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A New Family of Human Histone Deacetylases Related to *Saccharomyces cerevisiae* HDA1p*

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Histone deacetylases are the catalytic subunits of multiprotein complexes that are targeted to specific promoters through their interaction with sequence-specific DNA-binding factors. We have cloned and characterized a new human cDNA, HDAC-A, with homology to the yeast HDA1 family of histone deacetylases. Analysis of the predicted amino acid sequence of HDAC-A revealed an open reading frame of 967 amino acids containing two domains: a NH_2-terminal domain with no homology to known proteins and a COOH-terminal domain with homology to known histone deacetylases (42% similarity to RPD3, 60% similarity to HDA1). Three additional human cDNAs with high homology to HDAC-A were identified in sequence data bases, indicating that HDAC-A itself is a member of a new family of human histone deacetylases. The mRNA encoding HDAC-A was differentially expressed in a variety of human tissues. The expressed protein, HDAC-Ap, exhibited histone deacetylase activity and this activity mapped to the COOH-terminal region (amino acids 495–967) with homology to HDA1p. In immunoprecipitation experiments, HDAC-A interacted specifically with several cellular proteins, indicating that it might be part of a larger multiprotein complex.

Acetylation of core histones, first described by Allfrey and co-workers (1), has been correlated with transcription, chromatin assembly, DNA repair, and recombinational events (2–7). Transfer of an acetyl group from acetyl-CoA onto the ϵ-amino group of different lysines residues in the NH_2-terminal tail of core histones is a ubiquitous modification found in all eukaryotic species examined. Histone acetylation levels are controlled by the competing activities of histone acetyltransferases and histone deacetylases.

Cloning of the first histone acetyltransferase (8) and the first histone deacetylase (9) has led to the identification of a growing number of proteins with similar enzymatic activities (reviewed in Refs. 7 and 10). The characterization of the first histone acetyltransferase and the first histone deacetylase as homologues of *Saccharomyces cerevisiae* GCN5 and *S. cerevisiae* RPD3, respectively, two factors previously described genetically as transcriptional regulators, confirmed the long speculated role of histone modification in eukaryotic transcriptional regulation. Together with the demonstration that acetylation levels of nucleosomal histones change in discrete regions associated with certain promoters (11–13), these results established that chromatin is not only a structural scaffold responsible for DNA compaction in the eukaryotic nucleus but is also an active and dynamic participant in transcriptional regulatory mechanisms.

This model has been recently validated by the demonstration that the enzymatic activity of the yeast histone acetyltransferase GCN5 is necessary for the transactivational activity of this factor (14, 15). Similarly, mutation of amino acids critical for the histone deacetylase activity of RPD3 or HDAC1 reduced partially or totally their repressor activity (16, 17). Additional evidence for the involvement of histone acetylation in transcriptional regulation has come from studies with fungal toxins, such as trichostatin A and trapoxin, that specifically inhibit histone deacetylases. These inhibitors shift the dynamic equilibrium between histone acetylation and deacetylation toward the hyperacetylated state (18). Several reports established a correlation between hyperacetylation of histones and transcriptional activation; for example, both trichostatin A and trapoxin increase the transcriptional activity of the human immunodeficiency virus type 1 promoter *in vivo* and *in vitro* (19, 20). However, other studies have shown that histone deacetylases can also play a significant role in transcriptional silencing. In mammalian cells, inhibition of histone deacetylases by trichostatin A or trapoxin activates or represses a small fraction of cellular genes (21) and disruption of *RPD3* and *SIN3* in *S. cerevisiae* showed that both genes are required to fully activate or repress specific promoters (22–24).

The histone deacetylases identified can be grouped into three families: *S. cerevisiae* RPD3 and RPD3-related proteins in higher organisms, such as HDAC1, 2, and 3 (9, 25–28); *S. cerevisiae* HDA1 and the related *S. cerevisiae* HOS1, 2, and 3 (29, 30); and HD2 isolated from *Zea mays* (31), which presents no homology to the other two families. Biochemical and molecular biological studies in different systems have established that histone deacetylases are components of large multiprotein complexes that are targeted to promoter sites through their interaction with sequence-specific transcription factors (reviewed in Refs. 32 and 33).

In this report, we describe the identification and cloning of a human cDNA with a region of homology to yeast *HDA1*. The

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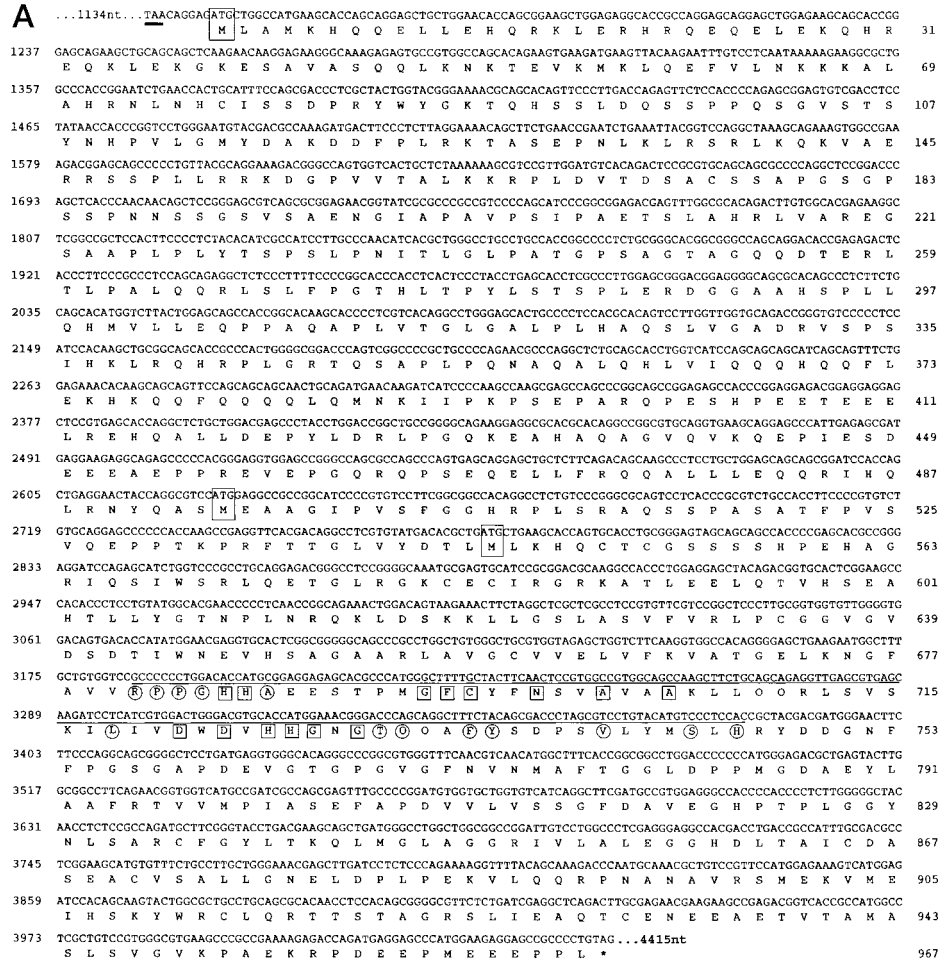

