Histone Deacetylase 3 Down-regulates Cholesterol Synthesis through Repression of Lanosterol Synthase Gene Expression*

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In vertebrates, a key step in the biosynthesis of cholesterol and steroid hormones is the conversion of (S)-2,3-oxidosqualene to lanosterol. The enzyme that catalyzes this complex cyclization/rearrangement step via the protosteryl cation intermediate is lanosterol synthase ((S)-2,3-epoxysqualene mutase (cyclizing, lanosterol forming), EC 5.4.99.7). Because of the crucial role that lanosterol synthase plays in cholesterol biosynthesis, there is great interest in the identification of drugs that target this enzyme for anticholesteremic purposes. Although most studies on lanosterol synthase in the past have focused on the structural and biochemical functions of this enzyme, almost nothing is known concerning how the synthesis of lanosterol synthase is regulated. Here, we report that histone deacetylase 3 (HDAC3) represses transcription from the lanosterol synthase promoter. Overexpression of HDAC3 decreases, whereas knockdown of HDAC3 by small interfering RNA increases, endogenous lanosterol synthase mRNA in cells. Similarly, in transient transfection assays, overexpression of HDAC3 decreases, whereas depletion of HDAC3 increases, expression of a reporter gene under the control of the lanosterol synthase promoter. Stable cell lines that overexpress HDAC3 show a decrease in lanosterol synthase mRNA and have lower cholesterol concentrations compared with parental cells. Extensive promoter analyses coupled with chromatin immunoprecipitation assays reveal that the transcription factor YY1 binds to and recruits HDAC3 to the lanosterol synthase promoter. Together, our results demonstrate that HDAC3 represses the synthesis of a key regulatory enzyme and reveal a novel mechanism by which the cholesterol biosynthetic pathway can be regulated.

Cholesterol, cholesterol metabolites, and biosynthetic precursors of cholesterol play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance, and calcium metabolism (1). Although important for many normal cellular functions, cholesterol can have negative effects when it reaches excess concentrations, contributing to several diseases (2), of which the most notable is atherosclerosis.

Cellular cholesterol homeostasis is achieved by influx and efflux and intracellular transport as well as by regulation of cholesterol synthesis (3). The rate-limiting enzyme in cholesterol synthesis is HMG-CoA reductase (HMGR),2 and high cholesterol accelerates degradation of HMGR (4). Another mechanism in the regulation of cholesterol synthesis involves the membrane-bound transcription factors sterol regulatory element-binding proteins (SREBPs). The promoters of sterol-regulated genes contain a sterol response element. SREBPs are synthesized as transcriptionally inactive endoplasmic reticulum transmembrane proteins. At high cholesterol levels within the cell, SREBPs remain in the endoplasmic reticulum in association with two proteins, SCAP and Insig (5). A decrease in cholesterol levels causes a conformational change in the sterol-sensing domain of SCAP, leading to the dissociation of Insig and allowing SREBP-SCAP to reach the Golgi (6). Two proteases in the Golgi release the active form of SREBP, which then translocates to the nucleus to activate transcription of genes needed for lipid synthesis (7, 8).

The cholesterol molecule is synthesized from acetate in a series of ~30 enzymatic reactions (for review, see Refs. 9–11). The first sterol intermediate, lanosterol, is formed by the condensation of the 30-carbon isoprenoid squalene, and subsequent enzymatic reactions are designated as the post-squalene half of the pathway. Because lanosterol is the parental compound of all steroids in mammals, precise control of lanosterol synthesis has obvious biological and medical significance.

Lanosterol synthase (EC 5.4.99.7; oxidosqualene-lanosterol cyclase; 2,3-epoxysqualene-lanosterol cyclase) catalyzes the cyclization of (S)-2,3-oxidosqualene to lanosterol in the reaction that forms the sterol nucleus. The human lanosterol synthase gene encodes a 732-amino acid protein with a predicted molecular mass of 83 kDa (12). The gene is located on chromosome 21q22.3 between marker D21S25 and 21qter (13). Mutation in the lanosterol synthase gene causes cholesterol defi-
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iciency, which leads to cataractogenesis in Shumiya cataract rats (14). Two crystal structures of human lanosterol synthase have been reported (15). The enzyme consists of two (α/α) barrel domains that are connected by loops and three smaller β-structures. A large active-site cavity is located in the center of the molecule between the two domains. Because lanosterol synthase plays a central role in the biosynthesis of cholesterol and other sterols, it is important to understand not only the structure and function of this enzyme but also its regulation.

Histone deacetylase (HDAC) enzymes catalyze the removal of acetyl moieties from the ε-amino groups of conserved lysine residues in the N-terminal tail of histones. The removal of this modification strengthens histone-DNA interactions and may generate specific docking surfaces for proteins that regulate chromatin folding and/or transcription. Transcriptional repression of many eukaryotic genes can be attributed to histone deacetylation. Interestingly, results from numerous recent studies overwhelmingly support the prediction that HDACs not only play crucial roles in gene transcription but also most likely affect all eukaryotic biological processes that involve chromatin.

