PCR mixture contained primers for the promoter region (URS1 in Fig. 1) and primers for the internal *LacZ* control; each value represents the molar ratio (average of two determinations ± error) of the promoter region to the *LacZ* region for that specific PCR mixture. A value of 1.0 indicates equimolar amounts of the promoter and *LacZ* fragments (as determined on total chromatin). The promoter region shows a slight preference for acetylated lysines 12 and 16 of histone H4 in the wild-type strain.

These results indicate that transcriptional repression is associated with decreased acetylation of histone H4 within the promoter region. The pattern of promoter-specific histone deacetylation (strongest effects at lysines 5 and 12, a moderate effect at lysine 16, and a minimal effect at lysine 8) is in excellent accord with the pattern previously observed with bulk chromatin in yeast cells (28).

Transcriptional repression is associated with promoter-specific deacetylation of histone H3. In addition to its ability to deacetylate specific residues of histone H4, Rpd3 also deacetylates histone H3 at lysines 9 and 14 (28). To analyze the acetylation status of histone H3, we carried out immunoprecipitation with antibodies to generally acetylated histone H3 tails. As shown in Fig. 3 (data quantitated in Table 1), the relative amount of promoter fragment in the wild-type strains is decreased twofold from that observed in mutant strains, indicating that the promoter region is relatively deacetylated at

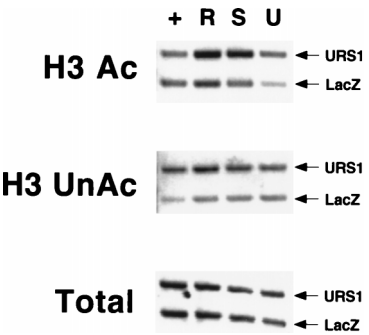

FIG. 3. Acetylation status of histone H3. Cross-linked and fragmented chromatin preparations from wild-type (+), *rpd3* (R), *sin3* (S), and *ume6* (U) strains were immunoprecipitated with the antibodies to generally acetylated (Ac) histone H3 or to nonacetylated (UnAc) H3; as a control, the analysis was performed prior to immunoprecipitation (Total). Recovered DNA was analyzed by quantitative PCR as described in the legend to Fig. 2.

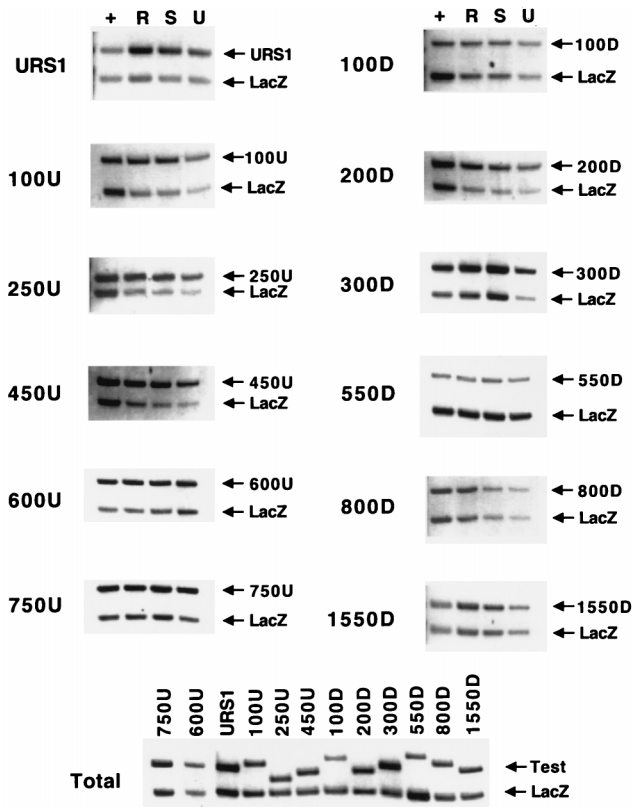

FIG. 4. Mapping the domain of localized histone deacetylation. Cross-linked and fragmented chromatin preparations from wild-type (+), *rpd3* (R), *sin3* (S), and *ume6* (U) strains were immunoprecipitated with the antibodies to histone H4 acetylated at lysine 5, and recovered DNA was analyzed by quantitative PCR. For each determination, the reaction mixture contained primers both for the indicated promoter (or flanking) region and for the internal control region corresponding to the *LacZ* structural gene (Fig. 1). Because individual PCRs are internally controlled, the relative level of histone acetylation in the indicated region is defined with respect to the level of histone acetylation within the *LacZ* region. These data are quantitated in Table 2 and expressed as the ratio of band intensities of the PCR fragments; the values are normalized to that obtained with the wild-type strain, which is defined as 1.0. The absolute level of band intensities reflects the amount of input DNA in each reaction mixture and is irrelevant to the analysis.