FIG. 1. **HDAC-A encodes a putative new histone deacetylase.** *A,* the nucleotide sequence and the deduced amino acid sequence corresponding to the longest ORF representing HDAC-A are shown. An upstream stop codon (TAA) immediately ahead of the start codon is *underlined*. The presumed start codon (ATG) is indicated by a *box*. Two other ATG codons that were used to generate NH_2-terminal truncations at amino acid positions, 495 and 545, are also indicated by a *box*. Residues conserved within different histone deacetylases and critical for enzymatic activity, as derived from Prodom Release 36, are highlighted (*box,* absolutely conserved; *circle,* very conserved; *underline,* histone deacetylase core region). *B*, alignment of the HDAC-A, NY-CO-9 (HDAC-B), HDA1, and RPD3 proteins. The sequence of NY-CO-9 is not complete. Proteins were aligned with PIMA (version 1.4) and printed with BOXSHADE (version 3.21). Identical residues are in *black*; conserved residues are in *gray*. *C*, schematic representations of HDAC-A, NY-CO-9 (HDAC-B), HDA1, and RPD3 are aligned. Homology values referring to HDAC-A for two different domains, NH_2- and COOH-terminal, are given in percent. Different regions of each protein are indicated by the corresponding amino acid positions.

protein encoded by this cDNA, HDAC-Ap, exhibits a catalytically active histone deacetylase domain *in vivo*. We also identified three additional human sequences with strong homology to HDAC-A that represent additional members of this new family of human histone deacetylases.

MATERIALS AND METHODS

Reagents and Cell Lines—293 cells were grown in Opti-MEM medium (Life Technologies) supplemented with 2% fetal bovine serum (Hyclone), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. HeLa cells were cultured on glass coverslips in Dulbecco's modified Eagle's medium, 10% fetal calf serum using the same supplementations. Trichostatin A was obtained from Wako Pure Chemical Industries, stored at 10 mg/ml in dimethyl sulfoxide at −20 °C, and diluted immediately before use.

Identification and Cloning of HDAC-A—A human brain cDNA library was constructed by reverse transcription of human whole brain poly(A)$^+$ RNA (CLONTECH) with reverse transcriptase (SuperScript II, Life Technologies) and a (dT)15 primer carrying the *Not*I site at the 5′ extremity (5′-pGACTAGTTCTAGATCGCGAGCGGCCGCCC(T)15–3′) according to the supplier's instructions (34). After ligation of the *Sal*I adaptor and *Not*I digestion, the resulting cDNAs were separated on 1% low melting point agarose gels, and cDNA fragments bigger than 9 kb[1] were recovered. These cDNA fragments were then ligated with the *Sal*I/*Not*I-digested pSPORT1 vector and introduced into *Eschericia coli* cells by electroporation (ElectroMax DH10B cells, Life Technologies). Plasmids were extracted from about 10,000 independent ampicillin-resistant colonies grown on agar plates by the standard alkaline/sodium dodecyl sulfate (SDS) method. The isolated plasmids were resolved by agarose gel electrophoresis, and those corresponding to the original fractionated size were retrieved. Clones were randomly selected for dideoxy sequencing with ABI PRISM cycle sequencing and ABI sequencers (Model 373A or 377 DNA, Perkin-Elmer).

Northern Blot Analysis—Multiple human tissue Northern blots and RNA master blots were obtained from CLONTECH. A ^{32}P-labeled probe corresponding to the HDAC-A cDNA was prepared with the Multiprime DNA labeling system (Amersham). Prehybridization, hybridization, and washing of blots were performed using ExpressHyb hybridization solution (CLONTECH) under high-stringency conditions according to standard methods (35). Membranes were also hybridized to a probe corresponding to the human glyceraldehyde-3-phosphate dehydrogenase cDNA to control for the relative amount of mRNA loaded in each lane. Relative expression levels were quantitated with a FUJIX BAS1000 PhosphorImager system and Mac Bas software (Fuji Photo Film Co., Ltd.).

