Cloning and characterization of human histone deacetylase 8

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Abstract To date, seven different human histone deacetylases (HDACs) have been identified, which fall into two distinct classes. We have isolated and characterized a cDNA encoding a novel human HDAC, which we name HDAC8. HDAC8 shows a high degree of sequence similarity to HDAC1 and HDAC2 and thus belongs to the class I of HDACs. HDAC8 is expressed in a variety of tissues. Human cells overexpressing HDAC8 localize the protein in sub-nuclear compartments whereas HDAC1 shows an even nuclear distribution. In addition, the HDAC8 gene is localized on the X chromosome at position q13, which is close to the XIST gene and chromosomal breakpoints associated with preleukemia.

Key words: Histone deacetylation; Transcriptional regulation; Cancer; X chromosome

1. Introduction

Reversible acetylation of the N-terminal histone tails is a key determinant of gene expression. The level of histone acetylation is determined by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Generally, hyperacetylation of histones results in transcriptional activation whereas deacetylation correlates with transcriptional silencing [1]. Interestingly, transcriptional activators are often associated with HAT activity (reviewed in [2,3]), whereas HDACs appear to form complexes with transcriptional repressors [4–7]. The HDAC core complex consists of HDAC1 and HDAC2 proteins associated with the RbAp46/48 proteins [6]. This core complex can further associate with proteins such as Sin3, MeCP2, SAP30 and SAP18 to form the Sin3–HDAC complex [6–8]. A second complex, the Mi-2/NuRD–HDAC complex, consists of the HDAC core complex plus MBD3, p66 and MTA-2 [6,7,9]. These HDAC complexes are recruited to the chromatin by corepressor proteins such as NcoR or SMRT and thus can specifically repress transcriptional activity [10,11].

Seven human HDACs have been described so far, falling into two classes: I (HDAC1–3) and II (HDAC4–7). The first HDAC, HDAC1, was identified by affinity purification using the inhibitor trapoxin [12]. Subsequently, several other HDACs have been identified mainly based on the sequence similarity to the prototype HDAC1. HDAC2 was identified in a yeast two-hybrid screen using transcription factor YY1 as a bait [13]. HDAC3 [14–16] appears to associate with other proteins to form a complex which is distinct from the HDAC1/2 complexes [17]. Recently, a second class of human HDACs has been identified whose C-terminal part shows significant sequence similarity to the *Saccharomyces cerevisiae* HDA1 HDAC [18–20]. HDAC4 and 5 have been shown to interact with nuclear corepressors independent of Sin3, whereas HDAC7 might act via multiple repression complexes [10,20]. HDACs are involved in cell-cycle progression and cellular differentiation, and their deregulation is associated with several cancers (reviewed in [21,22]).

Here we describe the cloning and characterization of human HDAC8, a novel class I HDAC. Using database searches, several overlapping expressed sequence tags (ESTs) were identified which show high similarity to class I HDACs. Tissue-specific transcription of the HDAC8 gene is different from the other class I HDACs. The HDAC8 gene is localized on the X chromosome at position q13, close to the X inactivation center and close to chromosomal breakage points associated with preleukemia.

2. Materials and methods

2.1. Bioinformatics strategy for the identification of HDAC ESTs from databases

A multiple sequence alignment was generated using the protein sequences of human HDAC1 (SwissProt Q13547), HDAC2 (SwissProt Q92769) and HDAC3 (SwissProt O15379). Based on the multiple sequence alignment, a similarity profile was calculated and applied to subsequent profile searches [23] in the SwissProt database [24]. Positive hits were used to optimize and refine the multiple alignment before searching in DNA databases. The EMBL DNA database and Incyte EST database were probed with the proframe search program. Searches were performed on a dedicated hardware accelerator (Bioccelerator, Compugen, Israel).

