Biochemical Analysis of Transcriptional Repression by Drosophila Histone Deacetylase 1*

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To study the mechanisms by which deacetylases regulate transcription by RNA polymerase II, we investigated the biochemical properties of purified recombinant Drosophila histone deacetylase 1 (dHDAC1, also known as dRPD3). We found that purified dHDAC1 and Gal4-dHDAC1 polypeptides possess substantial deacetylase activity. Thus, deacetylation by dHDAC1 does not require any additional cofactors. Gal4-dHDAC1, but not dHDAC1, was observed to repress transcription in vitro by about 2–3-fold from chromatin templates, but not from naked DNA templates, in a Gal4 site-dependent manner. This magnitude of repression is similar to that commonly seen by deacetylases in vivo, as assessed by treatment of cells with deacetylase inhibitors. Transcriptional repression by Gal4-dHDAC1 was blocked by the deacetylase inhibitor, FR901228, and thus, deacetylase activity correlates with repression. Single round transcription analyses showed that Gal4-dHDAC1 reduces the absolute number of productive initiation complexes with chromatin templates. Moreover, with chromatin templates that were assembled with completely purified components, Gal4-dHDAC1 was found to deacetylate nucleosomal histones as well as to repress transcription. These experiments provide biochemical evidence for the requirement of chromatin for transcriptional repression by dHDAC1 and further show that dHDAC1 acts to repress the transcription initiation process.

The precise control of gene transcription is essential for the proper growth and development of an organism. In eukaryotes, there are thousands of proteins that participate in the regulation of transcription by RNA polymerase II. These factors include sequence-specific DNA-binding proteins that interact with enhancer and silencer elements, the RNA polymerase II transcriptional machinery, chromatin remodeling factors, and enzymes that covalently modify histones and other proteins such as transcription factors (for reviews, see Refs. 1–14).

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There has recently been considerable effort devoted to the effects of histone acetyltransferases (HATs) as well as histone deacetylases (HDACs) upon transcriptional activity. In general, the acetylation of core histones correlates with transcriptional activation, whereas the deacetylation of core histones correlates with transcriptional repression. The importance of HATs and HDACs in the control of gene expression is well established, but the basic molecular mechanisms by which they act are a subject of current investigation. For instance, HATs can acetylate a variety of proteins other than core histones, and thus, it will be important to determine the importance of the acetylation of histones as well as nonhistone proteins.

Transcriptional studies of HDACs have been generally carried out in transiently transfected cells. In many instances, the treatment of cells with HDAC inhibitors, such as TSA or trichostatin A, has been found to result in a 2–4-fold increase in the transcription of HDAC-repressed genes (15–20), although in some cases, higher levels of transcriptional enhancement by HDAC inhibitors have been observed (21–23). LexA- or Gal4-HDAC fusion proteins have also been found to repress transcription in vivo (24–26), but the role of deacetylation in repression by these factors was not tested with HDAC inhibitors.

The ability of HDACs to repress transcription in vitro has not yet been tested. Hence, it is not yet known whether a chromatin template is required for repression by HDACs. It is possible, for instance, that HDACs repress transcription by acting through transcription factors or coregulators instead of the chromatin template. It is also not known whether HDACs repress the assembly of the transcription preinitiation complex or a post-initiation process such as transcriptional elongation.

To address these questions, we performed a biochemical analysis of Drosophila histone deacetylase 1 (dHDAC1, also known as dRPD3: Refs. 27 and 28). To this end, we prepared purified, recombinant dHDAC1 proteins that are highly active for the deacetylation of histones in vitro and then investigated the ability of these proteins to repress transcription by RNA polymerase II in vitro.

