View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Cell, Vol. 89, 349–356, May 2, 1997, Copyright ©1997 by Cell Press

Histone Deacetylases Associated with the mSin3 Corepressor Mediate Mad Transcriptional Repression

Carol D. Laherty,* Wen-Ming Yang,†
Jian-Min Sun,‡ James R. Davie,‡
Edward Seto,† and Robert N. Eisenman*
*Division of Basic Sciences
Fred Hutchinson Cancer Research Center
Seattle, Washington 98104
†H. Lee Moffitt Cancer Center and Research Institute
Department of Medical Microbiology and Immunology
University of South Florida
Tampa, Florida 33612
‡Department of Biochemistry and Molecular Biology
University of Manitoba
Winnipeg, Manitoba R3E OW3
Canada

Summary

Transcriptional repression by Mad–Max heterodimers requires interaction of Mad with the corepressors mSin3A/B. Sin3p, the S. cerevisiae homolog of mSin3, functions in the same pathway as Rpd3p, a protein related to two recently identified mammalian histone deacetylases, HDAC1 and HDAC2. Here, we demonstrate that mSin3A and HDAC1/2 are associated in vivo. HDAC2 binding requires a conserved region of mSin3A capable of mediating transcriptional repression. In addition, Mad1 forms a complex with mSin3 and HDAC2 that contains histone deacetylase activity. Trichostatin A, an inhibitor of histone deacetylases, abolishes Mad repression. We propose that Mad–Max functions by recruiting the mSin3–HDAC corepressor complex that deacetylates nucleosomal histones, producing alterations in chromatin structure that block transcription.

Introduction

Research on the mechanism of gene regulation has focused primarily on transcriptional activators. However, repression of transcription also plays a critical role in regulating gene expression (reviewed in Johnson, 1995). A system in which the functions of both activators and repressors are closely linked is the Max transcription factor network. Max is a widely expressed basic region-helix-loop-helix-leucine zipper (bHLH-Zip) protein that forms DNA-binding heterodimers with members of the Myc and Mad families of bHLH-Zip proteins (Blackwood and Eisenman, 1991; Prendergast et al., 1991; Ayer et al., 1993; Zervos et al., 1993). Although Myc–Max and Mad–Max heterodimers bind the same E-box-related DNA sequences, they elicit different transcriptional responses. Reporter genes are activated by Myc–Max, while Mad–Max represses their transcription (Amati et al., 1992; Kretzner et al., 1992).

Myc proteins are expressed in proliferating cells and are downregulated upon cell-cycle withdrawal or differentiation (reviewed in Henriksson and Lüscher, 1996). In contrast, Mad proteins are expressed at low levels in proliferating cells and are induced upon differentiation (Ayer and Eisenman, 1993; Hurlin et al., 1995b). Changes in the relative levels of Myc and Mad lead to a switch from Myc–Max to Mad–Max heterodimers during differentiation (Ayer and Eisenman, 1993; Hurlin et al., 1995a). It appears that Myc activates genes involved in promoting cellular proliferation, while Mad antagonizes Myc function by repressing the same genes. In agreement, Mad expression blocks Myc-induced transformation and cell-cycle progression (Lahoz et al., 1994; Chen et al., 1995; Koskinen et al., 1995; Roussel et al., 1996).

Mad represses transcription through direct interaction with mSin3A and mSin3B, mammalian homologs of the S. cerevisiae corepressor, Sin3p (Ayer et al., 1995; Schreiber-Agus et al., 1995). The Sin3 proteins are approximately 175 kDa in size and contain 4 imperfect repeats of a paired amphipathic helix (PAH) motif postulated to mediate protein–protein interactions (Wang et al., 1990). Mad family proteins (Mad1, Mxi1, Mad3, and Mad4), and another Max-binding repressor, Mnt, bind to PAH2 of mSin3 through a putative amphipathic helix in their N-termini, the mSin3 interaction domain (SID) (Ayer et al., 1995; Hurlin et al., 1995b, 1997; Schreiber-Agus et al., 1995). The SID is both required for Mad repression and sufficient to repress transcription (Ayer et al., 1996; Harper et al., 1996). The ability of Mad and Mnt to block Myc-dependent transformation and cell-cycle progression is lost in SID mutants, indicating a close link between biological function and repression (Koskinen et al., 1995; Roussel et al., 1996; Hurlin et al., 1997). mSin3A can repress transcription when fused to a DNA binding domain and can functionally substitute for the Mxi1 SID (Wang and Stillman, 1993; Ayer et al., 1996; Rao et al., 1996). These findings argue that Mad's biological activities require repression mediated by mSin3.

Studies in yeast have established that *SIN3* is genetically linked to the transcriptional repressor *RPD3*. Sin3p and Rpd3p regulate a common set of genes, and mutation of *RPD3* results in the same phenotype as mutation of *SIN3* (Vidal and Gaber, 1991; Stillman et al., 1994). Mutation of both *SIN3* and *RPD3* results in defects no more severe than those observed for either single mutant, suggesting that *SIN3* and *RPD3* function in the same pathway.

A possible mechanism underlying *SIN3* and *RPD3* transcriptional repression was suggested by the identification of two highly related mammalian homologs of yeast Rpd3, HD1 (HDAC1) and mRpd3 (HDAC2). HDAC1 was isolated using the histone deacetylase inhibitor, trapoxin, and subsequently shown to possess histone deacetylase activity (Taunton et al., 1996). HDAC2 was identified in a yeast two-hybrid screen as a corepressor that binds the YY1 transcription factor (Yang et al., 1996a). Given the genetic evidence linking *SIN3* with *RPD3* and the important role of histone acetylation in regulating transcription, we examined the possibility that mSin3 associates with HDAC1 and HDAC2 to mediate Mad repression.