2.2. Cloning of HDAC8 and generating recombinant cell lines

Clones containing ESTs encoding the human HDAC cDNA were obtained from the American Type Culture Collection (clones EST 182295 and EST 87343) and from Incyte Pharmaceuticals Inc. (clone 4284291). Two primers (forward: 5′-GGC TGC GGA ACG GTT TTA AGC-3′ based on EST 182295 sequence; reverse: 5′-GAA GCC AGC TGC CAC TTG ATG C-3′ based on Incyte clone 4284291 sequence) were designed. PCR reactions were performed on Quick-screen heart cDNA (Clontech) and the resulting 1320 bp fragment was cloned into the pCR2.1topo vector (Invitrogen) and sequenced. DNA sequencing was carried out on double-stranded plasmid DNA with dye-terminator chemistry as described by the manufacturer (Perkin Elmer/Applied Biosystems), and the products were resolved on an ABI Prism™ 377 Automated Sequencer. To obtain a full-length HDAC cDNA sequence, rapid amplification of cDNA ends (RACE) reactions were set up using Marathon-Ready

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cDNA from brain, kidney, prostate and ovary (Clontech) using the Adaptor Primer AP1 obtained from Clontech (5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′) and two nested RACE primers (5′ RACE primer: 5′-CTG ACC TTC TGG AGA TGC TGC AGA TAA GC-3′ and 3′ RACE primer: 5′-GCT GGG AGC TGA CAC AAT AGC TGG G-3′).

HDAC8/pcDNA6HisV5 was constructed by cloning the HDAC8 coding sequence upstream of and in-frame with the carboxyl-terminal V5 epitope and His sequence into pcDNA6HisV5 (Invitrogen). Using the same strategy, human HDAC1 (a kind gift of Dr. Yoichi Furukawa) was cloned into the same vector to yield plasmid HDAC1/pcDNA6HisV5. The constructs were verified by DNA sequencing of the insert and multiple cloning sites.

HEK293 cells were seeded in 150 mm dishes at a density of 6×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum, (heat-inactivated, Biowhittaker), 50 U/ml penicillin/50 μg/ml streptomycin, 1 mM L-glutamine (Life Technologies). Plasmid DNA (HDAC1/pcDNA6HisV5; HDAC8/pcDNA6HisV5 and pcDNA6HisV5) was transfected using Superfect transfection reagent according to the manufacturer's instructions (Qiagen). Thirty-six hours after transfection, selective medium was added containing 0.5 μg/ml blasticidin S HCl (Invitrogen). The medium was changed every 3–4 days until foci were detected. Cell foci were isolated and expanded. Overexpression of HDACs was verified by Western blot analysis using a V5 antibody. Two cell clones each for HDAC1 and HDAC8 and one clone for pcDNA6 (insert-less vector) were maintained.

2.3. Histone deacetylation assay

The activity of the overexpressed HDAC8 protein was measured by incubating a [^{3}H]acetyl-labelled fragment (amino acids 14–21) of histone H4 peptide (biotin-(6-aminohexanoic)Gly-Ala-[acetyl-^{3}H]Lys-Arg-His-Arg-Lys-Val-NH_2, 30 Ci/mmol, Amersham) and 1×HDAC buffer (25 mM Tris–HCl, pH 8.0, 10% glycerol, 50 mM NaCl) with 25 μl prepared extracts of each transfection for 60 min at 37°C in a total volume of 50 μl. Assays were performed in duplicate; 50 000 cpm of peptide was used per reaction. After incubation, the reaction was quenched with 1 M HCl and 0.16 M acetic acid. Released [^{3}H]acetic acid was extracted with 120 μl acetyl acetate and quantified by scintillation counting.