EXPERIMENTAL PROCEDURES

Transcription Factors and DNA Templates—His\(_{137}\)-tagged NF-κB p65 protein (29) was synthesized in Sf9 cells by using a recombinant baculovirus kindly provided by Dr. J. Hiscott (Lady Davis Institute, Montreal, Canada). The p65 protein was synthesized and purified essentially as described for His\(_{137}\)-tagged p300 (30, 31). FLAG-tagged Sp1 was purified by using a recombinant baculovirus that was kindly provided by Drs. Soojin Ryu and Robert Tjian (University of California, Berkeley, CA). Constructions that encode His\(_{137}\)-tagged versions of full-length dHDAC1 and Gal4-dHDAC1 were prepared from the dHDAC1 cDNA (27) that was generously provided by Drs. Andrew Barlow and Bryan Turner (University of Birmingham, United Kingdom). The resulting dHDAC1 and Gal4-dHDAC1 proteins were synthesized in Sf9 cells by using a baculovirus expression system (PharMingen) and purified by Ni(II) affinity chromatography (Ni-NTA resin; Qiagen). Gal4-dHDAC1 consists of the N-terminal 147 amino acid residues of the yeast Gal4 protein (which contains the DNA binding region) fused to the N terminus of full-length dHDAC1 protein. The plasmid pHIV is identical to pHIV except that it contains five tandem Gal4

1 The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; dHDAC1, Drosophila histone deacetylase 1; NF-κB, nuclear factor κB; p65, NF-κB p65 protein; TSA, trichostatin A; HIV, human immunodeficiency virus; Ni-NTA, nickel-nitrilotriacetic acid.
binding sites (derived from pGIE-0; Ref. 32) upstream of the HIV-1 promoter, with the nearest Gal4 site located 48 base pairs from the upstream NF-B site in the HIV-1 promoter.

**Histone Deacetylase Assays**—Histone deacetylase assays with free histones (as in Fig. 1) were performed as follows. Purified *Drosophila* core histones were prepared by the method of Bulger and Kadonaga (33) and then acetylated with [1-^14^C]acetic anhydride (ICN Radiochemicals), as described by Hebbes et al. (34). The resulting [^14^C]-acetyl histones were separated from the free labeled acetate and unreacted acetic anhydride by extensive dialysis. Histone deacetylase reactions were performed by incubation of the purified dHDAC derivatives (200 nm dHDAC1 or Gal4-dHDAC1) with [^14^C]-acetyl histones (20 μg/ml; 1.5 μM, pH 7.6, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. The reactions were terminated by the addition of HCl (to 0.82 M final concentration) and acetic acid (to 0.12 M final concentration) and the resulting [^14^C]-labeled acetic acid was extracted with ethyl acetate and quantitated with a liquid scintillation counter. The reported amounts of free [^14^C]-acetic acid obtained by subtracting the background levels of [^14^C]-acetic acid (in reactions with buffer only) from the amounts of [^14^C]-acetic acid observed with dHDAC1 or Gal4-dHDAC1. (The background levels of [^14^C]-acetic acid were typically about 13–20% of the total radiolabeled material.)

The deacetylation of nucleosomal histones (as in Fig. 4A) was assayed as follows. Purified *Drosophila* core histones (1 mg/ml; Ref. 33) were acetylated with purified Hi~6~tagged histone p300 (100 μM, pH 7.5, 30 and 311 μM acetyl-CoA) and 30 min at 30 °C. The Hi~6~tagged p300 was depleted from the sample with Ni-NTA resin (Qiagen), and the resulting p300-acetylated histones were assembled into chromatin with purified ACF and dNAP-1, as described by Ito et al. (35). This chromatin was incubated with purified dHDAC1 derivatives (100 nm Gal4-dHDAC1 or dHDAC1) for 30 min at 30 °C, digested with micrococcal nuclease, and immunoprecipitated with anti-acetyl-Lys9, Lys14, Lys16, or Lys18) histone H3 or anti-acetyl (Lys5, Lys8, Lys12, Lys16) histone H4 (Upstate Biotechnology). The resulting DNA fragments were resolved by 1.25% agarose gel electrophoresis and detected by Southern blot analysis.

The HDAC inhibitor FR901228 (36, 37) was a generous gift of Dr. Hidenori Nakajima (Fujisawa Pharmaceutical Co., Ltd., Ibaraki, Japan). Prior to use, FR901228 was dissolved in methanol to a concentration of 0.15 mg/ml and stored at 20 °C. The HDAC inhibitor TSA (Sigma catalog number T8552; Refs. 38 and 39) was dissolved in ethanol to a concentration of 1.5 mg/ml and then further diluted to a working stock solution of 0.1 mg/ml in methanol and stored at −20 °C. The HDAC inhibitor TSA (Sigma catalog number T8552; Refs. 38 and 39) was dissolved in ethanol to a concentration of 1.5 mg/ml and then further diluted to a working stock solution of 0.1 mg/ml in methanol and stored at −20 °C. The addition of FR901228 or TSA resulted in a final concentration of 0.1% (v/v) methanol or ethanol in the histone deacetylase or transcription reaction mixtures with buffers containing 0.1% methanol or ethanol were performed to ensure that the effects seen with FR901228 or TSA were due to the specific inhibitors instead of the buffer medium.