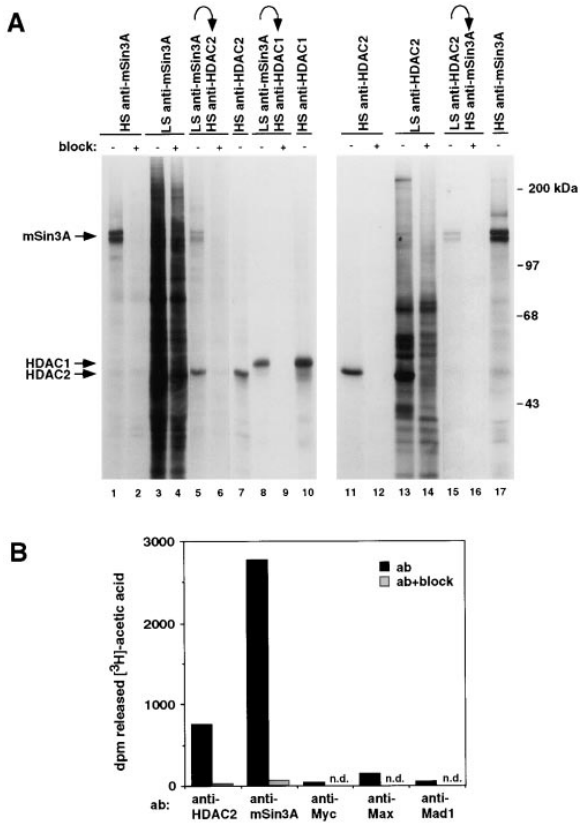

Figure 1. In Vivo Association of mSin3A with HDAC1 and HDAC2

(A) Immunoprecipitations of mSin3A and HDAC1/2 from [^{35}S]methionine-labeled cells were performed as described in Experimental Procedures using the antiserum indicated. LS indicates low stringency conditions, while HS indicates high stringency conditions. Curved arrows indicate the order in which the LS and HS conditions were employed. 293 cells were used for the left-hand portion of the panel; HeLa cells were used in the right-hand side. ''Block'' corresponds to the addition of cognate immunogen for the first antibody: GST-PAH2 for the left-hand portion and HDAC2 C-terminal peptide for the right-hand portion of the panel. Lanes 5, 6, 15, and 16 were exposed five times longer than the other lanes to demonstrate clearly the presence of HDAC1 and mSin3A. Bands corresponding to mSin3A, HDAC1, and HDAC2 are identified. Positions of the molecular mass markers are shown.

(B) Histone deacetylase assays were performed from HeLa cells using the antisera shown. When indicated, antisera were blocked with the C-terminal HDAC2 peptide for anti-HDAC2 serum and GST-PAH2 for anti-mSin3A serum. The amount of released [^{3}H]acetic acid correlates with immunoprecipitated histone deacetylase activity. n.d., not done.

Results

mSin3A Associates with Histone Deacetylases In Vivo

Immunoprecipitations were performed from [^{35}S]methionine-labeled 293 epithelial cells using an antibody against PAH2 of mSin3A. This antibody specifically recognizes the mSin3A doublet of ~150 kDa produced by in vitro translation of the cDNA for mSin3A and precipitates a 150 kDa doublet from lysates of labeled 293 cells under high stringency conditions (Figure 1A, lane 1). These peptides were not immunoprecipitated when the antiserum was preincubated with its cognate immunogen, a GST-PAH2 fusion protein, demonstrating the antibody specifically recognizes mSin3A proteins (Figure 1A, ''block'' cf. lanes 1 and 2).

To determine whether mSin3A associates with HDAC1 and HDAC2, immune and blocked anti-mSin3A precipitations were carried out under low stringency conditions (buffers lacking SDS and deoxycholate). While these immunoprecipitates showed increased background of nonspecific protein binding (Figure 1A, lanes 3 and 4), specific proteins in the complexes were identified following dissociation and reprecipitation under high stringency conditions (buffers containing SDS and deoxycholate). Low stringency anti-mSin3A precipitates were subjected to a second round of immunoprecipitation under high stringency conditions using affinity-purified antibodies against the C-termini of HDAC2 or HDAC1 (Taunton et al., 1996; see Experimental Procedures). Both HDAC2 and HDAC1 proteins were identified as components of low stringency mSin3A immunocomplexes (Figure 1A). Anti-HDAC2 recognized a 50 kDa protein that comigrated with HDAC2 immunoprecipitated under high stringency conditions (Figure 1A, lanes 5–7). Anti-HDAC1 reacted with a 55 kDa protein that comigrated with HDAC1 immunoprecipitated under high stringency conditions (Figure 1A, lanes 8–10). Neither HDAC1 nor HDAC2 was detected in low stringency anti-mSin3A precipitates using blocked serum, indicating that HDAC1 and HDAC2 require the presence of mSin3A for coprecipitation.

To confirm the interaction between mSin3A and HDAC2, low stringency anti-HDAC2 complexes from HeLa cells were dissociated and immunoprecipitated under high stringency conditions using anti-mSin3A serum. mSin3A specifically coprecipitated with HDAC2 in the presence of anti-HDAC2 serum but not the same antiserum blocked with the peptide immunogen, further demonstrating that mSin3A is associated with HDAC2 in vivo (Figure 1A, lanes 11–17).