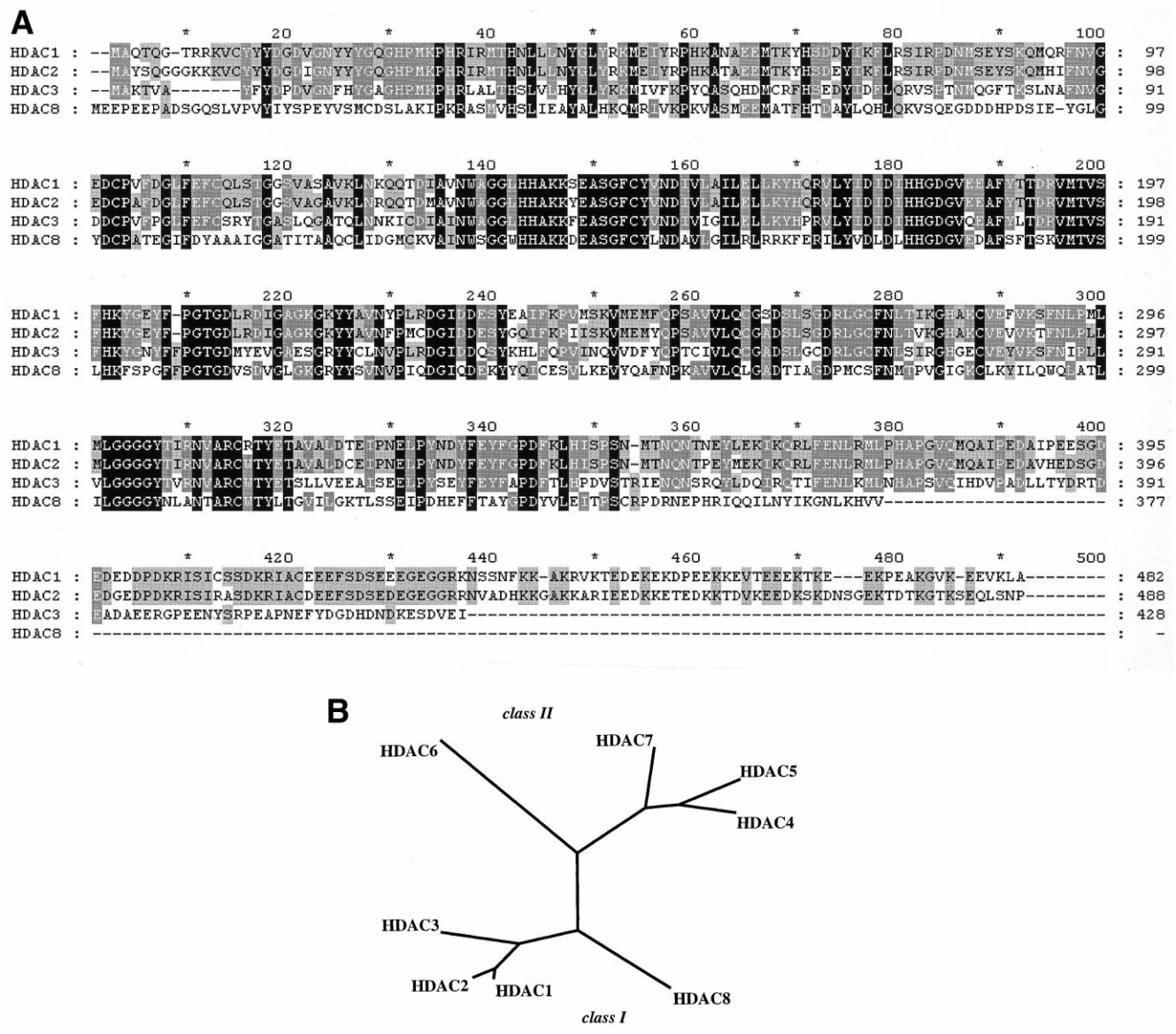

Fig. 1. A: Alignment of the amino acid sequence of human class I HDACs HDAC1 (SwissProt Q13457), HDAC2 (SwissProt Q92769), HDAC3 (SwissProt O15379) and HDAC8 (GenBank accession number AJ277724). Sequences were aligned using the ClustalW multiple sequence algorithm and conserved amino acid residues were annotated using the GeneDoc program. B: Phylogenetic tree of HDAC1 through HDAC8. Sequence alignment of HDAC1 through 8 alignment was imported into *TREECON* (version 1.3b) using the Phylip Input File format.