TABLE 2. Mapping the domain of repressed chromatin[a]

Promoter region	Yeast strain			
	Wild type	*rpd3*	*sin3*	*ume6*
750U	1.0	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.1
600U	1.0	1.3 ± 0.1	1.3 ± 0.4	1.6 ± 0.8
450U	1.0	1.6 ± 0.1	1.7 ± 0.5	3.0 ± 0.5
250U	1.0	2.1 ± 0.1	2.8 ± 0.2	3.1 ± 0.2
100U	1.0	2.4 ± 0.2	3.1 ± 0.1	3.3 ± 0.4
URS1	1.0	2.9 ± 0.1	2.5 ± 0.2	2.3 ± 0.7
100D	1.0	2.5 ± 0.3	2.6 ± 0.3	2.9 ± 0.2
200D	1.0	1.6 ± 0.1	1.8 ± 0.1	2.0 ± 0.2
300D	1.0	1.1 ± 0.2	0.9 ± 0.1	1.8 ± 0.1
550D	1.0	0.8 ± 0.1	0.9 ± 0.2	1.1 ± 0.3
800D	1.0	1.1 ± 0.1	0.9 ± 0.2	1.2 ± 0.1
1550D	1.0	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1

[a] Each entry represents analysis of cross-linked and fragmented chromatin from the indicated yeast strain that was immunoprecipitated with the antibodies to acetylated lysine 5 of histone H4. Each PCR mixture contained primers for the indicated part of the promoter region (see Fig. 1) and primers for the internal *LacZ* control; each value represents the ratio (average of two determinations ± error) of the promoter region to the *LacZ* region for that specific PCR mixture. Values are normalized to that obtained with the wild-type strain, which is defined as 1.0.

histone H3 in wild-type strains. As a control for this experiment, we analyzed chromatin immunoprecipitated with antibodies to nonacetylated tails of histone H3. As expected, the relative level of the promoter fragment was higher in the wild-type strain than in the mutant strains, providing independent evidence for decreased acetylation dependent on Ume6, Sin3, and Rpd3. Taken together, these experiments indicate that transcriptional repression is associated with promoter-specific deacetylation of histone H3.

Domain of localized histone deacetylation. The experiments described above indicate that, under conditions of transcriptional repression, histones H3 and H4 are preferentially deacetylated within a 288-bp region centered at the URS1 elements. To map the domain of localized histone deacetylation, we analyzed additional regions that either overlapped or flanked the region examined above (Fig. 1). These analyses were performed with chromatin immunoprecipitated with the antibody to acetylated lysine 5 of histone H4, and they utilized the *LacZ* fragment as an internal control.

As shown in Fig. 4 (data quantitated in Table 2), regions centered as far as 450 bp upstream or 200 bp downstream of the URS1 elements were preferentially deacetylated at lysine 5 of histone H4 under conditions of transcriptional repression. When normalized to the internal *LacZ* control, the relative intensities of bands corresponding to the probe regions were two- to threefold lower in the wild-type strain than in the mutant strains. In contrast, regions centered ≥600 bp upstream or ≥550 bp downstream of the promoter behaved indistinguishably in all four strains, indicating that histone acetylation in these regions was unaffected by transcriptional repression. A region centered 300 bp downstream of the URS1 elements showed a marginal, and possibly insignificant, effect (only observed in the *ume6* strain).