To ascertain whether the in vivo complexes containing mSin3A and HDAC1/2 possessed active histone deacetylase, low stringency immunocomplexes were assayed for their ability to deacetylate chicken histones (Hendzel et al., 1991; see Experimental Procedures). Both HDAC2 and mSin3A immunocomplexes contained significant histone deacetylase activity compared to precipitates using blocked antiserum (Figure 1B). The increased deacetylase activity associated with mSin3A compared to HDAC2 may be due to mSin3A's association with HDAC1 as well as HDAC2. No significant histone deacetylase activity was found associated with c-Myc, Max, or Mad1 immune complexes in these cells that do not express Mad1 (Figure 1B). These results demonstrate that mSin3A is associated with a functional histone deacetylase complex in vivo.

HDAC2 Binds a Conserved Repression Domain of mSin3A

To determine the region within mSin3A that interacts with HDAC2, 293 cells were transfected with plasmids expressing FLAG epitope-tagged HDAC2 (FLAG-HDAC2)

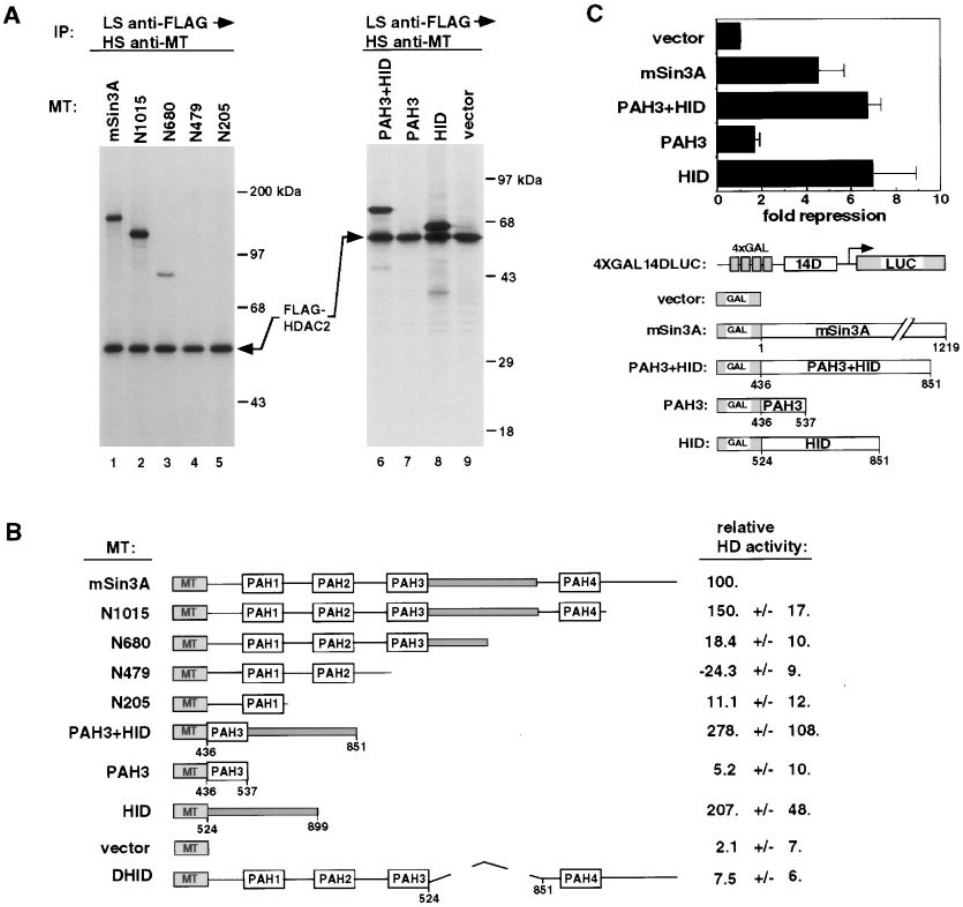

Figure 2. Identification of an mSin3A Repression Domain Required for Binding of HDAC2

(A) 293 cells were transfected with FLAG-HDAC2 and MT-mSin3A and deletion constructs as indicated. Cells shown in the left-hand side of the panel expressed a series of C-terminal truncations of mSin3; cells in the right-hand side expressed specific regions of mSin3A as indicated. Immunoprecipitations were performed on [^{35}S]methionine-labeled cells using antisera recognizing the epitope tags. High stringency (HS) anti-MT immunoprecipitations demonstrate expression of the MT-mSin3A constructs (data not shown). Low stringency (LS) anti-FLAG immunoprecipitations followed by HS anti-MT immunoprecipitation of associated MT-mSin3A constructs reveal those which bind FLAG-HDAC2. Bands corresponding to FLAG-HDAC2 are identified, and molecular masses are shown.

(B) A diagram of the Myc epitope-tagged mSin3A constructs is shown. Relative histone deacetylase (HD) activity associated with anti-MT immunoprecipitates from 293 cells transfected with each expression construct is also indicated. Average HD activity and standard deviations were obtained from at least two experiments performed in duplicate.

(C) Repression by Gal-mSin3A fusion proteins on the reporter, 4XGal14DLUC, was assayed in 293 cells transfected with the diagrammed reporter and expression constructs as indicated. Fold repression was calculated as the fold decrease in luciferase activity compared to the Gal vector. Values shown are the average and standard deviation of two experiments performed in duplicate.

and Myc epitope-tagged mSin3A (MT-mSin3A), or C-terminal deletion mutants (diagrammed in Figure 2B). MT-mSin3A and the mutants were expressed at similar high levels and were localized to the cell nucleus (data not shown). FLAG-HDAC2 complexes were immunoprecipitated under low stringency conditions using anti-FLAG antiserum. Following dissociation of the precipitate, MT-mSin3A was immunoprecipitated under high stringency conditions using 9e10 antibodies that recognize the Myc epitope (anti-MT). Both full-length MT-mSin3A and a mutant with amino acids 1016–1219 deleted (N1015) were associated with FLAG-HDAC2 (Figure 2A). Deletion of amino acids 681–1015 (N680) resulted in decreased HDAC2 binding, and further deletion of amino acids 480–680 (N479) abolished binding (Figure 2A). We further defined the segment important for binding by assaying smaller regions of mSin3A. Expression of PAH3 and the region between it and PAH4 (PAH3+HID) was sufficient for binding to FLAG-HDAC2 (Figure 2A). MT-HID, but not MT-PAH3, bound FLAG-HDAC2, demonstrating that the region between PAH3 and PAH4 is sufficient for interaction with HDAC2. We call this region of mSin3A the HDAC interaction domain (HID).