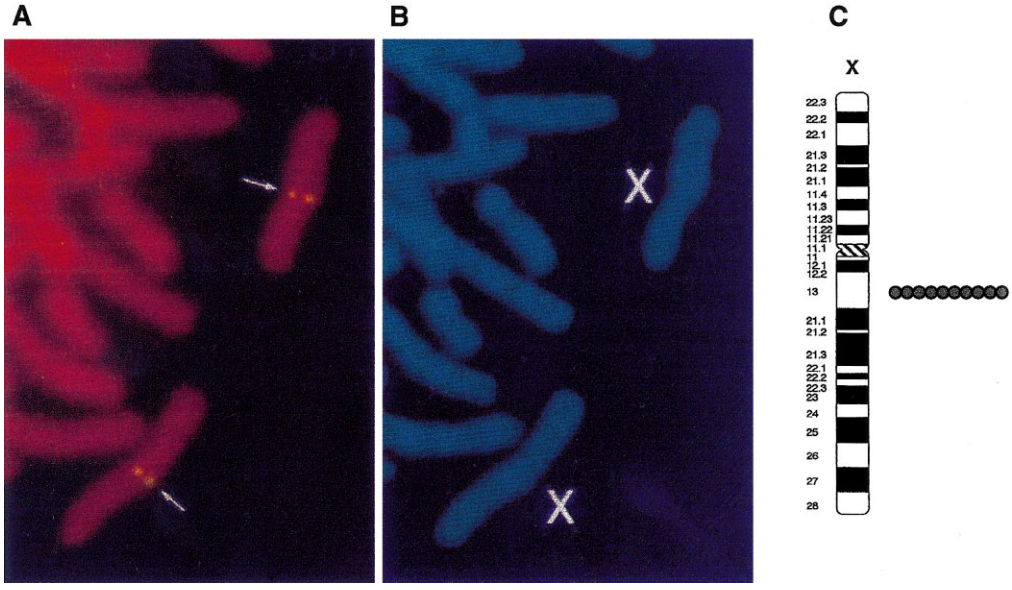

Fig. 2. Chromosomal localization of the HDAC8 gene. A: Metaphase chromosome spread labelled with fluorescent HDAC8 probe. B: The same mitotic figure stained with DAPI. C: Ideogram of the X chromosome. Cells were prepared from a female.

2.4. Western blot analysis

Standard protocols were followed. Lysed cell extracts or histone preparations were heat-denatured for 5 min in Laemmli sample buffer. Proteins were separated on a 12% sodium dodecyl sulphate–polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Millipore). After blocking with non-fat dried milk, the membrane was incubated in 1:5000 diluted anti-V5 mouse monoclonal IgG2a antibody (1.1 mg/ml, Invitrogen) followed by incubation with 1:1000 diluted anti-mouse horseradish peroxidase-conjugated IgG from sheep (Amersham). For histone Western blots, 0.75 μg/ml anti-acetylated histone H4 antibody or 250 ng/ml anti-acetylated histone H3 antibody (Upstate Biotechnology) were used as primary antibodies. Signals were detected using the ECL+Plus Western blotting detection system (Amersham).

2.5. Isolation of histones

Cells were grown to 70–80% confluence and treated for 4 h with 0.5 mM trichostatin A. After treatment, cells were washed and lysed in 40 ml NIB buffer (1% Nonidet P-40 in IB buffer (10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 3 mM $CaCl_2$ and 1 mM PMSF). Nuclei were collected ($500\times g$, 5 min) and washed twice in 40 ml NIB buffer followed by one wash with NIB buffer supplemented with 100 mM NaCl. Nuclei were high salt-extracted in 40 ml of 400 mM NaCl and IB buffer. The nuclear pellet was extracted twice in 10 volumes of 0.2 M H_2SO_4 for 90 min on ice and centrifuged (3500 rpm, 20 min). Supernatants were pooled and dialyzed at 4°C against 100 mM acetic acid.

2.6. Immunocytochemistry

HEK293 cells were seeded at a density of 0.5×10^6 cells/well in 6-well plates on cover glass slips coated with poly-L-Lysine and grown to 70–80% confluence. As indicated, cells were treated with 40 nM TSA. Cells were fixed with 2% paraformaldehyde for 30 min and washed for 5 min in phosphate-buffered saline (PBS) followed by 2 min treatment with 0.5% Triton X-100. Cells were washed for 5 min with 0.2% bovine serum albumin (BSA)/PBS and incubated for 120 min at 37°C in the presence of primary antibody (anti-V5 antibody (Invitrogen) diluted 1/200 in 0.2% BSA/PBS). After washing three times for 10 min with PBS, the secondary antibody was added (Fluorolink Cy3-labelled goat anti-mouse IgG(H+L), APBiotech) and proceeded according to the manufacturer's instructions. DAPI (4′,6′-diamidino-2′-phenylindole dichloride, Roche Diagnostics) staining of cells was carried out according to the manufacturer's instructions. Fluorescence signals were visualized using a fluorescence microscope