**Chromatin Assembly and in Vitro Transcription**—Transcription of S190-assembled chromatin (33, 40) was performed with HeLa nuclear extracts, essentially as described previously (30, 31), except that a 3-fold mass excess (relative to the template DNA prior to chromatin assembly) of pUC18 was added to the reaction medium after chromatin assembly to remove any additional core histones. Transcription of chromatin that was assembled with purified recombinant ACf, purified recombinant dNAP-1, purified core histones, DNA, and ATP was carried out as described by Jiang et al. (41), except that the core histones were pre-acetylated with purified recombinant p300 and acetyl CoA, as described above for the nucleosomal histone deacetylase assay. Quantitation of the DNA was carried out with a PhosphorImager (Molecular Dynamics). All reaction conditions were performed in duplicate, and each experiment was performed a minimum of two (but typically, several) independent times to establish the reproducibility of the results. The amounts of transcription are reported as the mean ± S.D., relative to a reference indicated by parentheses.

**RESULTS AND DISCUSSION**

To study the mechanisms by which histone deacetylases regulate transcription by RNA polymerase II, we synthesized *Drosophila* HDAC1 (dHDAC1) and Gal4-dHDAC1 in Sf9 cells by using a baculovirus expression system and then purified the proteins to near homogeneity (Fig. 1A). dHDAC1 is in the Rpd3-like family of histone deacetylases and is also known as dRPD3 (27, 28). Gal4-dHDAC1 consists of the Gal4 DNA-binding domain fused to the N terminus of full-length dHDAC1.

The purified recombinant dHDAC1 and Gal4-dHDAC1 were then tested for histone deacetylase activity. For these assays, we prepared [^14^C]-acetyl histones by incubation of purified *Drosophila* core histones (which are mostly unacytlated) with [1-^14^C]acetic anhydride under conditions that result in the acetylation of essentially all of the unmodified lysine residues in the core histones (34). With these [^14^C]-acetyl histones, we observed that purified dHDAC1 and Gal4-dHDAC1 can deacetylate about 38 and 15%, respectively, of the total acetyl lysine residues at a molar ratio of histone polypeptides to dHDAC1 (or Gal4-dHDAC1) of ~7.5:1 (Fig. 1B). These findings thus indicate that the purified dHDAC1 protein is highly active for deacetylation of free histones in the absence of any other cofactors.

We also observed that the HDAC activity of the recombinant proteins is completely inhibited by 10 mM sodium butyrate, 150 ng/ml (500 nM) TSA, or 100 ng/ml (185 nM) FR901228 (Fig. 1B). Whereas sodium butyrate and TSA are well known HDAC inhibitors, FR901228 has recently been found to be a potent and specific inhibitor of histone deacetylases (36, 37). In fact, FR901228 inhibits HDAC activity at a lower concentration than TSA (37). Therefore, based on the potency, specificity, and chemical stability of FR901228, we chose to use this compound for the inhibition of the HDAC activity.

Next, we tested the ability of the dHDAC1 proteins to modulate transcription by RNA polymerase II. To this end, we used a chromatin transcription system based on the *Drosophila* S190 chromatin assembly extract (30–32, 40, 42). Two different plasmids were used as reporter templates: pPH, which contains the HIV-1 long terminal repeat promoter; and pG65 HIV, which is identical to pHIV except that it contains five Gal4 binding sites upstream of the HIV-1 promoter (Fig. 2). (To minimize steric effects of the binding of Gal4-dHDAC1 to the promoter, the Gal4 sites were placed upstream of the HIV-1 promoter.) The plasmids were assembled into chromatin with the S190 extract and then subjected to *in vitro* transcription analysis with a HeLa nuclear extract. Transcription was activated by Sp1 and NF-kB p65 proteins, which were added along with the dHDAC1 derivatives subsequent to chromatin assembly. As shown in Fig. 2, Gal4-dHDAC1, but not dHDAC1,
Gal4-dHDAC1, but not dHDAC1, represses transcription in vitro with chromatin templates. Chromatin was assembled onto template DNAs containing either five (pG5HIV) or zero (pHIV) Gal4 sites located upstream of the promoter region of the HIV-1 long terminal repeat. Where indicated, purified dHDAC1 (100 nM), Gal4-Gal4 sites located upstream of the promoter region of the HIV-1 long terminal repeat. Where indicated, purified dHDAC1 (100 nM), Gal4-dHDAC1 (100 nM), Sp1 (10 nM), and NF-xB p65 (100 nM) were added to the chromatin templates, and transcription was carried out with a HeLa nuclear extract. The resulting transcripts were detected by primer extension analysis, and the reverse transcription products are shown. Transcriptional activity is reported as relative to that observed with Sp1 and NF-xB p65 in the absence of any dHDAC derivative, which is designated as “(100)”.