Plasmids—To generate COOH-terminal epitope-tagged constructs of different histone deacetylases, we used polymerase chain reaction (PCR) amplification with a reverse primer containing the sequence for the FLAG peptide. First the following two primers were used to amplify HDAC3: forward, 5′-CCGGATCCGAATTCACCATGGCCAAGACCGTGGCC-3′; backward, 5′-GCTCTAGA**TTA***CTTGTCATCGTCGTCCTTGTAGTC*TCCTCCGAATTCAATCTCCACATCGCTTTCC-3′, (restriction sites are underlined; the sequence encoding the FLAG epitope is italic, and the stop codon is bold). The PCR product was digested with *Bam*HI and *Xba*I and inserted into corresponding sites of the pcDNA3.1(+) vector (Invitrogen) using standard protocols (35, 36). The region coding for HDAC3 was then replaced with PCR fragments encoding each of the other histone deacetylases by subcloning into new *Eco*RI sites that had been engineered into the PCR primers. The following oligonucleotides were used for PCR amplification: HDAC1 forward, 5′-CGGAATTCACGATGGCGCAGACGCAGGGCAC-3′, backward, 5′-CGGAATTCGGCCAACTTGACCTCCTCCTTG-3′; murine HDAC2 (a gift from Dr. Ed Seto) forward, 5′-CGGAATTCACCATGGCGTACAGTCAAGGAG-3′, backward, 5′-CGGAATTCAGGGTTGCTGAGTTGTTCTG-3′; HDAC-A forward, 5′-CGGAATTCCAGGAGATGCTGGCCATGAAG-3′, backward, 5′-CGGAATTCCAGGGGCGGCTCCTCTTC-3′; HDAC-A1–544 forward, 5′-CGGAATTCACCATGCTGGCCATG-

[1] The abbreviations used are: kb, kilobase pair(s); PCR, polymerase chain reaction; ORF, open reading frame.

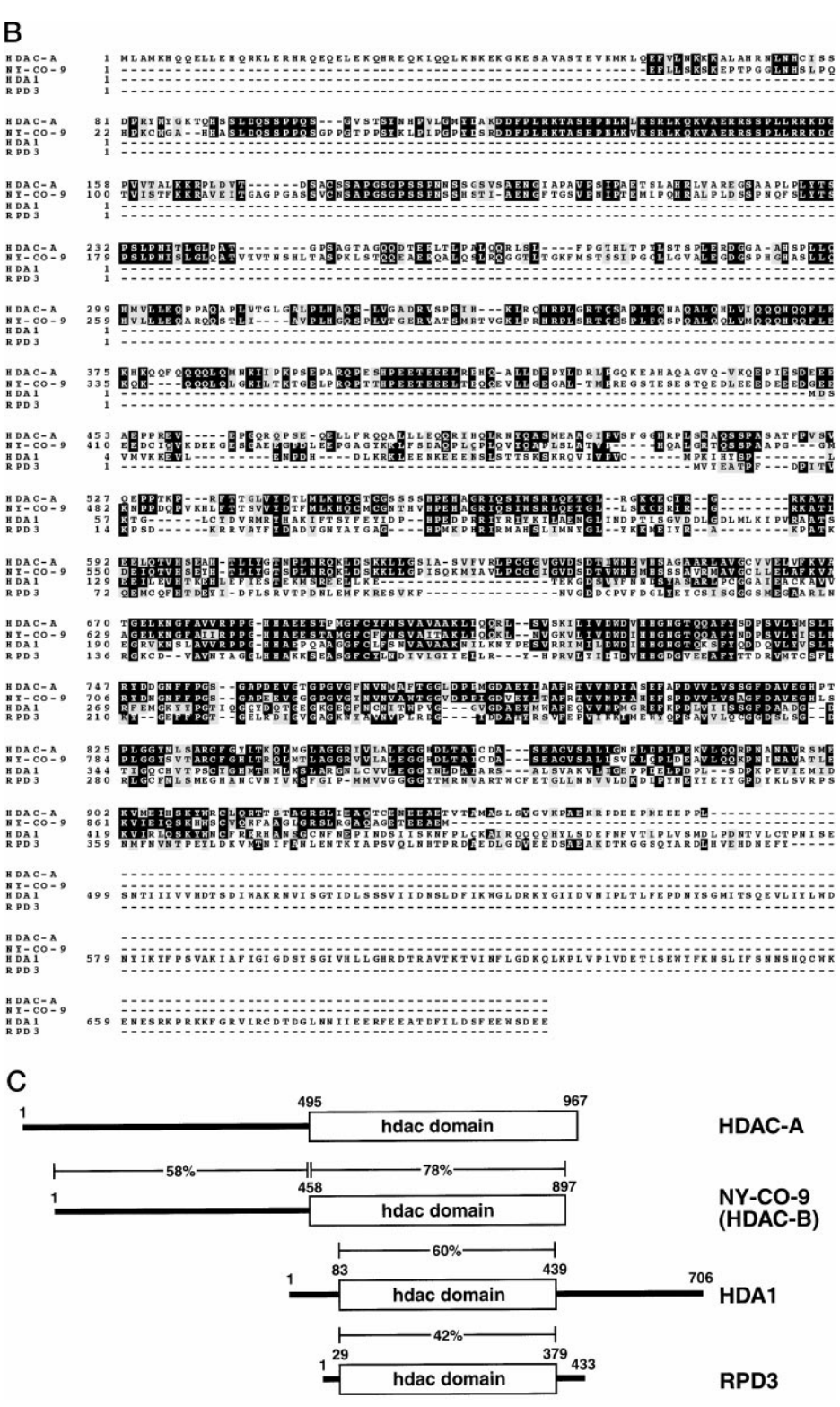

FIG. 1—*continued*

AAGCACC-3′, backward, 5′-CGGAATTCCAGCGTGTCATACACGAGGCC-3′; HDAC-A495–967 forward, 5′-CGGAATTCACCATGGAGGCCGCCGGCATC-3′; HDAC-A544–967 forward, 5′-CGGAATTCACCATGCTGAAGCACCAGTGCACC-3′.

Subcellular Localization of HDAC-A—HeLa cells growing on coverslips were transfected using LipofectAMINE (Life Technologies). After 48–72 h, cells were fixed with 1.0% paraformaldehyde in phosphate-buffered saline for 5 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline and stained as described (37). The primary antibody, anti-FLAG (M2, Sigma), was used at a 1:1000 dilution. Imaging was done on a Leica DM-R epifluorescence microscope with a 100 × or a 63 × 1.4 N.A. PlanApo lens. Images were collected digitally with a cooled 12-bit CCD (Princeton MicroMax). Digital deconvolution was performed as described previously (37).