In humans, HDACs are divided into three categories (for review, see Ref. 16); that is, the class I RPD3-like proteins (HDAC1, HDAC2, HDAC3, and HDAC8), the class II HDA1-like proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), and the class III SIR2-like proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7). The class II enzymes can be further divided into two subclasses: IIA (HDAC4, HDAC5, HDAC7, and HDAC9) and IIB (HDAC6 and HDAC10). HDAC11, the most recently discovered human HDAC, uniquely shares sequence homology with the catalytic domains of both class I and II HDAC enzymes. The class III proteins do not exhibit any sequence similarity to HDAC family members from class I or II and differ from other HDACs in that they require the cofactor NAD⁺ for activity.

In this study using expression profiling to identify genes whose expression is potentially regulated by HDACs, we found that lanosterol synthase gene expression is activated by the HDAC inhibitor trichostatin A and repressed selectively by HDAC3. Furthermore, our data suggest that the transcription factor YY1 targets HDAC3 to the lanosterol synthase gene promoter and modifies the acetylation status of histones to repress lanosterol synthase expression. These findings confirm and extend the importance of HDACs in transcription regulation and present a potential novel mechanism by which lanosterol synthase and ultimately cholesterol synthesis can be regulated by HDAC3.

EXPERIMENTAL PROCEDURES

Cell Cultures—HeLaS3 and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium and modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO₂. CHO cells were cultured in a 1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. SRD-13A cells, kindly provided to us by Michael Brown, were cultured in the same media as described for CHO, supplemented with 5 μg/ml cholesterol, 50 μM sodium mevalonate, and 20 μM sodium olate as described (17).

To establish stable cell lines, cells were cultured at 90% confluence in 6-well dishes and then transfected with 4 μg of a plasmid expressing FLAG-HDAC3 plus 200 ng of a plasmid that expresses Genetin (G418) resistance. Control cells were transfected with a plasmid expressing FLAG-HDAC3 alone. One day later, cells were subcultured and grown in the presence of 400 μg/ml G418 (Invitrogen). After about 2 weeks, single G418-resistant colonies were obtained by serial dilution in 24-well dishes. Colonies then were maintained in a medium containing 200 μg/ml of G418 and analyzed individually for expression of FLAG-HDAC3.

Microarray Analysis—Microarray analysis was performed using the Affymetrix GeneChip instrument system following protocols established by Affymetrix, Inc. Total cellular RNA was prepared with TRIzol reagent (Invitrogen) and further purified using an RNeasy kit (Qiagen). RNA was converted to double-stranded cDNA using an oligo(dT)₂₄ primer containing a T7 RNA polymerase recognition sequence, and the resulting double-stranded cDNA was transcribed into biotin-labeled cRNA using T7 RNA polymerase. Biotinylated cRNA was hybridized to the Affymetrix human genome U133 Plus 2.0 array set, which contains ~47,000 full-length human genes and EST clusters. Hybridized RNA was visualized by staining the biotinylated cRNA with streptavidin-conjugated phycoerythrin. Scanned chip images were analyzed using GeneChip algorithms.

Reverse Transcription-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen), and 1 μg of the material was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. One-tenth of the cDNA generated was used as a template for each PCR reaction. Primers used were 5’-GGCAGACGTGACCTACCT-3’ (sense) and 5’-GAAA-GTGACCACCAACTC-3’ (antisense) for human lanosterol synthase and 5’-GCTGTCGTCGCAACCGCTC-3’ (sense) and 5’-CAACACATGATCCTGGTGACTCT-3’ (antisense) for human β-actin. Signal intensities were obtained from ethidium bromide-stained gels and qualified using the ImageQuant 5.2 software (GE Healthcare).