Histone deacetylase activity associated with MT-mSin3A and the deletion mutants correlated with HDAC2 binding (Figure 2B). Deletion of amino acids 681–1015, which reduced HDAC2 binding, also reduced associated histone deacetylase activity, while further deletions exhibited no coprecipitated activity. The HID region that bound HDAC2 was associated with wild-type levels of

histone deacetylase activity, while PAH3 lacked deacetylase activity (Figure 2B). In addition, deletion of the HID from mSin3A resulted in a complete loss of associated histone deacetylase activity, consistent with our observation that this mutant demonstrates substantially reduced binding to FLAG-HDAC2 (Figure 2B; data not shown). These data map the HDAC2 binding domain of mSin3A to the conserved HID region adjacent to PAH3.

We examined whether mSin3A's association with histone deacetylases was sufficient for repression by expressing PAH3 and the HID as Gal4 fusion proteins. Both constructs containing the HID (PAH3+HID and HID) repressed transcription, demonstrating the HID region is capable of repression (Figure 2C). Repression by mSin3A and the HID required Gal4 DNA binding sites in the reporter plasmid (Ayer et al., 1996; data not shown). Deletion of the HID from mSin3A did not inhibit mSin3A repression, however, suggesting that mSin3A may possess alternative means of repressing transcription in the absence of HDAC (data not shown). Nonetheless, our results indicate that association with histone deacetylases is sufficient for repression.

Mad1 Forms a Ternary Complex with mSin3A and HDAC2

We examined the ability of Mad1 to form a complex with mSin3A and HDAC2. 293 cells were transfected with plasmids expressing FLAG-HDAC2 and Mad1 or Mad1-Pro, a Mad1 mutant containing proline substitutions in the SID that does not bind mSin3A or repress transcription (Ayer et al., 1995). Following low stringency immunoprecipitation with anti-FLAG antibodies, the immune complexes were analyzed by immunoblotting with anti-mSin3A and anti-Mad1 sera. Endogenous mSin3A and ectopically expressed Mad1 and Mad1Pro were detected in whole-cell lysates (Figure 3A, lanes 7–9). mSin3A was evident in immunocomplexes from cells expressing FLAG-HDAC2 but not the empty FLAG vector, demonstrating its interaction with HDAC2 (Figure 3A, lanes 1–6). Mad1 was also detected in anti-FLAG immune complexes from cells expressing FLAG-HDAC2 (Figure 3A, lane 5). Importantly, Mad1Pro was not coprecipitated with FLAG-HDAC2 (Figure 3A, lanes 5 and 6). The requirement of a SID strongly suggests that mSin3 is required for Mad1's association with HDAC2. These data, together with the fact that HDAC2 and Mad bind to separate regions of mSin3, indicate that Mad1, mSin3A, and HDAC2 form a ternary complex.

Association of Mad with histone deacetylase activity was examined in 293 cells transfected with plasmids expressing Mad1, HA-tagged Mad1, or HA-tagged Mad3. Deacetylase assays of anti-Mad1 or anti-HA (12CA5) immune complexes demonstrated that deacetylase activity was associated with both Mad1 and Mad3 (Figure 3B). No activity was precipitated by control sera. These results indicate that Mad–mSin3–HDAC2 complexes contain functional histone deacetylase activity.

Mad Repression Requires Histone Deacetylase Activity

To determine if associated histone deacetylase activity is required for Mad repression, we examined the effect

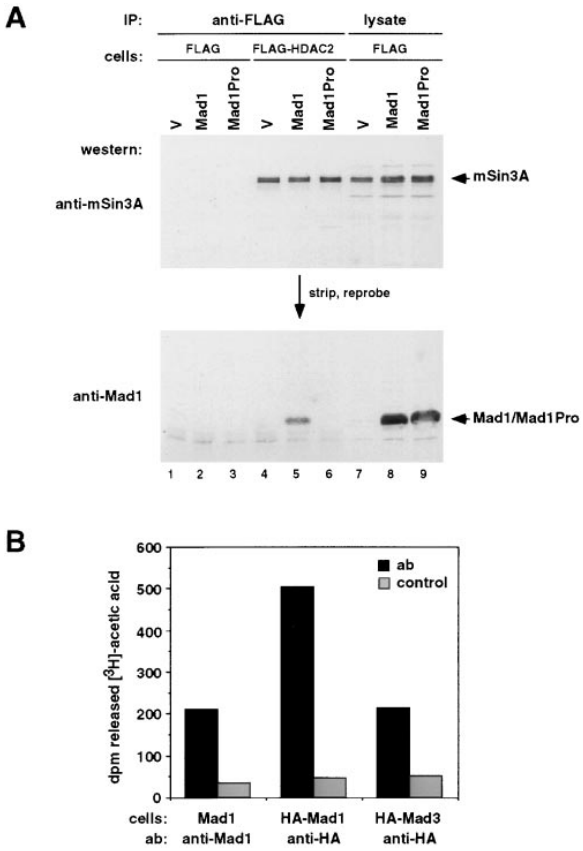

Figure 3. Ternary Complex Formation Between Mad1, mSin3, and HDAC2

(A) 293 cells were transfected with FLAG vector or FLAG-HDAC2 and the indicated Mad1 expression constructs. Western blot analysis was performed on low stringency anti-FLAG immunoprecipitates or whole-cell lysate. Following Western analysis using anti-mSin3A serum, the blot was stripped and reprobed with anti-Mad1 antiserum. Bands corresponding to mSin3A and Mad1 or Mad1Pro are identified.