Immunoprecipitation: Histone Deacetylase Assays—293 cells (5×10^7 cells) were transfected with LipofectAMINE. After 36 h, the cells were harvested and lysed in low stringency lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) in the presence of a protease inhibitor mixture (Boehringer Mannheim). Protein concentrations of extracts were normalized with a modified Lowry assay (Bio-Rad). To control for expression of different constructs, Western blot analysis was performed with the enhanced chemiluminescence procedure (Amersham) as described (28). Protein G–Sepharose was preincubated with bovine serum albumin at 10 mg/ml to reduce nonspecific

TABLE I

HDAC-A belongs to a new family of human histone deacetylases

Human sequences with homology to HDAC-A were identified using BLAST and TBLASTN algorithms searching nonredundant nucleid acid data bases on the GenomNet WWW server (Human Genome Center, University of Tokyo). Accession numbers for cDNA sequences, genomic sequences, and representative EST clones are given. Sequence alignment for genomic clones of HDAC-C and HDAC-D were performed and manually aligned by using the HDAC-A sequence as template.

	Synonym	Genomic location	Accession no.	Genomic sequence	dbEST
HDAC-A	KIAA0288		AB006626		AA101045 N27858 AA3274322
HDAC-B	NY-CO-9 KIAA0600	Chr. 17	AF039691 AB011172	AC004150	AA194873 AA116072 H69274
HDAC-C		Chr. 7p15-p21		AC004994 AC004744 AC002410	AA405905 AA287983
HDAC-D		Chr. 12q31		AC004466	W04418 H09529 N64095

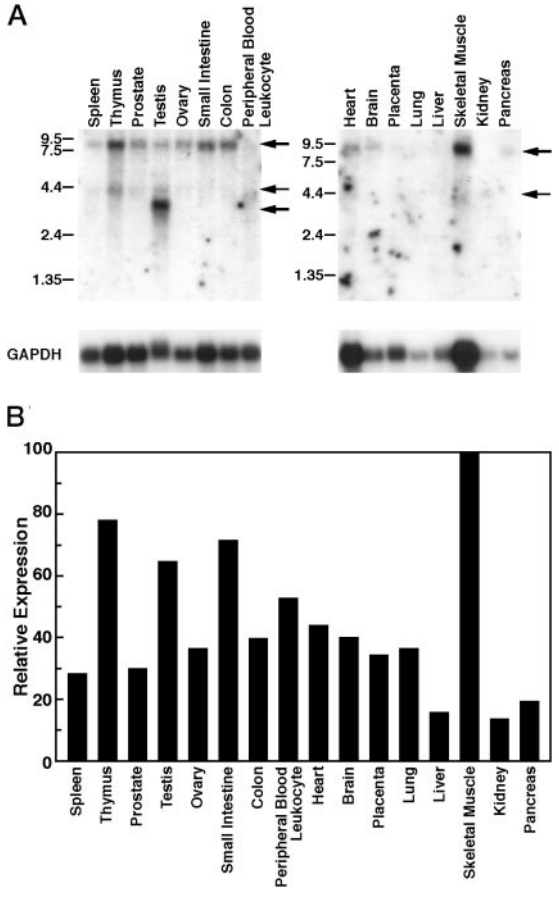

FIG. 2. **Tissue-specific expression of HDAC-A.** *A,* Northern blot analysis of HDAC-A was performed with mRNA isolated from different human tisues. Hybridization to human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is shown as control. The *arrow* indicates the HDAC-A mRNA; an additional band in testis and a series of weaker bands possibly corresponding to a degradation product are also indicated. Molecular weights are shown on the left. *B,* quantitative Northern dot blot analysis for the expression of HDAC-A. Expression levels are relative to the expression level in the tissue with maximum expression (skeletal muscle).

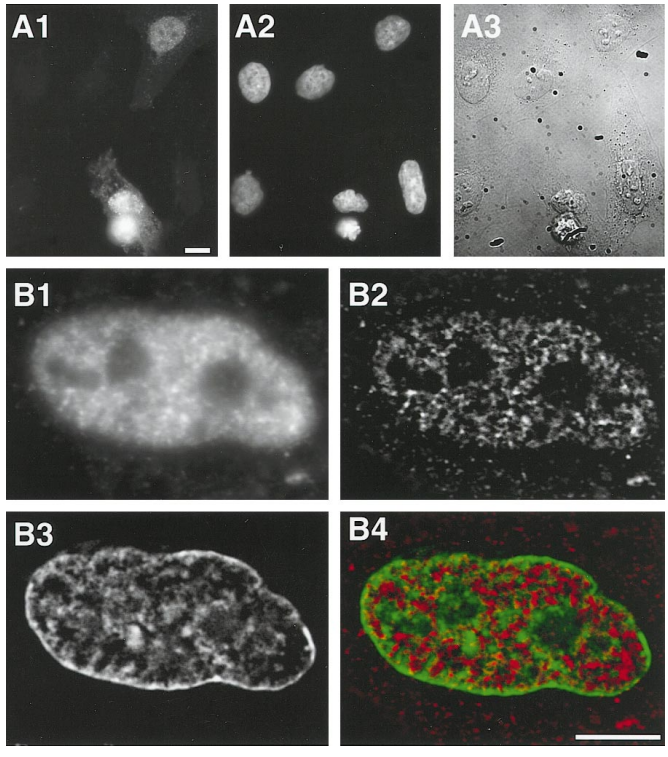

FIG. 3. **HDAC-Ap is a predominantly nuclear protein with a distinct subnuclear localization.** *A*, a field of randomly growing HeLa cells that were transfected with an expression vector encoding FLAG-tagged HDAC-A is shown. *Panel A1* shows staining with the anti-FLAG antibody. *Panel A2* shows staining of DNA using DAPI. *Panel A3* shows the corresponding DIC image. The cell at the bottom is in mitosis and exhibits a diffuse cellular distribution of HDAC-A–FLAG. The upper two cells are in interphase and show predominantly nuclear staining. The *scale bar* represents 10 μm. *B,* HDAC-A–FLAG accumulates in discrete foci within the interchromatin space. The nucleus of a HeLa cell transiently expressing HDAC-A–FLAG was imaged by collecting serial z-sections at 0.4-μm intervals. A single optical section near the center of the cell nucleus is shown. *Panel B1* shows anti-FLAG staining before digital deconvolution. *Panel B2* shows the corresponding digitally deconvolved optical section. *Panel B3* shows the corresponding digitally deconvolved DAPI section. *Panel B4* shows a composite image in which DAPI is *green* and HDAC-A–FLAG is *red*. The *scale bar* represents 10 μm.