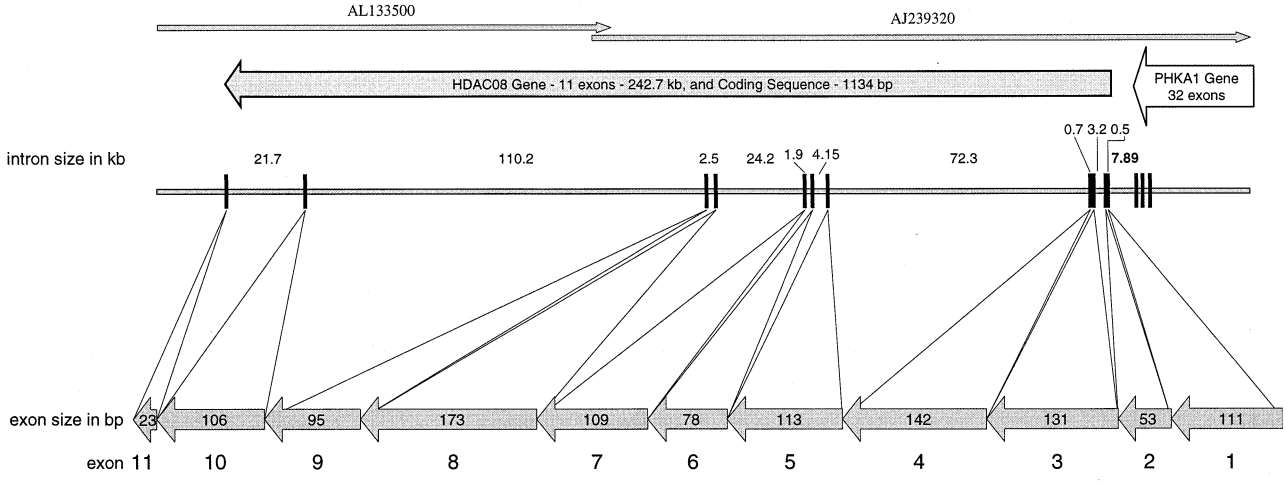

Fig. 3. Structure of the HDAC8 gene. Genomic sequences AL133500 and AJ239320 are indicated, sizes of introns are in kb, exon sizes are in bp.

(Axiovert 135, Zeiss). Cy3 fluorescence was visualized at 520 nm for 10 s, DAPI staining at 376 nm for 10 s. Images were recorded and analyzed using MERLIN software (Life Science Resources).

2.7. Immunoprecipitation of HDACs

Cells were lysed in IPH buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.2 mM PMSF) and left on ice for 20 min followed by centrifugation for 20 min at 10 000×*g* at 4°C. 5 μg anti-V5 antibody (Invitrogen) was added to the supernatant and rotated for 3 h at 4°C. 50% protein A/G Sepharose beads (Sigma) was added and incubated at 4°C for 1 h. Beads were washed and resuspended in three volumes of IPH buffer. Deacetylation assays were performed as above.

2.8. Northern blotting

Two human multiple tissue Northern blots and a cancer cell line blot (all from Clontech and containing 2 μg poly(A)$^+$ RNA per lane) were probed with a 948 bp fragment corresponding to the HDAC8 open reading frame, labelled with [^{32}P]dCTP by random priming using the *redi*prime II DNA labelling system (Amersham).

2.9. Fluorescence in situ hybridization studies (FISH)

Determination of the chromosomal localization of the HDAC8 gene was carried out by SeeDNA Inc. (Ont., Canada). Lymphocytes isolated from human blood were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum and phytohemagglutinin at 37°C for 68–72 h. The lymphocyte cultures were treated with bromodeoxyuridine (0.18 mg/ml) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the bromodeoxyuridine block and recultured at 37°C for 6 h in α-MEM with thymidine (2.5 mg/ml). Cells were harvested and slides were prepared by using standard procedures including hypotonic treatment, fixation and air-drying.

A HDAC8 cDNA fragment was gel-purified and biotinylated with dATP using the BRL BioNick labelling kit (15°C, 1 h) [25]. The procedure for FISH detection was performed as previously described [25,26]. Briefly, slides were baked at 55°C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 2×SSC for 2 min at 70°C followed by dehydration with ethanol. Probes were denatured at 75°C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulphate. Probes were loaded on the denatured chromosomal slides. After overnight hybridization, slides were washed and the signal detected. FISH signals and the DAPI banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping data to chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes [27].