These results indicate that the apparent domain of localized histone deacetylation extends ~450 bp upstream and ~200 bp downstream from the URS1 elements. However, to map the actual domain of localized histone deacetylation, it is necessary to consider the lengths of the fragmented chromatin and the PCR product. In Fig. 5, we provide a theoretical method for determining the extent of the actual domain; the only assumption of this method is that chromosomal fragmentation by sonication occurs with no sequence specificity. Consider the situation of an actual domain of 1 bp (defined here as position 0), chromatin fragments of 400 bp, and a PCR product of 300 bp. If the PCR product is centered at position 0, there are 100 distinct fragments that contain the actual domain (i.e., position 0) and hence can be used as the template to generate the product. A similar result is obtained with 300-bp PCR products centered as far as position +150 or −150, indicating that an actual domain of 1 bp would correspond to an apparent domain of 300 bp. In fact, the apparent domain extends further in both directions, because 50 distinct fragments containing position 0 would be identified with PCR products centered at ±200; i.e., the apparent domain "signal" at ±200 is half maximal. Calculations for this and related situations that differ only in chromatin fragment size (ranging from 350 to 550 bp) are presented graphically in Fig. 5.

In the actual experiment depicted in Fig. 4, chromatin fragments ranged from 180 to 550 bp, with an average of 350 bp, and PCR products ranged from 260 to 310 bp. We estimated the relative molar amounts of DNA fragments in 50-bp intervals (i.e., 300, 350, 400, and so on to 550) by ethidium bromide

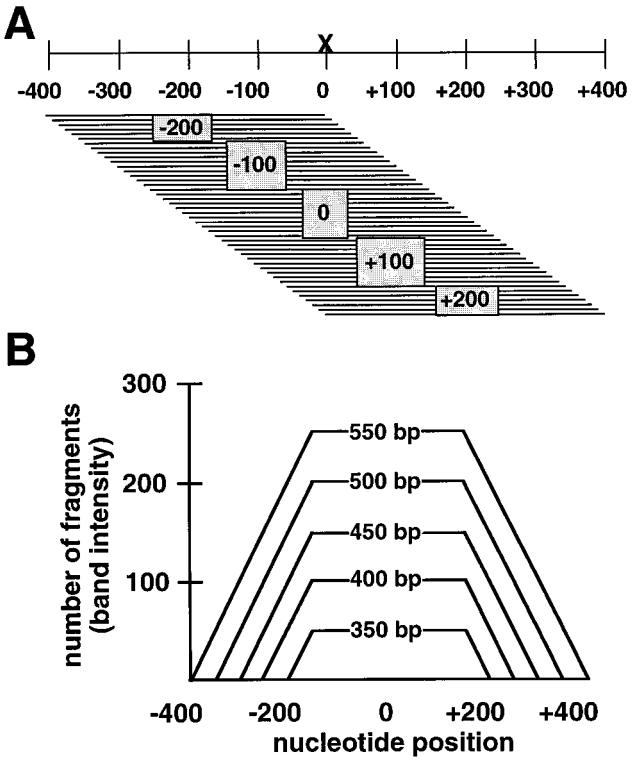

FIG. 5. Theoretical approach for determining the extent of the actual domain. (A) The diagrammed situation contains an actual domain of 1 bp (X) located at position 0 (shown within a region that extends from −400 to +400). Horizontal lines below the coordinate scale indicate 400-bp chromosomal DNA fragments that contain position 0; there are 400 such fragments. The subset of DNA fragments that are detectable as 300-bp PCR fragments (defined by the central position of the PCR fragment) are indicated by the shaded boxes. (B) The graphs represent various situations in which the length of the chromosomal fragments (350 to 550 bp) is indicated; in all cases, the PCR fragments are 300 bp. The number of distinct chromosomal DNA fragments containing position 0 that can be detected by 300-bp PCR fragments (*y* axis) is shown as a function of the central position of the PCR fragment (*x* axis). For any PCR fragment (as defined by the location of the central base pair), the number of distinct chromosomal DNA fragments is directly related to the expected experimental signal. This approach assumes that chromosomal fragmentation is random with respect to nucleotide position.

staining of the fragmented chromatin sample and used this information to normalize the calculated data in Fig. 5. Given these parameters, an actual domain of 1 bp would give an apparent domain of 400 to 500 bp. Assuming that the domain of localized histone deacetylation is contiguous, the observed domain of approximately 650 bp corresponds to an actual domain of approximately 150 to 250 bp or a region of one to two nucleosomes.