binding, and extracts were precleared for immunoprecipitation as described (28). Precleared lysates were immunoprecipitated by incubation with the M2 anti-FLAG antibody (Sigma) at 10 mg/ml overnight at 4 °C. As a control, immunoprecipitations were also performed with the M2 anti-FLAG antibody after a 2-h preincubation at room temperature with a synthetic peptide corresponding to the FLAG epitope (100-fold molar excess). Immune complexes were recovered by adding 20 μl of the preblocked 50% protein G–Sepharose slurry for 4 h at 4 °C and washing

three times with low stringency lysis buffer, twice with lysis buffer containing 0.5 M NaCl, and twice with histone deacetylase buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10% glycerol). For inhibition studies, the immunoprecipitated complexes were preincubated with trichostatin A (400 nM) in histone deacetylase buffer for 30 min at 4 °C. Beads were resuspended in 30 μl of histone deacetylase buffer containing 20,000 cpm of an acetylated H4 peptide. Histone deacetylase activity was determined after incubation for 2 h at 37 °C, as described (28).

Coimmunoprecipitation—Twenty-four hours after transfection, 293 cells (2×10^7) were metabolically labeled with [^{35}S]EXPRE^{35}S^{35}S protein labeling mixture (NEN Life Science Products Inc.). Cells were starved by a 20-min incubation in methionine- and cysteine-free Dulbecco's modified Eagle's medium (Life Technologies). Labeling was done in methionine- and cysteine-free 90% Dulbecco's modified Eagle's medium and 10% Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum containing 0.15 mCi/ml radiolabeled amino acids ([^{35}S]Met, [^{35}S]Cys). Cells were harvested after 16 h and lysed as described above. Lysates were divided after preclearing and immunoprecipitated with M2 antibody or preblocked M2 antibody (Sigma) for 2 h at 4 °C. Protein G-Sepharose slurry (50%, 20 μl) was added for 1 h at 4 °C, and immunoprecipitates were washed three times with low stringency lysis buffer, three times with the same buffer supplemented with 500 mM NaCl and 0.5% sodium deoxycholate, and once with phosphate-buffered saline. Immune complexes were eluted from the beads with phosphate-buffered saline, containing 1% SDS for 15 min at room temperature and analyzed by SDS–polyacrylamide gel electrophoresis on 10% gels. After electrophoresis, the gels were fixed, enhanced with Amplify (Amersham), dried, and exposed for autoradiography.

RESULTS

Cloning of a Novel Human cDNA Encoding a Protein with Similarity to the S. cerevisiae HDA1p—A cloning strategy was developed to identify new long cDNAs (34). Random sequencing of a size-selected (>9 kb) human brain cDNA library identified a new cDNA encoding a putative protein with striking homology to a yeast histone deacetylase, HDA1 (see Fig. 1, *A* and *B*). Sequence analysis of this cDNA, referred to as HDAC-A hereafter, revealed an open reading frame (ORF) of 2,901 base pairs encoding a putative protein of 967 amino acids (Fig. 1*A*). This ORF is preceded by a 1134-nucleotide untranslated 5′ region and followed by a 4415-nucleotide untranslated domain. The molecular mass of the derived protein is 105 kDa, and the calculated isoelectric point is 6.7. A stop codon (TAA) is located in-frame immediately upstream of the presumed start codon. Although, several potential initiation sites are located in the 5′-untranslated region upstream of the putative translation initiation site (data not shown), the resulting ORFs are probably too short to yield a protein *in vivo*. Since the deduced ORF encodes a functional protein, this mRNA belongs to a growing number of transcripts that do not use the first ATG codon to initiate translation (38).

Alignment of HDAC-Ap with other histone deacetylases, such as HDA1p and RPD3p, showed that HDAC-Ap contains two distinct domains. The COOH-terminal domain (amino acids 495–967) displayed homology to known histone deacetylases, whereas the NH_2-terminal domain (amino acids 1–494) exhibited no homology to known proteins (Fig. 1*B*). Comparison of HDAC-Ap with HDA1p and RPD3p revealed a high degree of homology to HDA1p in the catalytical core region (43% identity and 60% similarity), whereas the homology to RPD3p in a similar region was significantly lower (25% identity and 43% homology) (Fig. 1*C*).

HDAC-A Belongs to a Larger Family of Similar Sequences—Using the BLAST and TBLASTN algorithms to search for other sequences with homology to HDAC-A, we identified another human cDNA, NY-CO-9. This cDNA was recently cloned as an autoantigen from serum from patients with colon cancer (39). The deduced amino acid sequence of this cDNA, which we propose to call HDAC-B showed high overall homology (58% identity, 67% similarity) to the HDAC-A protein, particularly in the conserved catalytic histone deacetylase domain. The