For mRNA analysis in CHO and SRD-13A cells, RNA was extracted using TRIzol reagent (Qiagen), and cDNA was obtained with the iScript cDNA synthesis kit (Bio-Rad). Target mRNA was quantitated using MyIQ single color real time PCR detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). Lanosterol synthase primers (sense 5’-CCCTAAGACTATTGTCGCTCT-3’ and antisense 5’-TTAGGGTGTGGTGAT-3’), hmg-CoA reductase primers (sense 5’-GACTGTGAGTGAAGTGTGTC-3’ and antisense 5’-ATGGAGCCGTGATGAGTT-3’), squalene epoxidase primers (sense 5’TCCAGAGATGGAGAGAA-3’ and antisense 5’-TGATATGTGCGCTGACAG-3’), mevalonate kinase primers (sense 5’-ATCGGTATTAAGCAGGTGTG-3’ and antisense 5’-GATTGCCAGGTACAGGATGA-3’), and GAPDH primers (sense 5’-ATGGCCCTCCGTGTTCTC-3’ and anti-
TTCCCTCTGTCTGCTA-3' (sense) and 5'-AATTTGTGAGGAGGGTCCCGAG-3' (antisense), -1212/ -915 5'-AGTTGAGGTGGGTAGAAGGA-3' (sense) and 5'-GCCCTAGGAAGAAATAGG-3' (antisense), -1545/ -1195 5'-AGTTGAGGTGGGTAGAAGGA-3' (sense) and 5'-CCTACCCCAGGCCAATCTCC-3' (antisense), -1800/ -1305 5'-CCTAGCTTGGCCAGGTCTGCA-3' (sense) and 5’-GGCGGGGTTTATGACAATCAA-3’ (antisense). pBS/U6-Y1 was constructed by inserting an oligodeoxynucleotide corresponding to nucleotides 1095–1114 of YY1 cDNA (NM003403.3) (sense 5’-GCGGAGAAGAGCAGTACGAA-3’ and antisense 5’-AGCTTTCTGCAACCTGTCTGCTC-3’ ) into pBS/U6 vector. The second inverted sequence then was inserted into the intermediate plasmid to generate the final product. All constructs were confirmed by DNA sequencing. Expression plasmids for class I and class II FLAG-HDACs (20), FLAG-HDAC3 deletions (21), and FLAG-YY1, anti-GAPDH, and anti-TFIIB were purchased from Santa Cruz Biotechnology, and anti-HMGR was purchased from Cell Signalingtechnology.

Transient Transfection and Luciferase Reporter Assays—All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For reporter assays, cells grown in 6-well dishes were harvested with 250 μl of passive lysis buffer (Promega). Protein concentrations of all samples were determined using Bradford reagent (Bio-Rad), and the relative light unit values were measured in a luminometer using the luciferase kit from Promega. All assays were carried out in triplicate, and Western blots were performed to evaluate protein expressions.

Chromatin Immunoprecipitation (ChIP) Assays and Double-Chip Assays—ChIP studies were performed as described (23) with modifications. All steps were carried out at 4 °C. Cultures of HeLa, HD3.3, HepG2, and HD3.8 cells grown on 100-mm dishes were incubated for 10 min with 1% formaldehyde (Sigma) under gentle agitation. Cross-linking was stopped by the addition of 0.125 mM glycerol for 5 min. The cells were washed with 10 ml of PBS, scraped off in the same volume of PBS, and collected by centrifugation at 1000 × g for 5 min. The cell pellet was resuspended in lysis buffer (50 mM Heps (pH 7.8), 20 mM KCl, 3 mM MgCl2, 0.1% Nonidet P-40, and a mixture of protease inhibitors) and incubated for 10 min on ice. The cell extract was collected by centrifugation at 1000 × g for 5 min, resuspended in sonication buffer (50 mM Heps (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a mixture of protease inhibitors), and incubated for 10 min on ice. To reduce the length of the chromatin fragments to ~500 bp (confirmed by electrophoretic analysis and PCR), the extract was sonicated using 20 10-s pulses at 5 watts with a 10-s rest between pulses. After centrifugation at 16,000 × g, the supernatant was collected, frozen in liquid nitrogen, and kept at -80 °C. An aliquot was used for A260 measurements. Cross-linked extracts (6 units A260) were resuspended in a sonication buffer to a final volume of 500 μl. The samples were precleared by incubation with normal mouse IgG plus protein G-agarose (Santa Cruz Biotechnology) for 2 h at 4 °C with agitation. After centrifugation at 1000 × g for 5 min, the supernatant was collected and immunoprecipitated with either an anti-FLAG agarose-conjugated (Sigma) or anti-HDAC3 antibody. The immunocomplexes (except from FLAG/agarose conjugates) were recovered with...
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A

Genes down-regulated by over-expression of HDAC3
Genes up-regulated by knock-down of HDAC3
Genes up-regulated by TSA

B

Relative lanosterol synthase signal

C

Fold over control signal

D

RT-PCR
LS

RT-PCR
β-actin

Control HDAC3 HDAC3 siRNA Control HDAC3 HDAC3 siRNA

HeLa HepG2

75 kDa 50 kDa 37 kDa 27 kDa 75 kDa 50 kDa 37 kDa 27 kDa

Control HDAC3 HDAC3 siRNA Control HDAC3 HDAC3 siRNA
the addition of 30 µl of protein-A agarose beads and subsequent incubation for 2 h at 4 °C with agitation. The complexes were washed twice with sonication buffer, twice with sonication buffer plus 500 mM NaCl, twice with LiCl buffer (20 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, and 0.5% Triton X-100), and twice with TE buffer (2 mM EDTA and 50 mM Tris-HCl (pH 8.0)), with the solution incubated at each washing for 5 min at 4 °C. The protein-DNA complexes were eluted by incubation with 100 µl of elution buffer (50 mM NaHCO₃ and 1% SDS) for 15 min at 65 °C. After centrifugation at 1000 g for 5 min, the supernatant was collected and incubated with 10 µg of RNase A per ml for 1 h at 42 °C. NaCl was added to the mixture to a final concentration of 200 mM and incubated at 65 °C to reverse the cross-linking. The proteins were digested with 200 µg/ml of proteinase K for 2 h at 50 °C. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation using glycogen as a precipitation carrier. The primers used were the same used for cloning the segments of the lanosterol synthase promoter. For double-ChIP assays, the immunoprecipitated complexes obtained by the first ChIP were eluted by incubation for 30 min at 37 °C in 25 µl of 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with sonication buffer and subjected to the ChIP procedure.