3. Results and discussion

3.1. Cloning and sequence analysis of HDAC8

Based on the sequences of identified EST sequences, PCR primers were designed to amplify the full-length HDAC8 cDNA. Several 5′ and 3′ RACE reactions were performed in order to verify start and stop codon of the predicted open reading frame for the HDAC cDNA (Fig. 1A). The proposed open reading frame of HDAC8 contains 377 amino acids with a predicted molecular mass of 42 kDa. Alignment of the amino acid sequence of HDAC8 to HDAC1, HDAC2 and HDAC3 reveals high sequence similarity to the class I HDACs (54% to HDAC1 and HDAC2; 39% to HDAC3; Fig. 1A). Similarity to class II HDACs is lower, but phylogenetic tree analysis places HDAC8 at the boundary with class II HDACs (Fig. 1B). The first 34 N-terminal amino acids of HDAC8 are considerably different from those of HDAC1–3, as are the 30 C-terminal amino acids (Fig. 1A), possibly indicating a special role for both N-terminus and C-terminus in the specific function of HDAC8. A distinct stretch of mainly acidic amino acids (HDAC8 position 83–95) is not present in HDAC1–3, which could be an important motif for protein/protein interactions. The central part of the protein is highly conserved between the four class I HDACs, displaying all seven homology regions previously identified [18]. Histidine and aspartic acid residues shown to be important for catalytic activity and proper folding of HDAC1 are conserved in all human class I HDACs, including HDAC8 [17]. We conclude that HDAC8 represents a novel human class I HDAC.

3.2. Chromosomal localization and structure of the HDAC8 gene

The chromosomal position of the HDAC8 gene was determined using FISH. Under the conditions used, the FISH detection efficiency was approximately 64% for the HDAC8 probe (among 100 checked mitotic figures, 64 showed signals on one pair of chromosomes) (Fig. 2A). The DAPI banding pattern was used to establish that the HDAC8 gene localizes to the long arm of the X chromosome (Fig. 2B). The detailed position was further determined based upon the analysis of 10 photographs leading to the conclusion that the HDAC8 gene is located on human chromosome X, region q13 (Fig. 2C).

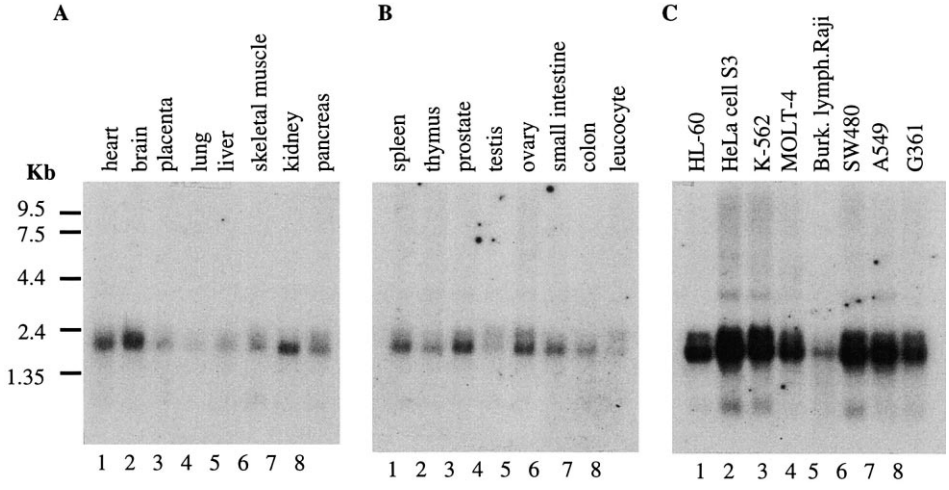

Fig. 4. Northern blot analysis of HDAC8. RNA source is indicated above the lanes, 2 μg poly(A)$^+$ RNA is loaded in each lane.