represses transcription by about 2-fold from pG5HIV, but not from pHIV. Thus, transcriptional repression by dHDAC1 requires its recruitment to the template, which occurs, in this instance, via the Gal4 protein fusion and Gal4 binding sites. It is additionally important to note that dHDAC1 was found to have higher HDAC activity than Gal4-dHDAC1 (Fig. 1B), and hence, the inability of dHDAC1 to repress transcription was not due to the lack of intrinsic HDAC activity.

We further examined the repression of transcription by the Gal4-dHDAC1 protein as follows. First, we found that repression is blocked by the HDAC inhibitor, FR901228, and that repression is not observed with Gal4-(1–147) alone (which corresponds to the Gal4 portion of Gal4-dHDAC1) (Fig. 3A). Thus, repression requires the dHDAC1 protein and correlates with the deacetylase activity. Second, we observed that Gal4-dHDAC1 does not repress transcription from naked DNA templates (Fig. 3B). Hence, repression by Gal4-dHDAC1 requires chromatin and is not due to an effect upon the intrinsic transcription process.

To determine the step in the transcription process that is affected by Gal4-dHDAC1, we carried out single round transcription reactions. In these experiments, we used the detergent Sarkosyl, which inhibits the assembly of the transcription preinitiation complex but not the elongation of the transcriptionally engaged polymerase (45–45). Chromatin transcription reactions were performed as described in the legend to Fig. 2 and then Sarkosyl was added immediately (10 s) after the initiation of transcription by the addition of ribonucleoside 5'-triphosphates. These experiments revealed that Gal4-dHDAC1 mediates about 3.5-fold repression of the assembly of productive transcription preinitiation complexes (Fig. 3C). Moreover, repression by Gal4-dHDAC1 was found to be inhibited by FR901228 as well as dependent upon the presence of Gal4 sites in the DNA template. Therefore, these results indicate that Gal4-dHDAC1 represses the transcription initiation process.

To investigate further the requirement for chromatin for repression by Gal4-dHDAC1, we tested the effect of Gal4-dHDAC1 upon chromatin templates that were assembled with completely purified components instead of the crude S190 extract. In these experiments, periodic nucleosome arrays were assembled with purified recombinant ACF, purified recombinant dNAP-1, purified core histones, plasmid DNA (pG5HIV or pHIV), and ATP, as described previously by Ito et al. (35). The chromatin that is assembled with the purified factors is not subject to potential alteration by undefined factors that are present in the S190 extract.

We first tested the ability of dHDAC1 and Gal4-dHDAC1 to deacetylate histones that are packaged into chromatin. With chromatin containing five Gal4 sites, Gal4-dHDAC1 deacetylates nucleosomal histones H3 and H4 more efficiently than dHDAC1 (Fig. 4A). This preferential deacetylation by Gal4-dHDAC1 relative to dHDAC1 was not seen with a template (pHIV) lacking Gal4 sites (data not shown). In addition, the deacetylation of nucleosomal H3 and H4 was inhibited by FR901228 (Fig. 4A). We thus conclude that Gal4-dHDAC1 can deacetylate nucleosomal histones when the protein is recruited to the chromatin template.

Next, we examined the effects of Gal4-dHDAC1 upon transcription of chromatin that is assembled with the purified components. Chromatin was assembled onto pG5HIV (five Gal4 sites) or pHIV (no Gal4 sites), as described above for the chromatin deacetylase assays (Fig. 4A) and then transcribed...
with a HeLa nuclear extract supplemented with 15 μM acetyl-CoA, as described by Jiang et al. (41).

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