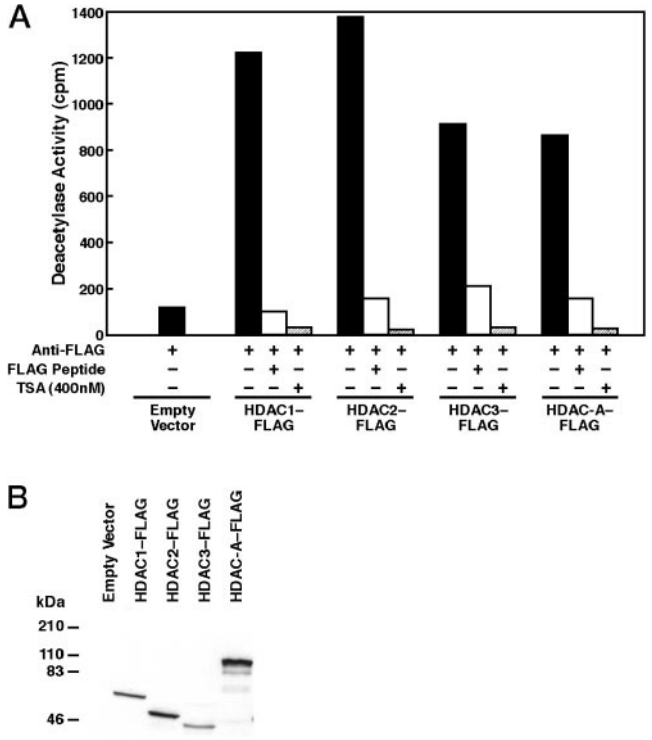

FIG. 4. **HDAC-A is a functional histone deacetylase.** *A*, 293 cells were transfected with plasmids encoding different FLAG-tagged histone deacetylases. Cellular extracts were immunoprecipitated with a monoclonal antibody recognizing the FLAG epitope and assayed for deacetylase activity on a ^{3}H-acetylated H4 peptide substrate in the absence or presence of trichostatin A (*TSA*, 400 nM). Counts for released [^{3}H]acetate are given for a representative experiment. Control immunoprecipitations were performed with anti-FLAG antibody preincubated with a 100-fold molar excess of a synthetic peptide corresponding to the FLAG epitope. *B*, cellular extracts from 293 cells transfected with FLAG-tagged histone deacetylases were analyzed by Western blot with an anti-FLAG antibody.

cDNA clone described by Scanlan and co-workers (independently isolated by us –KIAA0600) is truncated at the 5′ and 3′ extremities in comparison to HDAC-A. However, the genomic sequence of HDAC-B was identified on chromosome 17 (Human Genome Project, GenBank accession no. AC004150) and regions corresponding to the 5′ and 3′ extremities of the HDAC-A ORF were found. Therefore, we expect the full-length HDAC-B protein to be similar in size to HDAC-A. Two additional genomic sequences located on human chromosomes 7p15-7p21 and 12q31, respectively, also showed high sequence similarity to HDAC-A (Table I). Expressed sequence tags corresponding to these genomic clones were identified (Table I), and it is therefore likely that these encode additional HDAC-A homologues. We propose to call these new cDNAs HDAC-C and HDAC-D.

Differential Expression of the HDAC-A Transcript in Different Human Tissues—To explore the expression of HDAC-A in different tissues, we performed Northern blot analysis (Fig. 2*A*) with a radiolabeled probe corresponding to the HDAC-A cDNA. A single species of 8.4 kb was detected in all tissues examined except testis, where a smaller species of 3.4 kb was present as well. The size of this transcript is in good agreement with the size of the cloned cDNA (8459 nucleotides). A weaker band corresponding to 4.2 kb in many tissues examined might represent a degradation product of the HDAC-A mRNA. To compare HDAC-A expression in a wider array of tissues and to obtain more information on transcript abundance, we used normalized mRNA master dot blots (CLONTECH). Quantification of the dot blots showed that HDAC-A was highly expressed

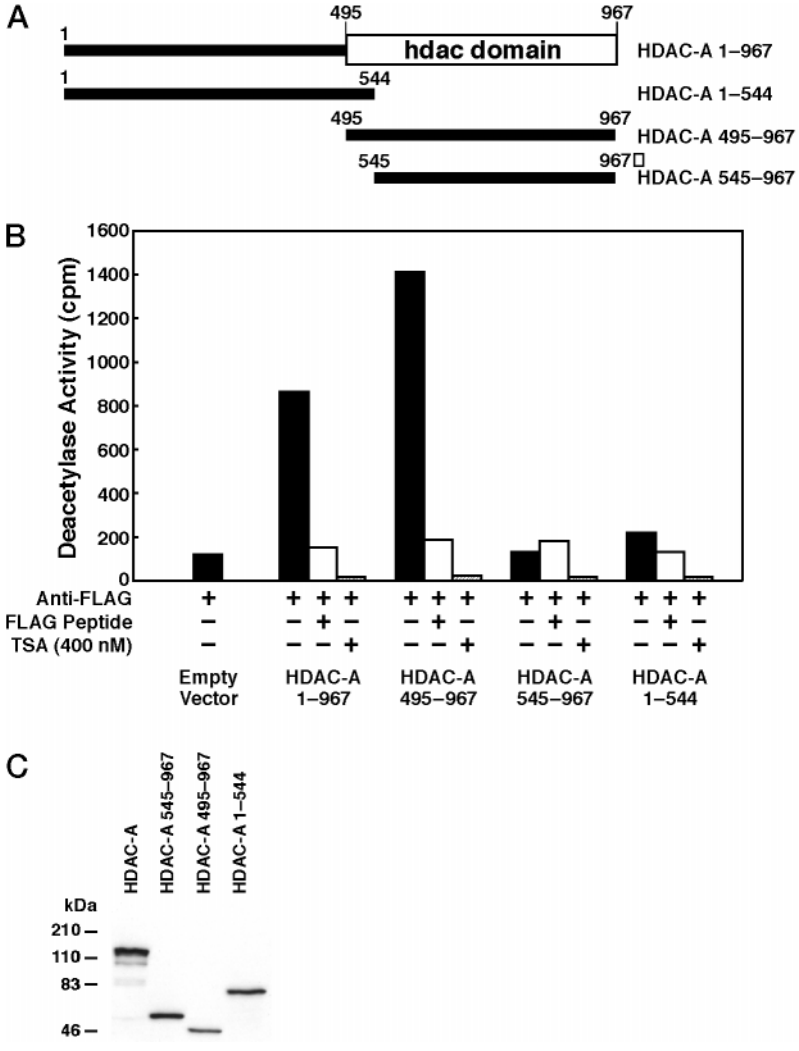

FIG. 5. **Histone deacetylase activity maps to a COOH-terminal domain of HDAC-A.** *A,* schematic representation of different constructs used to map different domains of HDAC-A. *B,* transfections and histone deacetylase immunoprecipitation analysis was performed as described in the legend to Fig. 4*A*. *C,* Western blot analysis of cellular extracts with anti-FLAG antibody after transfection of FLAG-tagged HDAC-A constructs. *TSA*, trichostatin A.

in skeletal muscle, thymus, and small intestine. Heart, colon, brain, ovary, peripheral blood leukocytes, prostate, pancreas, spleen, and lung showed intermediate expression, while liver, placenta, and kidney showed very low expression (Fig. 2*B*).