Cellular Cholesterol Quantification—Cholesterol concentrations were determined using a previously published protocol (24) with modifications. Briefly, cells were cultured up to 90% confluence in 100-mm dishes. Monolayers were washed with PBS, allowed to dry, then incubated in 200 µl of chloroform:methanol (2:1) solution containing 5 mM butyl hydroxytoluene and 10 mg/ml cholesteryl heptadecanoate and cholesteryl propyl ether as internal standards. Chloroform:methanol extracts were dried under nitrogen. Subsequently, free and total cholesterol were determined by high-performance liquid chromatograph (HPLC) before and after saponification using methanolic KOH as previously described (24). Dry extracts were reconstituted in 1.5 ml of acetonitrile by sonication (15 min) and filtered by Millipore 0.22-µm filters, and cholesterol and cholesteryl esters were separated using reverse phase HPLC at 35 °C on a C-18 column (Supelco) using an eluent of acetonitrile-isopropanol 30:70 (v/v) and 210-nm HPLC-diode array detection (Shimadzu SPD-M10Avp). The concentrations were calculated by integrating areas under the curve using proper sterol standards and corrected by internal standard area. Values are expressed as a ratio between the total cholesterol and total protein present in each sample.

Detection of Metabolically Labeled Cholesterol—Thin-layer chromatographic detection of metabolically labeled lipids was performed as previously described (25). HeLa cells were transfected with either a plasmid expressing HDAC3 or an empty vector using Lipofectamine 2000. Twenty-four hours after transfection, cells were incubated with 1 mM [2-14C]acetate for an additional 24 h and then harvested. Cell pellets were incubated with 2 ml of 40% aqueous potassium hydroxide solution and 2 ml of ethanol for 45 min at 80 °C and then brought to room temperature. After extraction with hexanes, the extracts were evaporated to dryness, resuspended in 100 µl of hexane-chloroform (70:30 v/v), and spotted onto a silica gel thin-layer chromatography plate that was developed using petroleum ether/diethyl ether/acetic acid (60:40:1). Authentic samples of cholesterol (10–100 µg) were spotted onto adjacent lanes, and migration positions were visualized by exposing the developed and dried plate to iodine vapor. The plate was then exposed to BioMax MR film (Eastman Kodak Co.) for 6 days. Spots corresponding to cholesterol were scraped from plates into scintillation vials and quantified in a scintillation counter. Samples were normalized by protein concentrations from an aliquot saved before the organic extractions. Final values are expressed as pmol of [2-14C]cholesterol × h⁻¹ × mg⁻¹ of protein. For controls, fatty acid synthesis was measured as described (26). Briefly, aqueous phases from first lipid extractions were acidified with HCl and then extracted three times with petroleum ether. Samples were evaporated, resuspended in chloroform, and then spotted onto a silica gel thin-layer chromatography plate using heptane/diethyl ether/acetic acid (90/30/1) as solvent. Fatty acids (Rf 0.30) were visualized by iodine vapor and quantified as described for [2-14C]cholesterol.

RESULTS

HDAC3 Represses Lanosterol Synthase Expression—Initially, the goal of our study was to identify HDAC3-regulated genes that not only are repressed in response to overexpression of HDAC3 but also de-repressed in response to the knockdown of HDAC3 and activated by trichostatin A treatment (Fig. 1A). HDAC3 was overexpressed or knocked down by RNAi in HeLa cells, and expression profiles from these cells were obtained with microarray analyses. One of the genes repressed by HDAC3 encodes for lanosterol synthase (Fig. 1B; see the supplemental table, Affymetrix code 211018_at). As expected, treatment of cells with a general class I and class II HDAC inhibitor, trichostatin A, resulted in the activation of lanosterol synthase. Similarly, knockdown of HDAC3 by siRNA resulted in an increase in lanosterol synthase expression. Interestingly, although HDAC3 can modulate expression of several different enzymes in the cholesterol synthetic pathway, it appeared that lanosterol synthase was uniquely down-regulated by HDAC3. In fact, with the exception of lanosterol synthase, all other genes encoding enzymes involved in the synthesis of cholesterol that we have examined were up-regulated by HDAC3 (Fig. 1C).