DISCUSSION

Targeted recruitment of the Sin3-Rpd3 complex causes localized histone deacetylation in vivo. In a previous work, we demonstrated that the URS1-binding protein Ume6 represses transcription by recruiting the Sin3-Rpd3 histone deacetylase complex to promoters; conversely, repression by the Sin3-Rpd3 complex does not occur unless it is targeted to specific promoters (18). Similarly, a variety of mammalian DNA-binding repressors or corepressors inhibit transcription by recruiting a related Sin3-Rpd3 complex (1, 7, 13, 15, 18, 21–24, 35). Further, histones are physiological substrates for Rpd3 histone deacetylase (28), and catalytic activity of Rpd3 is important for Ume6-dependent transcriptional repression (19). Taken together, these observations provide strong evidence that transcriptional repression occurs by locally perturbing chromatin structure.

Here, we directly show that targeted recruitment of the Sin3-Rpd3 histone deacetylase complex by the Ume6 repressor is associated with localized histone deacetylation in vivo. In wild-type cells, a Ume6-repressible promoter is preferentially deacetylated at histone H4 (lysines 5 and 12 and to a lesser extent lysine 16) and histone H3 (lysines unspecified). Two lines of evidence indicate that such localized histone deacetylation is directly caused by recruitment of the Sin3-Rpd3 complex and is mechanistically relevant for transcriptional repression. First, the specificity of localized histone deacetylation (i.e., the lysines and histones affected) is in excellent accord with the properties of Rpd3 histone deacetylase in bulk chromatin (28). Second, mutant strains lacking the DNA-binding repressor (Ume6), the corepressor necessary for recruitment (Sin3), or the deacetylase itself (Rpd3) show relatively increased acetylation in the promoter region. Taken together, these observations indicate that targeted recruitment of the Sin3-Rpd3 complex and local perturbation of chromatin structure by histone deacetylation are the physiological mechanisms for transcriptional repression by Ume6. After the present work was initially submitted for publication, similar results were published for the *INO1*, *IME2*, and *SPO13* promoters (29).

We have occasionally noted that histone acetylation in the promoter region appears somewhat less pronounced in *rpd3* strains than in *sin3* and *ume6* strains. Although this effect is marginal (and perhaps not significant), it is interesting in light of our previous suggestion of a secondary, albeit quantitatively minor, Rpd3-independent mechanism of repression (18, 19). Perhaps, this Rpd3-independent mechanism of transcriptional repression also involves histone deacetylation by one of the four Rpd3-like proteins in yeast.

The domain of localized histone deacetylation is highly localized to a region of one to two nucleosomes. As defined by the acetylation status of lysine 5 of histone H4, the apparent domain of localized histone deacetylation spreads for ~650 bp. Probes centered 450 bp upstream and 200 bp downstream from the URS1 elements show clear evidence of histone deacetylation dependent on Ume6, Sin3, and Rpd3, whereas probes further upstream or downstream do not (with the possible exception of the probe centered 300 bp downstream, which shows a very marginal and perhaps insignificant effect). As discussed under Results, this apparent domain of approximately 650 bp corresponds to an actual domain of localized histone deacetylation of approximately 150 to 250 bp. This result depends on the reasonable, but unproven, assumption that our chromatin fragmentation method breaks DNA at random (or near-random) positions within the genome. Given that nucleosomes are spaced approximately 160 to 170 bp apart in yeast, the domain of localized histone deacetylation covers approximately one to two nucleosomes (Fig. 6).

The limited spread of histone deacetylation from the site of recruitment suggests that localized chromatin modification is an inherent property of the Sin3-Rpd3 complex that is relatively insensitive to the presence or absence of other promoter elements. Further, our results suggest that the tethered Sin3-Rpd3 complex has a limited degree of flexibility that permits it to modify the nucleosome at the recruitment site and perhaps the neighboring nucleosome. The precise range of action of the Sin3-Rpd3 complex could be affected by the location of the URS1 elements with respect to the nucleosome dyad and/or by the specific promoter. Although we cannot exclude the possibility that the Sin3-Rpd3 complex can act at greater distances,