(B) Histone deacetylase activity was assayed from immunoprecipitates of 293 cells transfected with the Mad expression constructs indicated. Control ab was preimmune for Mad1 or anti-MT sera for HA-Mad1 and HA-Mad3. HD assays were performed in duplicate from two independent samples, and the values shown are the averages.

of trichostatin A (TSA), a specific inhibitor of histone deacetylases (Yoshida et al., 1995). Mad repression was measured using proteins containing the Mad1 repression domain (SID) fused to c-Myc. A previous study demonstrated that both transcriptional activation and transformation of c-Myc are inhibited by fusion with the wild-type Mad1 SID (MadMyc), but not the SID containing proline substitutions (MadProMyc) (Ayer et al., 1996). 293 cells were transfected with plasmids expressing Myc, MadMyc, or MadProMyc, and a luciferase reporter plasmid containing four copies of the Myc–Max binding site (CACGTG) upstream of the SV40 early promoter (pGL2M4LUC; Figure 4). Myc transactivated the reporter 3.5-fold compared to the empty vector, and fusion of a functional SID to Myc abolished the 3.5-fold

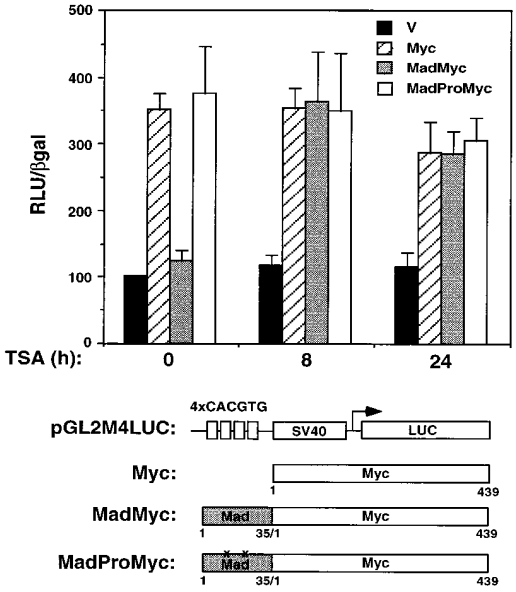

Figure 4. Effect of Trichostatin A on Mad1 Repression

293 cells were transfected with the pGL2M4Luc reporter and the diagrammed Myc expression constructs. Transfections were divided into three samples and treated with trichostatin A (TSA) for the indicated times. Luciferase assays were performed and standardized to β-gal activity expressed from a cotransfected control plasmid. The data shown are the average and standard deviation of three independent experiments.

transactivation (Figure 4). When the transfected cells were treated with TSA for 8 or 24 hr, MadMyc activated transcription to the same levels as Myc or MadProMyc, indicating that the Mad1 SID lost its ability to repress transcription (Figure 4). These data suggest that Mad1 repression requires histone deacetylase activity, presumably through formation of a ternary complex with mSin3 and HDAC1/2.

Discussion

While it has been known for years that changes in chromatin structure are of fundamental importance in gene regulation, it is only recently that genetic and biochemical studies have merged to elucidate one mechanism for the dynamic alteration of chromatin structure: acetylation and deacetylation of histones (reviewed in Davie and Hendzel, 1994; Kingston et al., 1996). Generally, there is a correlation between the level of histone acetylation and the transcriptional activity of a gene (reviewed in Wolffe and Pruss, 1996). Acetylation of nucleosomal histones is thought to induce an open chromatin conformation, which allows the transcription machinery access to promoters. Transcriptional activation has been directly linked to acetylation of histones. The yeast coactivator, Gcn5p, the mammalian coactivators, p300 and CBP, and $TAF_{II}250$ possess histone acetyltransferase activity (Bannister and Kouzarides, 1996; Brownell et al., 1996; Mizzen et al., 1996; Ogryzko et al., 1996; Yang et al., 1996b). In this study, we provide evidence that transcriptional repression can occur through targeting of histone deacetylases by a sequence-specific DNA-binding protein.

mSin3–HDAC Corepressor Complex

The identification of a mammalian homolog of yeast Rpd3p as a histone deacetylase led to the hypothesis that Sin3 represses transcription through a mechanism involving histone deacetylation (Wolffe, 1996). We have demonstrated that endogenous mSin3A and the two related mammalian histone deacetylases, HDAC1 and HDAC2, are associated in vivo. Low stringency immunoprecipitaion using mSin3A antiserum coprecipitated HDAC1/2 and histone deacetylase activity, demonstrating that the mSin3A–HDAC2 complex is functional (Figure 1). We have not observed direct binding of mSin3A to HDAC2 in vitro, suggesting that other proteins may be required for this interaction. The large size of both Sin3 and histone deacetylase complexes implies that a number of other proteins are necessary components (Carmen et al., 1996; M. M. Kasten et al., submitted). The identification of proteins associated with the mSin3–HDAC repression complex will allow further characterization of the functional interactions regulating its assembly and activity at the molecular level.