FIGURE 1. Analysis of the effect of HDAC3 on genes involved in the cholesterol synthesis pathway. A, Illustration of HDAC3 target genes. B and C, data collected from HeLa cells using the microarray Affymetrix GeneChip were plotted showing -fold increase/decrease over control. T h e y a x denotes no differences from the control value. For panel C, the Enzyme Commission number is shown next to each enzyme name. D, upper panel, HeLa cells were transfected with vector alone or plasmids expressing FLAG-tagged HDAC3 or RNAi for HDAC3. Total RNA was isolated 36 h after transfection and cDNA was synthesized using specific antisense oligos for lanosterol synthase (LS) or β-actin. CDNA was used for amplification of specific sequences for lanosterol synthase or β-actin. Signal intensities obtained from ethidium bromide-stained gels were quantified using the ImageQuant 5.2 software (GE Healthcare). RT, reverse transcription. Lower panel, cells were treated identically as in the upper panel, lysed 36 h after transfection, and subjected to SDS-PAGE followed by Western blots with the indicated antibodies to monitor protein expression and equal loading.
To confirm that HDAC3 repressed expression of lanosterol synthase, reverse transcription-PCR was performed using mRNA derived from HeLa and HepG2 cells with either overexpression or knockdown of HDAC3. As shown in Fig. 1D, compared with mock-transfected control cells, overexpression of HDAC3 resulted in a decrease in luciferase activity both in HeLa and HepG2 cells. In contrast, expression of HDAC3 siRNA, but not HDAC2 siRNA, caused an increase in luciferase activity.

Although our data convincingly showed that HDAC3 repressed lanosterol synthase, it did not rule out the possibility that other class I and class II HDAC members also could regulate lanosterol synthase. To test that possibility, we transfected HeLa cells with all class I and class II HDAC-expressing plasmids together with pLS-Luc, and luciferase assays were performed. As shown in Fig. 2A, overexpression of class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10) and HDAC11 had no appreciable effect on luciferase activity. For class I HDACs, we found that HDAC1 and HDAC3, but not HDAC2 or HDAC8, repressed the lanosterol synthase promoter. At this time we do not know whether HDAC1 siRNA activated the lanosterol synthase promoter, and we do not yet know the exact mechanism by which HDAC1 repressed lanosterol synthase. Whatever the mechanism, unlike HDAC3, we found that overexpression of HDAC1 under these conditions resulted in non-selective repression of a large number of different promoters, both related and unrelated to cholesterol synthesis (data not shown). Thus, we focused our further analysis solely on HDAC3.

Overexpression of HDAC3 Decreases Intracellular Cholesterol—Because HDAC3 specifically down-regulated lanosterol synthase but not other enzymes in the cholesterol synthetic pathway, we focused our studies to address the consequences of lanosterol synthase repression by HDAC3. In particular, we evaluated if overexpression of HDAC3 could affect intracellular cholesterol levels. We established two stable cell lines overexpressing FLAG-HDAC3 from HeLa (HD3.3) and HepG2 (HD3.8) cells (Fig. 3A). These cells expressed less lanosterol synthase reporter in pGL3Basic, and the resultant plasmid (pLS-Luc) was transfected into HeLa and HepG2 cells in the presence of expression plasmids for HDAC3. As shown in Fig. 2A, compared with mock-transfected control cells, overexpression of HDAC3 resulted in a decrease in luciferase activity both in HeLa and HepG2 cells. In contrast, expression of HDAC3 siRNA, but not HDAC2 siRNA, caused an increase in luciferase activity.

To study the mechanisms by which HDAC3 repressed lanosterol synthase, we isolated a human genomic DNA fragment containing 1.8 kilobases of the lanosterol synthase promoter. The cloned genomic DNA was inserted upstream of the luciferase reporter in pGL3Basic, and the resultant plasmid (pLS-Luc) was transfected into HeLa and HepG2 cells in the presence of expression plasmids for HDAC3. As shown in Fig. 2A, compared with mock-transfected control cells, overexpression of HDAC3 resulted in a decrease in luciferase activity both in HeLa and HepG2 cells. In contrast, expression of HDAC3 siRNA, but not HDAC2 siRNA, caused an increase in luciferase activity.