HDAC-A Is a Predominantly Nuclear Protein with a Distinct Subnuclear Localization—To examine the subcellular localization of the HDAC-A protein, HeLa cells were transfected with a HDAC-A-FLAG fusion construct, and indirect immunofluorescence was performed with the anti-FLAG M2 antibody, as described (37). HDAC-Ap was found predominantly in the interphase cell nucleus (Fig. 3*A*). As expected, HDAC-Ap shows diffuse localization during mitosis, when the nuclear compartment is disintegrated (*bottom* of Fig. 3*A1*). In the interphase cell nucleus, HDAC-Ap is excluded from nucleoli and accumulates in discrete foci (Fig. 3*B*). Similar chromatin-depleted foci have been reported for the localization of endogenous HDAC1p (37). Like HDAC1p, HDAC-Ap is depleted near the periphery of the cell nucleus and excluded from heterochromatic territories.

HDAC-A Is a Histone Deacetylase—To determine whether HDAC-Ap possesses intrinsic histone deacetylase activity, plasmids expressing FLAG-tagged fusion proteins were transfected into 293 cells. After transfection, a new protein of approximately 120 kDa was detected by Western blots with the anti-FLAG antibody (Fig. 4*B*). We used the anti-FLAG antibody to immunoprecipitate HDAC-Ap and assayed for histone deacetylase activity with a synthetic peptide corresponding to the NH_2-terminal tail of histone H4 as a substrate (28). Similar immunoprecipitations were performed after transfection of plasmids encoding FLAG-tagged versions of HDAC1, mHDAC2, and HDAC3 as controls. Material immunoprecipitated with the anti-FLAG antibody in cells transfected with the HDAC-A–FLAG plasmid demonstrated histone deacetylase activity (Fig. 4*A*). In contrast, immunoprecipitated material from cells transfected with the corresponding empty vector plasmid yielded no detectable histone deacetylase activity. No histone deacetylase activity was detected when immunoprecipitation was performed with the anti-FLAG antibody preincubated with an excess of a synthetic peptide corresponding to the FLAG epitope. As reported for other histone deacetylases, the enzymatic activity of HDAC-Ap was inhibited by trichostatin A. These results demonstrate that HDAC-Ap is a functional enzyme with histone deacetylase activity *in vivo*.

To map the catalytical domain of HDAC-A, we generated deletion constructs of HDAC-A either from the NH_2 or COOH terminus guided by the homology to other histone deacetylases, especially HDA1 (Figs. 1*A* and 5*A*). A fragment corresponding to amino acids 495–967 of HDAC-Ap, the region homologous to HDA1p, exhibited significant histone deacetylase activity, which was reproducibly higher than the activity of the full-length protein (Fig. 5*B*). A fragment of HDAC-Ap corresponding to amino acids 545–967 exhibited no histone deacetylase activity, indicating that the short region between amino acids 495 and 545 is critical for enzymatic activity. As expected, the amino-terminal portion of HDAC-Ap, amino acids 1–544, had no histone deacetylase activity. The higher histone deacetylase

activity of the fragment containing amino acids 495–967 in comparison to the full-length protein is consistent with the possibility that the amino-terminal region of HDAC-Ap contains a negative regulatory element.

HDAC-A Is Part of a Multiprotein Complex—Both HDAC1p and HDAC2p are part of a large multiprotein complex, and the *S. cerevisiae* histone deacetylase HDA1p represents the enzymatic component of the ~350-kDa HDA complex in yeast (29, 30). To determine whether HDAC-Ap is also part of a multiprotein complex, we performed coimmunoprecipitation experiments after transfection of a HDAC-A–FLAG fusion construct into 293 cells. Analysis of immunoprecipitated material by SDS–polyacrylamide gel electrophoresis identified a predominant 119-kDa band, in agreement with the predicted molecular mass of HDAC-Ap of 105 kDa. Several cellular proteins were found to coprecipitate with HDAC-Ap. Preincubation of the anti-FLAG antiserum with the corresponding FLAG peptide before immunoprecipitation showed that HDAC-Ap specifically interacts with proteins with molecular masses of 290, 137, 64, 50, 27, 25, 24, and 20 kDa, (Fig. 6). The band corresponding to 95 kDa seems to be a degradation product of HDAC-A–FLAG, since it was also detected in Western blot analysis of transfected 293 cells with the anti-FLAG antibody (Figs. 4*B* and 5*C*). The stringency of the washes used in these immunoprecipitations suggests that the immunoprecipitated proteins form a stable multiprotein complex *in vivo.* Preliminary experiments using gel filtration and density gradient centrifugation analysis to fractionate cellular extracts indicated that HDAC-Ap segregates as a higher molecular mass species (~220 kDa on sucrose gradients and ~600 kDa or gel filtration) than predicted based on its molecular mass.[2] These observations are consistent with the possibility that HDAC-Ap is part of a multiprotein complex in the cell nucleus.