HDAC2 binds the region of mSin3A between PAH3 and PAH4 that we call the HID. The primary seqence of the HID is well conserved between mammalian mSin3A and mSin3B, Drosophila Sin3, and yeast Sin3p, exhibiting up to 50% identity between the mammalian and yeast proteins (Ayer et al., 1995; D. Pauli and G. Pannetta, personal communication). However, previously identified interaction motifs are not evident within this region. The HID region of mSin3A is capable of mediating transcriptional repression, providing strong evidence that the interaction of mSin3 with HDAC1 and HDAC2 is functionally important (Figure 2). HDAC2 also repressed transcription when fused to a heterologous DNA binding domain, suggesting that interaction with HDAC is sufficient for repression (Yang et al., 1996a). Our observation that inhibition of histone deacetylation by trichostatin A abolished Mad repression demonstrates that histone deacetylation is the principle mechanism of Mad repression. However, our finding that mSin3A can repress transcription in the absence of the HID region suggests that mSin3 contains an alternative repression domain active in the absence of bound HDAC.

In addition to the Mad proteins, at least three distinct types of transcriptional repressors appear to utilize the Sin3–HDAC corepressor. Ume6, a yeast DNA-binding repressor regulating meiosis, binds Sin3p and requires *SIN3* and *RPD3* for repression (Kadosh and Struhl, 1997 [this issue of *Cell*]). In addition, mSin3 is required for transcriptional repression by nuclear hormone receptors. In the absence of ligand, nuclear receptors bind the corepressor, NCoR, which represses transcription through direct interaction with the mSin3–HDAC corepressor complex (Heinzel et al., 1997). Finally, YY1 repression is likely mediated by direct interaction with HDAC2 (Yang et al., 1996a). As observed for transcriptional activation, the regulation of histone acetylation

appears to be a common mechanism underlying repression.

Mad–Max Repression

The finding that Mad1 forms a ternary complex with mSin3 and HDAC that contains histone deacetylase activity suggests that histone deacetylation is the biochemical basis of Mad repression. Although we have linked Mad repression with histone deacetylation, we have not shown that Mad mediates deacetylation of histones within chromatin. In addition, the extent of hypoacetylation and DNA inaccessibility surrounding Mad target genes has not yet been addressed. However, previous studies have suggested that hyper- or hypoacetylation can be a localized event compatible with the activation or repression of specific genes (Van Lint et al., 1996; reviewed in Wolffe and Pruss, 1996). We speculate that HDAC1/2 will exhibit a localized effect on nucleosomes associated with target promoters, while other deacetylases might exert a long range effect required for sustained silencing of large regions of the genome.

Characterization of the known components of the mSin3A–HDAC2 complex reveals that mSin3A and HDAC2 are stable nuclear proteins, as is Max (data not shown). This contrasts sharply with the extremely labile nature of Mad1 ($t_{1/2} = 20$ min). mSin3 is widely expressed during mouse development, while Mad genes display restricted expression patterns (Hurlin et al., 1995a; C. Queva and R. N. E., unpublished data). mSin3A and HDAC2 are highly expressed in a variety of cell lines (C. D. L. and R. N. E., unpublished data). In contrast, Mad proteins are expressed at low levels in proliferating cells but are strongly induced during differentiation (Ayer and Eisenman, 1993). Based on the functional relationship we have established for Mad, mSin3, and HDAC1/2 and their biochemical characteristics and expression patterns, we propose the following model of Mad transcriptional repression. The stable nuclear mSin3–HDAC corepressor complex is brought to specific Mad–Max target genes upon expression of Mad during differentiation, cell-cycle withdrawal, or specific stages of development. While tethered to DNA by Mad, mSin3-associated HDAC deacetylates proximal histones, leading to an altered chromatin structure that prevents transcriptional initiation of Mad–Max target genes. This model implies that Mad expression and DNA binding are the rate-limiting steps governing the repression of Mad target genes.

The model also has implications for Myc transcriptional activation. Since Mad antagonizes Myc function both biologically and transcriptionally at Myc–Max binding sites, our results raise the possibility that Myc may drive histone acetylation. While this idea is attractive in that it proposes a balance between Myc- and Mad-regulated transcription occurring through the opposing actions of histone acetytransferases and deacetylases, there is no data to support the model. However, recent reports suggest that inappropriate histone acetylation can activate genes driving cell growth and lead to tumor formation (Borrow et al., 1996; Reifsnyder et al., 1996; reviewed in Roth, 1996). These studies and the work reported here demonstrate that histone acetylation and deacetylation are fundamental regulatory mechanisms governing cell proliferation and differentiation.

Experimental Procedures

Antibodies against HDAC2

A peptide corresponding to amino acids 478–488 of HDAC2 (KGAKSEQLSNP) coupled to keyhole limpet hemacyanin was injected subcutaneously into New Zealand white rabbits (0.1 mg of peptide per injection). The resulting antibodies were immunoaffinity purified on a peptide column. This anti-HDAC2 serum recognizes human and mouse HDAC2 as single 50 kDa bands by Western blot.

Immunoprecipitations and Western Blots

Immunoprecipitations were performed as described (Blackwood et al., 1992). Cells were lysed in high stringency Ab buffer (20 mM Tris [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% SDS, 0.5% deoxycholate) or low stringency LS buffer (PBS containing 0.1% NP-40) containing protease inhibitors, sonicated, clarified by centrifugation, and immunoprecipitated at 4°C with the indicated antibodies and protein A- or protein G-Sepharose CL4B (Sigma). High stringency immunoprecipitations were washed once with Ab buffer, once with RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS), once with high salt buffer (2 M NaCl, 10 mM Tris [pH 7.4], 1% NP-40, 0.5% deoxycholate), and once with RIPA buffer. Low stringency immunoprecipitations were washed four times with LS buffer.