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FIGURE 3. Quantification of cholesterol in HDAC3 overexpressing cells. A, Western blots to assess protein expression in HD3.3 and HD3.8 stable cell lines compared with parental cells. Anti-GAPDH blots were used as loading controls. B, total RNA were purified from HD3.3 and HD3.8 stable cell lines and parental cells, and reverse transcription-PCR were performed to quantify lanosterol synthase (LS) mRNA. β-Actin was used as a loading control. C, HD3.3 and HD3.8 stable cell lines and parental cells were cultured in low-cholesterol media containing 2.5% fetal calf serum. At about 90% confluence, cells were harvested and processed to determine cholesterol concentrations by HPLC using cholesteryl propyl ether as the internal standard. Values are expressed as the ratio between total cholesterol and total protein in each sample. The data are expressed as the mean ± S.D. (denoted by error bars) from three separate experiments. D, HeLa cells were transfected with either a plasmid expressing HDAC3 or an empty vector (Control). After incubation with 2-[14C]acetate, lipids were extracted, and samples were spotted onto a silica gel thin-layer chromatography plate. Migration positions were visualized by exposing the developed and dried plate to iodine vapor and exposing the plate to film. Spots previously identified as cholesterol or fatty acids were scrapped into scintillation vials and quantified in a scintillation counter. Samples were normalized by protein concentration from an aliquot saved before the organic extractions. The data are expressed as the mean ± S.D. from three separate independent experiments. A representative result is displayed in the right panel. E, proteins derived from HD3.3 and HD3.8 stable cell lines and parental cells were subjected to Western blot analysis using an antibody specific for HMGR. Anti-GAPDH blots were done to assess equal loading. F, CHO (upper panel) and SRD-13A (lower panel) cells were transfected with vector alone or plasmids expressing FLAG-tagged HDAC3 or RNAi for HDAC3. Total RNA was isolated 36 h after transfection, and cDNA was synthesized and used for amplification of specific sequences. Products were quantified using MyiQ single color real time PCR detection system and iQ SYBR green Supermix. The data are expressed as mean ± S.D. from three separate experiments.
synthase mRNA compared with parental cells as assayed using reverse transcription-PCR (Fig. 3B). More significantly, these HDAC3 overexpressing cells showed lower intracellular cholesterol concentrations compared with parental cells (Fig. 3C), suggesting that HDAC3 plays a critical role in the regulation of cholesterol biosynthesis through repression of lanosterol synthase.

To confirm that HDAC3 affects cholesterol biosynthesis, we used standard [14C]acetate labeling studies to examine changes in cholesterol and post-squalene sterol intermediates in response to HDAC3. As shown in Fig. 3D, left and right panels, overexpression of HDAC3 resulted in a significant decrease in cholesterol and sterol intermediates in HeLa cells. As a control,
we showed that HDAC3 had negligible effects on fatty acids synthesis (middle panel).

High cholesterol accelerates degradation of HMGCR, the rate-limiting enzyme in cholesterol synthesis by promoting association of its sterol-sensing domain with Insig (27). This negative feedback could explain our observation that, whereas HDAC3 repressed lanosterol synthase gene expression, it paradoxically activated HMGCR (Fig. 1C). Western blots revealed that the protein levels of HMGCR indeed were higher in HD3.3 and HD3.8 cells compared with parental cell lines (Fig. 3E).

In contrast to lanosterol synthase, several genes in the cholesterol biosynthetic pathway unexpectedly appear to be up-regulated by HDAC3 (Fig. 1C), which could be a reflection of counter regulation by SREBPs. To examine the contribution of SREBPs on HDAC3-mediated regulation of cholesterol synthetic genes, we examined the effects of HDAC3 on gene expression of several enzymes in the pathway using a mutant CHO cell line, SRD-13A, that cannot cleave SREBPs due to mutations in SCAP (17). As shown in Fig. 3F, overexpression of HDAC3 in SRD-13A resulted in different effects ranging from repression of lanosterol synthase and squalene epoxidase, to no effect on mevalonate kinase, to a slight activation of HMG-CoA reductase.

Multiple Regions of the Lanosterol Synthase Promoter Respond to HDAC3 Repression—To define the region on the lanosterol synthase promoter responsible for transcriptional repression by HDAC3, we examined the effects of overexpression and knockdown of HDAC3 using reporter plasmids containing deletion mutations of the lanosterol synthase promoter. Signal intensities obtained from ethidium bromide-stained gels were quantified using the ImageQuant 5.2 software (GE Healthcare). Right panels show the PCR results for serial dilutions of the input material used for ChIPs.
FIGURE 6. Effects of YY1 on Lanosterol Synthase promoter activity. A, schematic representation of the lanosterol synthase (LS) promoter with putative transcription factor binding sites. The arrow indicates the transcriptional initiation site. B and C, lanosterol synthase promoter or different fragments of the lanosterol synthase promoter fused to the β-actin promoter upstream of the luciferase reporter gene were transfected into HeLa cells with or without plasmids expressing HDAC3, HDAC3 siRNA, YY1, YY1 siRNA. Luciferase activity was determined 36 h after transfection. Results from the average of three independent experiments ± S.D. are shown. Protein expressions in transfected cells were assessed by Western blots, and representative blots are shown (bottom panel).
the fact that endogenous HDAC3 is highly expressed and already saturated in HeLa cells. Like HeLa cells, siRNA knockdown of HDAC3 resulted in an increase in luciferase activity in all deletion mutants examined in HepG2 cells. However, more importantly, overexpression of HDAC3 in HepG2 cells significantly repressed all lanosterol synthase promoter deletion mutants. Thus, our results clearly suggest that multiple regions of the lanosterol synthase promoter can confer repression by HDAC3.