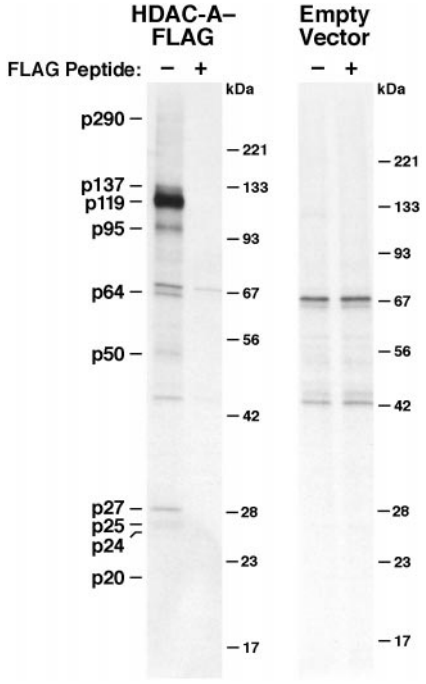

FIG. 6. **HDAC-A is part of a multiprotein complex.** 293 cells were transfected with an expression vector encoding FLAG-tagged HDAC-A or the control empty vector. Cellular proteins were metabolically labeled with a combination of [^{35}S]methionine and [^{35}S]cysteine and immunoprecipitated with monoclonal anti-FLAG antibody in the absence or presence of an excess of the FLAG-epitope peptide. Immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis on 10% gels. Proteins specifically interacting with HDAC-A are indicated by their molecular weight. Molecular weight markers are indicated on the *right* in kilodaltons.

DISCUSSION

We have cloned and analyzed a cDNA encoding the first member of a new family of human histone deacetylases. The members of this new family are more closely related to the *S. cerevisiae* protein HDA1p than to RPD3p. To distinguish this new family from a previously identified group of human histone deacetylases with highest homology to RPD3, HDAC1, 2, and 3 (9, 25–28), we propose to call the newly identified cDNAs HDAC-A, B, C, and D. HDAC-Ap is expressed in different tissues and is predominantly localized to the cell nucleus. HDAC-Ap exhibits histone deacetylase activity *in vivo* that maps to a COOH-terminal region of the protein.

HDAC1p and HDAC2p are thought to exert their effects as part of large multiprotein complexes. These complexes contain mSin3 (22, 40–42), the corepressors N-Cor and/or SMRT (43–45), RbAp48 and RbAp46 (9), SAP30 and SAP18 (41, 46, 47), and Mi-2 (48–50). They can be recruited to promoters by sequence-specific DNA-binding proteins such as Mad-Max (40, 41, 47), the unliganded nuclear hormone receptors (43–45), DP1-E2F and the Rb family of transcription factors (13, 51–53), transcriptional repressors containing a BTB/POZ domain (54, 55), CBF1/RBP-Jκ, the mammalian homolog of *Drosophila* Suppressor of Hairless (56), CtBP (57), homeodomain-containing repressors like Rpx, POU domain proteins such as Pit-1 (58), and MeCP2, a methylcytosine-binding protein involved in DNA methylation (reviewed in Ref. 59). The transcription factor YY1 interacts directly with HDAC2p but also with a recently identified new human RPD3 ortholog, HDAC3p (26). Despite its high degree of homology to HDAC1 and 2, HDAC3p coimmunoprecipitates with distinct factors and appears to participate in a different multiprotein complex.[3] In yeast, two different histone deacetylase complexes (HDA and HDB) have been defined biochemically (29, 30). The ~600-kDa HDB complex is the yeast equivalent of the human HDAC1/HDAC2-containing complex, and these two complexes share several subunits, including RPD3, RbAp48, SIN3, and SAP30 (22, 46).

The HDA complex is ~350 kDa and contains at least three different proteins with molecular masses of 75, 73/72, and 71 kDa (29, 30). The only component analyzed in detail so far is HDA1. Owing to the stringency employed in the purification protocol, it is not clear whether additional factors are associated with this core complex under less stringent conditions. Despite their sequence similarity to HDA1, nothing is known about the biology of the protein products of the HOS genes.

The evolutionary conservation of different histone deacetylase complexes suggests that they serve distinct and important functions in transcriptional regulation. As demonstrated for the HDAC1·HDAC2 complex, recruitment by different transcription factors can target the histone deacetylase activity to distinct promoters under different physiological conditions. In *S. cerevisiae,* the RPD3-containing complex specifically targets genes regulated by the UME6 transcription factor; however, these genes are not affected by a corresponding deletion of HDA1 (11). It is likely that the HDA1 complex is targeted to different sites by as yet unidentified sequence-specific DNA-binding factors. These two different histone deacetylase families might also exhibit different specificities in term of their enzymatic targets (different core histones or/and different lysine residues (29)). Differences in substrate use could add another level of specificity, since it is known that the acetylation states of specific lysine residues of histone H3 and H4 are

[2] W. Fischle and E. Verdin, unpublished observations.

[3] W. Fischle, S. Emiliani, and E. Verdin, unpublished observations.

associated with distinct biological functions (for review, see Ref. 32). The growing list of nonhistone proteins that are also regulated by acetylation, such as p53 (60, 61), EKLF (62), TFIIE, and TFIIF (63), also raises the possibility that so-called "histone" deacetylases might target nonhistone proteins for deacetylation. However, the nuclear localization of HDAC-Ap and its targeting to similar subnuclear granules, as observed for HDAC1, 2, and 3 (37),[4] suggests that these proteins might be functionally related.

Future effort will be devoted to defining the factors that interact with HDAC-Ap and the enzymatic specificity of HDAC-Ap in comparison to HDAC1, 2, and 3. It is anticipated that these studies will increase our emerging understanding of the role of histone deacetylation in transcriptional regulation.

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Note Added in Proof—After this manuscript was accepted for publication, Verdel and Khochbin (64) reported the identification of related cDNAs in mice. Their cDNA called mHDA1 is highly homologous to HDAC-B reported in this manuscript, whereas their cDNA called mHDA2 does not correspond to either HDAC-A, -B, -C, or -D.

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NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

A New Family of Human Histone Deacetylases Related to *Saccharomyces cerevisiae* HDA1p

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