For radioimmunoprecipitation experiments, cells were metabolically labeled for 3 hr with [^{35}S]methionine (DuPont-NEN). When indicated, nonimmunoreactive components of immune complexes were eluted with Ab buffer and reprecipitated under high stringency conditions. For histone deacetylase assays, 10 cm dishes of HeLa or 293 cells were lysed in LS buffer, immunoprecipitated, washed 3 times with LS buffer, and resuspended in HD buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol).

Immunoprecipitated proteins were resolved on SDS–polyacrylamide gels and examined by autoradiography or immunoblot analysis. Semidry transfer to Nitrobind membranes (MSI) was performed by standard procedures (Brasier and Fortin, 1995). Membranes were probed with the indicated antisera in TNT buffer (0.15 M NaCl, 25 mM Tris [pH 7.5], 0.05% Tween 20) containing 1% nonfat dry milk. The membrane was washed four times in TNT buffer and incubated with horseradish peroxidase-conjugated donkey anti-rabbit (Amersham) or rabbit anti-mouse (Zymed) secondary antibodies. The membrane was washed three times with TNT buffer, once with TNT buffer containing 0.25% Tween 20, and analyzed by ECL procedures (Amersham).

Histone Deacetylase Assays

Histone deacetylase assays were performed as described (Hendzel et al., 1991). Immunoprecipitated complexes were incubated for 30 min at 37°C with 180 mg (150,000 dpm) of acid-soluble histones isolated from [^{3}H]acetate-labeled chicken erythrocytes. The reaction was terminated with acetic acid (0.12 N) and HCl (0.72 N). Released [^{3}H]acetic acid was extracted with ethyl acetate and quantitated by liquid scintillation counting. Samples were assayed in duplicate at least twice, and the nonenzymatic release of label was subtracted to obtain the final value.

Subcloning and Mutagenesis

Fusion proteins were constructed by standard PCR and cloning techniques. Myc epitope-tagged mSin3A was generated by cloning the mSin3A cDNA into the pCS2+MT vector (Turner and Weintraub, 1994). Carboxy-terminal deletion mutants were generated using internal and polylinker restriction enzyme sites. MT-PAH3+HID, MT-PAH3, MT-HID, Gal-PAH3+HID, Gal-PAH3, and Gal-HID were constructed by PCR and sequenced. FLAG epitope-tagged HDAC2 was generated by subcloning the HDAC2 cDNA in the pME18S vector (Shiio et al., 1992).

Transfection and Luciferase Assays

293 cells were maintained in DMEM with 10% fetal bovine serum (Hyclone). Transfections and luciferase assays were performed by standard procedures (Brasier and Fortin, 1995). For experiments using trichostatin A (TSA), transfected cells were split into 3 dishes 24 hr after transfection. One plate remained untreated; the others were treated with TSA (100 ng/ml) for 24 or 8 hr prior to harvesting. Transfection efficiencies were normalized using a cotransfected β-galactosidase plasmid.

Acknowledgments

We thank Angela Bush for technical assistance; C. Queva, P. Gallant, L. Madisen, Y. Shiio, B. Kelly, and S. Coates for reagents; P. Hurlin and C. Grandori for advice; D. Ayer and S. Schreiber for reagents and communicating results prior to publication; and K. Struhl, D. Stillman, T. Heinzel, and G. Rosenfeld for communicating results prior to publication. We are grateful to D. Gottschling and C. Grandori for critical readings of the manuscript. This work was supported by an NIH/NCI grant to R. N. E., a Medical Research of Canada grant to J. R. D., an NSF grant to E. S., and a postdoctoral fellowship to C. D. L. from the Ladies Auxiliary to the Veterans of Foreign Wars.

Received January 9, 1997; revised March 20, 1997.

References

Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I., and Land, H. (1992). Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature *359*, 423–426.

Ayer, D.E., and Eisenman, R.N. (1993). A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. Genes Dev. *7*, 2110–2119.

Ayer, D.E., Kretzner, L., and Eisenman, R.N. (1993). Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell *72*, 211–222.

Ayer, D.E., Lawrence, Q.A., and Eisenman, R.N. (1995). Mad–Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell *80*, 767–776.

Ayer, D.E., Laherty, C.D., Lawrence, Q.A., Armstrong, A.P., and Eisenman, R.N. (1996). Mad proteins contain a dominant transcription repression domain. Mol. Cell. Biol. *16*, 5772–5781.

Bannister, A.J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641–643.

Blackwood, E.M., and Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA binding complex with Myc. Science *251*, 1211–1217.

Blackwood, E.M., Luscher, B., and Eisenman, R.N. (1992). Myc and Max associate in vivo. Genes Dev. *6*, 71–80.

Borrow, J., Stanton, V.P., Jr., Andresen, J.M., Becher, R., Behm, F.G., Chaganti, R.S.K., Civin, C.I., Disteche, C., Dube, I., Frischauf, A.M., et al. (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nature Genet. *14*, 33–41.

Brasier, A.R., and Fortin, J.J., eds. (1995). Current Protocols in Molecular Biology, Vol. 3 (New York: John Wiley and Sons, Inc.).

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843–851.

Carmen, A.A., Rundlett, S.E., and Grunstein, M. (1996). HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex. J. Biol. Chem. *271*, 15837–15844.

Chen, J., Willingham, T., Margraf, L.R., Schreiber-Agus, N., DePinho, R.A., and Nisen, P.D. (1995). Effects of the MYC oncogene antagonist, MAD, on proliferation, cell cycling and the malignant phenotype of human brain tumor cells. Nature Med. *1*, 638–643.

Davie, J.R., and Hendzel, M.J. (1994). Multiple functions of dynamic histone acetylation. J. Cell. Biochem. *55*, 98–105.

Harper, S.E., Qiu, Y., and Sharp, P.A. (1996). Sin3 corepressor function in Myc-induced transcription and transformation. Proc. Natl. Acad. Sci. USA *93*, 8536–8540.