To analyze the effect of HDAC3 on different regions of the lanosterol synthase promoter, we fused different segments of the lanosterol synthase promoter to a minimal β-actin promoter, which is non-responsive to HDAC3, upstream of the luciferase reporter. As shown in Fig. 4B, siRNA knockdown of HDAC3 consistently resulted in an increase in the luciferase activity of all promoter fragments, with the fragments containing −1212/−912 and −1545/−1195 possessing the most pronounced effect.

**HDAC3 Is Recruited to the Lanosterol Synthase Promoter**—To determine whether the repressive effect of HDAC3 on the lanosterol synthase promoter was a result of direct recruitment of HDAC3 to the promoter, we performed ChIP assays covering different segments of the promoter in HeLa as well as HD3.3 cells. Although small quantities of HDAC3 were detected binding throughout the lanosterol synthase promoter, we found that more HDAC3 was present in two segments: −1212/−912 and −1545/−1195 (Fig. 5). These results fit well with the observation that these two fragments are the chief targets of transcription repression.

**Repression of the Lanosterol Synthase Promoter Is Mediated by YY1**—Many DNA binding transcription factors associate with HDAC3 to modify histones and produce alterations in chromatin structure that block transcription (28). We performed a thorough inspection of the lanosterol syn-

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**FIGURE 7. Identification of YY1 and HDAC3 binding regions on the lanosterol synthase promoter.** Cross-linked chromatin prepared from HeLa cells transfected with FLAG-YY1 was precipitated with the indicated antibodies. A fraction of each immunoprecipitated product was subjected to a second immunoprecipitation. PCR analysis was performed using the same primers described in Fig. 5 that were specific for different regions of the lanosterol synthase promoter.
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A

B

FIGURE 8. Analysis of the HDAC3-YY1 interaction. A, HeLa cells were transfected with plasmids that express either wild type FLAG-HDAC3 or FLAG-HDAC3 deletion mutants. Lysates were prepared from transfected cells and immunoprecipitated (IP) with anti-FLAG antibody followed by Western blot (WB) analysis with anti-YY1 (top panel). In separate experiments, HeLa cells were transfected with plasmids that express either wild type FLAG-HDAC3 or FLAG-HDAC3 deletion mutants plus a plasmid that expresses hemagglutinin-tagged YY1. Lysates were prepared from transfected cells and immunoprecipitated with anti-hemagglutinin antibody followed by Western blot analysis with anti-FLAG (second panel from the bottom). Protein expression and immunoprecipitation efficiencies were assessed by Western blot with the indicated antibodies. LU, luciferase units. B, HeLa cells were co-transfected with pLS-Luc and plasmids expressing wild type or HDAC3 deletion mutants. Luciferase activity was measured 36 h after transfection, and signals were normalized to protein concentrations. Results are tabulated from the average of three independent transfections ± S.D.

Next, using ChIPs and double-ChIPs, we determined if YY1 interacted with the lanosterol synthase promoter simultaneously with HDAC3. In ChIP assays we found that FLAG-YY1 preferentially bound to −1212/−912, −1545/−1195, and −1800/−1307 in the lanosterol synthase promoter (Fig. 7, lane 6). However, in a double-immunoprecipitation with HDAC3 first followed by a second immunoprecipitation with anti-FLAG, we found that FLAG-YY1 bound more to segments −1212/−912 and −1545/−1195 (Fig. 7, lane 3). Likewise, in a reciprocal experiment with anti-FLAG immunoprecipitation first followed by a second immunoprecipitation with anti-HDAC3, HDAC3 bound slightly more to −1212/−912 and −1545/−1195 (Fig. 7, lane 7). We conclude that YY1 binds to at least three regions on the lanosterol synthase promoter, with −1212/−912 and −1545/−1195 being the most critical for recruitment of HDAC3.

YY1 Interacts with the C Terminus of HDAC3—Consistent with our earlier observations (29), HDAC3 interacted with YY1 (Fig. 8A). To define the exact region of HDAC3 that interacts with YY1, we performed additional co-immunoprecipitation assays using different segments of HDAC3 fused to the FLAG epitope. As shown in Fig. 8A, C-terminal deletion mutants of HDAC3 (1–122, 1–180, 1–313, and 1–373) did not bind YY1, whereas fragments that contain the C-terminal domain of HDAC3 (122–428 and 180–428) interacted well with YY1. In functional assays, whereas overexpression of HDAC3 C-terminal deletion mutants had no effect on transcription of the lanosterol synthase promoter, overexpression of wild type or the C-terminal portion of HDAC3 effectively repressed the lanosterol synthase promoter (Fig. 8B). Taken together, our results suggest that YY1 recruits HDAC3 to repress lanosterol synthase expression by a specific interaction with the C-terminal domain of HDAC3.