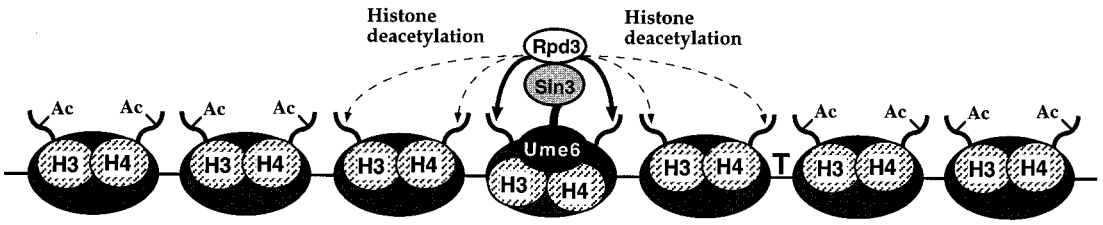

FIG. 6. Creation of a repressive chromatin domain by targeted recruitment of the Sin3-Rpd3 histone deacetylase complex. The Ume6 repressor binds URS1 (shown as occurring in the context of a nucleosomal template) and recruits the Sin3-Rpd3 corepressor complex to the promoter. As a consequence, histones H3 and H4 (lysines 5 and 12 and to a lesser extent lysine 16) are deacetylated ("Ac" does not appear) over a range of one to two nucleosomes from the site of recruitment. Thick arrows indicate sites of frequent histone deacetylation, whereas dashed arrows indicate histone tails that are infrequently modified. Nucleosomes further downstream and upstream are not specifically deacetylated (Ac). This region of local histone deacetylation is defined with respect to the promoter analyzed in this paper; it includes the UAS element but probably ends upstream of the TATA elements (T). Analogous regions of other Sin3-Rpd3 repressed promoters might vary in length and position. The TATA elements are indicated in the spacer region for clarity of the figure; there is no information on the nucleosomal position of these TATA elements in vivo. Although transcriptional repression is associated with the generation of a domain of localized histone deacetylation, the figure is not intended to suggest any particular mechanism of repression (e.g., inhibiting access of activators, TFIID, or the Pol II holoenzyme or inhibiting the communication between these components).

our results suggest that such long-range effects occur at a low frequency.

Transcriptional repression by localized histone deacetylation. The domain of modified chromatin, though not precisely defined, includes the UAS element, but it probably ends prior to the TATA elements (Fig. 6). Although the magnitude of histone deacetylation in individual experiments is modest (two- to threefold), the overall effect on chromatin structure is likely to be more substantial, because at least two histones (H3 and H4) and multiple lysine residues are affected. The simplest model for transcriptional repression is that localized histone deacetylation generates a repressive chromatin structure that inhibits the binding of activator proteins or TFIID to their cognate promoter elements. In this regard, histone acetylation increases TBP binding to TATA elements within nucleosomal templates in vitro (16). However, in the promoter examined here, we disfavor a direct effect on TBP binding, because the domain of localized histone deacetylation is unlikely to extend as far as the TATA elements. Alternatively, locally deacetylated chromatin might not reduce the accessibility of activators or TBP per se but rather might interfere with the communication of these components with each other or with the Pol II holoenzyme. For example, a locally repressive chromatin structure might inhibit the DNA looping that is presumed to occur when activators are bound relatively far away from the TATA and initiator elements. More detailed information on the mechanism of transcriptional repression will require measurements of promoter occupancy of activators, TFIID, and Pol II holoenzyme in vivo.

ACKNOWLEDGMENTS

We thank David Allis for antibodies to acetylated and nonacetylated histone H3 and David Allis, Oscar Aparicio, Steve Bell, and Laurent Kuras for technical advice on the chromatin immunoprecipitation procedure.

This work was supported by research grant GM 53720 to K.S. from the National Institutes of Health.