Heinzel, T., Lavinsky, R.M., Mullen, T.-M., Söderström, M., Laherty, C.D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S.N., Davie, J.R., et al. (1997). N-Cor, mSin3, and histone deacetylase-containing complexes in nuclear receptor and Mad repression. Nature *387*, in press.

Hendzel, M.J., Delcuve, G.P., and Davie, J.R. (1991). Histone deacetylase is a component of the internal nuclear matrix. J. Biol. Chem. *266*, 21936–21942.

Henriksson, M., and Lüscher, B. (1996). Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer Res. *68*, 109–182.

Hurlin, P.J., Foley, K.P., Ayer, D.E., Eisenman, R.N., Hanahan, D., and Arbeit, J.M. (1995a). Regulation of Myc and Mad during epidermal differentiation and HPV-associated tumorigenesis. Oncogene *11*, 2487–2501.

Hurlin, P.J., Queva, C., Koskinen, P.J., Steingrimsson, E., Ayer, D.E., Copeland, N.G., Jenkins, N.A., and Eisenman, R.N. (1995b). Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-Myc-dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. *14*, 5646–5659.

Hurlin, P.J., Quéva, C., and Eisenman, R.N. (1997). Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. Genes Dev. *11*, 44–58.

Johnson, A.D. (1995). The price of repression. Cell *81*, 655–658.

Kadosh, D., and Struhl, K. (1997). Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell, this issue.

Kingston, R.E., Bunker, C.A., and Imbalzano, A.N. (1996). Repression and activation by multiprotein complexes that alter chromatin structure. Genes Dev. *10*, 905–920.

Koskinen, P.J., Ayer, D.E., and Eisenman, R.N. (1995). Repression of Myc-Ras cotransformation by Mad is mediated by multiple protein-protein interactions. Cell Growth Differ. *6*, 623–629.

Kretzner, L., Blackwood, E.M., and Eisenman, R.N. (1992). Myc and Max proteins possess distinct transcriptional activities. Nature *359*, 426–429.

Lahoz, E.G., Xu, L., Schreiber-Agus, N., and DePinho, R.A. (1994). Suppression of Myc, but not E1a, transformation activity by Max-associated proteins, Mad and Mxi1. Proc. Natl. Acad. Sci. USA *91*, 5503–5507.

Mizzen, C.A., Yang, X.-J., Kokubo, T., Brownell, J.E., Bannister, A.J., Owen-Hughes, T., Workman, J., Berger, S.L., Kouzarides, T., Nakatani, Y., and Allis, C.D. (1996). The $TAF_{II}250$ subunit of TFIID has histone acetyltransferase activity. Cell *87*, 1261–1270.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953–959.

Prendergast, G.C., Lawe, D., and Ziff, E.B. (1991). Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and Ras cotransformation. Cell *65*, 395–407.

Rao, G., Alland, L., Guida, P., Schreiber-Agus, N., Chen, K., Chin, L., Rochelle, J.M. Seldin, M.F., Skoultchi, A.I., and DePinho, R.A. (1996). mSin3A interacts with and can functionally substitute for the amino terminal repression of the Myc antagonist Mxi1. Oncogene *12*, 1165–1172.

Reifsnyder, C., Lowell, J., Clarke, A., and Pillus, L. (1996). Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nature Genet. *14*, 42–49.

Roth, S.Y. (1996). Something about silencing. Nature Genet. *14*, 3–4.

Roussel, M.F., Ashmun, R.A., Sherr, C.J., Eisenman, R.N., and Ayer,

D.A. (1996). Inhibition of cell proliferation by the Mad1 transcriptional repressor. Mol. Cell. Biol. *16*, 2796–2801.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A.I., and DePinho, R.A. (1995). An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell *80*, 777–786.

Shiio, Y., Yamamoto, T., and Yamaguchi, N. (1992). Negative regulation of Rb expression by the p53 gene product. Proc. Natl. Acad. Sci. USA *89*, 5206–5210.

Stillman, D.J., Dorland, S., and Yu, Y. (1994). Epistatic analysis of suppressor mutations that allow HO expression in the absence of the yeast SWI5 transcriptional activator. Genetics *136*, 781–788.

Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408–411.

Turner, D.L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. *8*, 1434–1437.

Van Lint, C., Emiliani, S., and Verdin, E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Express. *5*, 245–253.

Vidal, M., and Gaber, R.F. (1991). RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae. Mol. Cell. Biol. *11*, 6317–6327.

Wang, H., and Stillman, D.J. (1993). Transcriptional repression in Saccaromyces cerevisiae by a SIN3-LexA fusion protein. Mol. Cell. Biol. *13*, 1805–1814.

Wang, H., Clark, I., Nicholon, P.R., Herskowitz, I., and Stillman, D.J. (1990). The Saccaromyces cerevisiae *SIN3* gene, a negative regulator of *HO*, contains four paired amphipathic helix motifs. Mol. Cell. Biol. *10*, 5927–5936.

Wolffe, A.P. (1996). Histone deacetylase: a regulator of transcription. Science *272*, 371–372.

Wolffe, A.P., and Pruss, D. (1996). Targeting chromatin disruption: transcription regulators that acetylate histones. Cell *84*, 817–819.

Yang, W.-M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996a). Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc. Natl. Acad. Sci. USA *93*, 12845–12850.

Yang, X.-J., Ogryzko, V.V., Nishikawa, J., Howard, B.H., and Nakatani, Y. (1996b). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature *382*, 319–324.

Yoshida, M., Horinouchi, S., and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays *17*, 423–430.

Zervos, A.S., Gyuris, J., and Brent, R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc–Max recognition sites. Cell *72*, 223–232.