DISCUSSION

Inhibitors of cholesterol biosynthesis have great potential as therapeutic tools to lower plasma cholesterol levels and reduce the risks of coronary heart disease. Traditionally, pharmacological inhibition of cholesterol synthesis has been primarily achieved by inhibition of one of the first enzymes in the chole-
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A specific group of drugs, known as statins, competitively block enzymatic activity of HMGR (30). Many studies have shown that statins have a multitude of other actions, being involved in endothelial function, cell proliferation, inflammatory responses, immunological reactions, platelet function, and lipid oxidation (31). Although statins efficiently reduce serum cholesterol levels, recent research indicates that statins affect not only synthesis of cholesterol, but also, through inhibition of HMGR, affect other intermediates in the cholesterol pathway and, thus, may lead to many undesirable effects. In fact, it has been hypothesized that the use of pravastatin might increase the incidence of extrahepatic cancers (32, 33).

One of the alternative strategies for the regulation of cholesterol synthesis is to alter the synthesis and, therefore, the activity of lanosterol synthase, which acts in the post-squalene half of the cholesterol pathway. In one study, pharmacologically active doses of a lanosterol synthase inhibitor produced less adverse effects than statins in hamsters (34). Several lanosterol synthase inhibitors have been developed, but their potential use as agents to reduce the risk of hypercholesterolemia are far from completely defined (25, 35, 36).

In this study, we discovered a novel approach to inhibit lanosterol synthase. We found that HDAC3 effectively represses the transcription of lanosterol synthase. Our data showing that cells overexpressing HDAC3 have a lower intracellular cholesterol level reveal a possible alternative mechanism by which cholesterol synthesis can be regulated. Mechanistically, our results argue that transcription factor YY1 recruits HDAC3 to a specific region of the lanosterol synthase promoter to repress transcription.

Previous studies from the Osborne (37–39) and Edwards (40) laboratories have shown that YY1 transcriptionally represses SREBP-regulated genes. YY1 is a 414-amino acid Krüppel-related human C2H2 zinc finger transcription factor that binds to the (C/g/a)(G/t)(C/t/a)CATN(T/a)(T/g/c) consensus DNA element located in promoters and enhancers of many cellular and viral genes (41, 42). YY1 is effective both as an activator and as a repressor depending on its relative concentration, its binding partners, and on promoter context. YY1 is expressed ubiquitously and is highly conserved among different species (43–46). Using GenBank™ data base analysis, we estimate that over 8% of all cellular gene promoters contain at least one YY1 consensus-binding site. It is unclear at this time what dictates the ability of YY1 to recruit HDACs to selective promoters, such as the lanosterol synthase promoter, but not other promoters that contain YY1 binding sites. Whatever the mechanism, the presence of ubiquitous YY1 binding sites coupled with the ability of HDAC3 to repress expression of a multitude of genes in addition to lanosterol synthase may limit the potential therapeutic usefulness of activating HDAC3 activity.

Previously, we demonstrated that YY1 interacts with HDAC1, HDAC2, and HDAC3 in vitro and in vivo (29, 47). In addition, we showed that overexpression of HDAC2 enhances the ability of YY1 to repress transcription of a reporter construct containing YY1 binding sites (47). However, our analysis suggests that HDAC2 is clearly not involved in the regulation of the lanosterol synthase promoter. It is unclear at this time what signals govern the ability of YY1 to selectively recruit HDAC3, but not HDAC2, to the lanosterol synthase promoter. What our results clearly advocate is that not only do different HDACs have different histone substrate specificities, but different HDACs also have different promoter specificities.

Another interesting outcome of our study is our finding that transcription factor YY1 binds to the C terminus of HDAC3, suggesting that YY1 most likely targets HDAC3 to the lanosterol synthase promoter via the C terminus of HDAC3. Of the human class I HDACs, HDAC3 is more similar to HDAC1 and HDAC2 than to HDAC8. Uniquely, HDAC3 lacks a small segment found at the extreme N termini of HDAC1, HDAC2, and HDAC8. Regions at the C termini of HDAC1 (residues 399–482) and HDAC2 (residues 400–488) also are absent in HDAC3, and the last 34 residues of HDAC3 have no similarity to any known proteins. In future experiments it would be useful to determine whether other HDAC3 binding transcription factors also interact with HDAC3 via the unique HDAC3 C-terminal domain.

Our finding that HDAC3 represses lanosterol synthase adds to the growing list of genes that are now known to be regulated by HDAC3 in association with other cellular factors. Examples of proteins encoded by genes that are regulated by HDAC3 include the thyroid hormone receptor (48), growth/differentiation factor 11 (20), interferon-γ, STAT1 (49), matrix metalloproteinase-2 (50), Delta-like 1 (51), tumor necrosis factor (52), osteocalcin (53), and Bmal1 (54). A more complete understanding of the mechanisms and biological consequences of HDAC3 repression on lanosterol synthase and understanding why HDAC3 exert effect on the synthesis of multiple different proteins with seemingly unrelated functions will be an active area of future research.

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