REFERENCES

1. **Alland, L., R. Muhle, H. Hou, J. Potes, L. Chin, N. Schreiber-Agus, and R. A. DePinho.** 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature **387:**49–55.
2. **Allfrey, V., R. M. Faulkner, and A. E. Mirsky.** 1964. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. USA **51:**786–794.
3. **Aparicio, O. M., D. M. Weinstein, and S. P. Bell.** 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. Cell **91:**59–69.
4. **Bannister, A. J., and T. Kouzarides.** 1996. The CBP co-activator is a histone acetyltransferase. Nature **384:**641–643.
5. **Braunstein, M., A. B. Rose, S. G. Holmes, C. D. Allis, and J. R. Broach.** 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. **7:**592–604.
6. **Braunstein, M., R. E. Sobel, C. D. Allis, B. M. Turner, and J. R. Broach.** 1996. Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. Mol. Cell. Biol. **16:**4349–4356.
7. **Brehm, A., E. A. Miska, D. J. McCance, J. L. Reid, A. J. Bannister, and T. Kouzarides.** 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391:**597–601.
8. **Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, and C. D. Allis.** 1996. *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell **84:**843–851.
9. **Chen, H., R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M. L. Privalsky, Y. Nakatani, and R. M. Evans.** 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell **90:**569–580.
10. **Grant, P. A., L. Duggan, J. Cote, S. M. Roberts, J. E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C. D. Allis, F. Winston, S. L. Berger, and J. L. Workman.** 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complexes. Genes Dev. **11:**1640–1650.
11. **Grunstein, M.** 1997. Histone acetylation in chromatin structure and transcription. Nature **389:**349–352.
12. **Guarente, L., and T. Mason.** 1983. Heme regulates transcription of the *cyc1* gene of *S. cerevisiae* via an upstream activation site. Cell **32:**1279–1286.
13. **Hassig, C. A., T. C. Fleischer, A. N. Billin, S. L. Schreiber, and D. E. Ayer.** 1997. Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell **89:**341–347.
14. **Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, and M. Grunstein.** 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell **80:**583–592.
15. **Heinzel, T., R. M. Lavinsky, T.-M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W.-M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387:**43–48.
16. **Imbalzano, A. N., H. Kwon, M. R. Green, and R. E. Kingston.** 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. Nature **370:**481–485.
17. **Janknecht, R., and T. Hunter.** 1996. A growing coactivator network. Nature **383:**22–23.
18. **Kadosh, D., and K. Struhl.** 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell **89:**365–371.
19. **Kadosh, D., and K. Struhl.** 1998. Histone deacetylase activity of Rpd3 is important for transcriptional repression *in vivo*. Genes Dev. **12:**797–805.
20. **Kuo, M.-H., J. Zhou, P. Jambeck, M. E. A. Churchill, and C. D. Allis.** 1998. Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes *in vivo*. Genes Dev. **12:**627–639.
21. **Laherty, C., W.-M. Yang, J.-M. Sun, J. R. Davie, E. Seto, and R. N. Eisenman.** 1997. Histone deacetylases associated with the mSin3 corepressor mediate Mad:Max transcriptional repression. Cell **89:**349–356.
22. **Luo, R. X., A. A. Postigo, and D. C. Dean.** 1998. Rb interacts with histone deacetylase to repress transcription. Cell **92:**463–473.
23. **Magnaghijaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J. P. Levillain, F. Troalen, D. Trouche, and A. Harelbellan.** 1998. Retinoblastoma

protein represses transcription by recruiting a histone deacetylase. Nature **391:**601–605.

24. **Nagy, L., H.-Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans.** 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell **89:**373–380.
25. **Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani.** 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell **87:**953–959.
26. **Pazin, M. J., and J. T. Kadonaga.** 1997. What's up and down with histone deacetylation and transcription? Cell **89:**325–328.
27. **Pogo, B. G. T., V. G. Allfrey, and A. E. Mirsky.** 1966. RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. Proc. Natl. Acad. Sci. USA **55:**805–812.
28. **Rundlett, S. E., A. A. Carmen, R. Kobayashi, S. Bavykin, B. M. Turner, and M. Grunstein.** 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes. Proc. Natl. Acad. Sci. USA **93:**14503–14508.
29. **Rundlett, S. E., A. A. Carmen, N. Suka, B. M. Turner, and M. Grunstein.** 1998. Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature **392:**831–835.
30. **Silverman, N., J. Agapite, and L. Guarente.** 1994. Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. Proc. Natl. Acad. Sci. USA **91:**11665–11668.
31. **Spencer, T. E., G. Jenster, M. M. Burcin, C. D. Allis, J. Zhou, C. A. Mizzen, N. J. McKenna, S. A. Onate, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature **389:**194–198.
32. **Struhl, K.** 1998. Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. **12:**599–606.
33. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional corepressor complex. Nature **369:**758–761.
34. **Wade, P. A., D. Pruss, and A. P. Wolffe.** 1997. Histone acetylation: chromatin in action. Trends Biochem. Sci. **22:**128–132.
35. **Yang, W.-M., C. Inouye, Y. Zeng, D. Bearss, and E. Seto.** 1996. Transcriptional repression by YY1 is mediated by interaction with the mammalian homolog of the yeast global regulator RPD3. Proc. Natl. Acad. Sci. USA **93:**12845–12850.
36. **Zhang, Y., R. Iratni, H. Erdjument-Bromage, P. Tempst, and D. Reinberg.** 1997. Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell **89:**357–364.