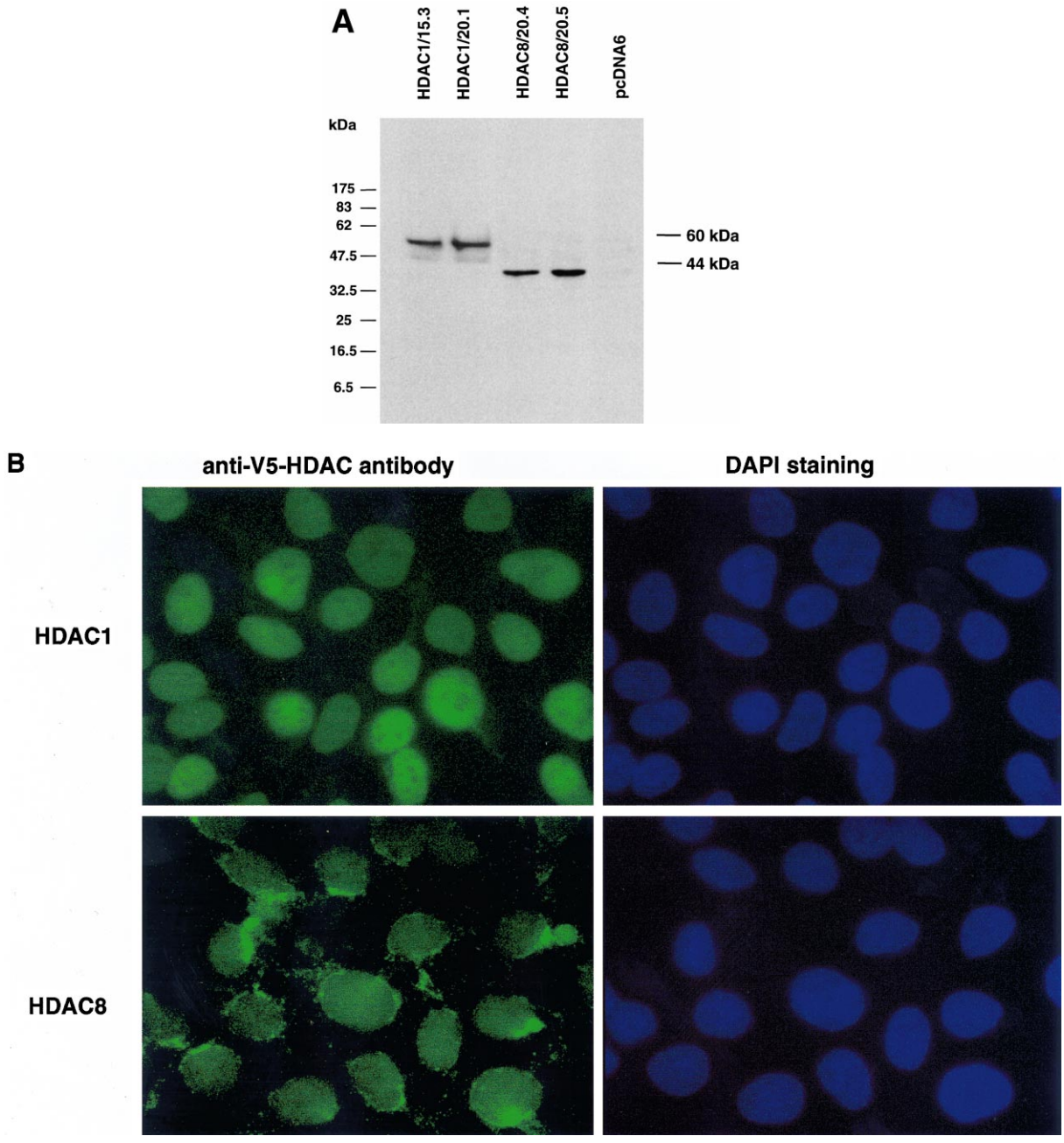

Fig. 5. A: Western blot analysis of HDAC overexpression in HEK293 cells. Total cell extracts from HEK293 cell clones constitutively overexpressing HDAC1 (clone HDAC1/15.3 and clone HDAC1/20.1) and HDAC8 (clone HDAC8/20.4 and clone HDAC8/20.5) were subjected to Western blot analysis using an anti-V5 antibody (Invitrogen). HEK293 clone pcDNA6 is transformed with the insert-less vector and serves as a control. B: Localization of recombinant HDACs in HEK293 cells. V5 C-terminal tagged HDAC1 (cell clone 15.3) and HDAC8 (cell clone 20.5) were detected using an anti-V5 antibody (left panels); nuclear staining using DAPI (right panels).

Furthermore, using the Blast program, two genomic DNA sequences were identified (GenBank accession numbers AJ239320 and AL133500) which contain fragments of the HDAC8 cDNA sequence (Fig. 3). 5′ exons of HDAC8 are located on sequence AJ239320, 3′ exons on the sequence AL133500. Both sequences have an identical overlap of 8442 bp and thus can be assembled into one contig, which contains the full-length HDAC8 gene (Fig. 3). From start to stop codon, 11 sequences are identical to consecutive segments of the HDAC8 cDNA sequence. Their boundaries comply with standard exon–intron splice site rules, and their added length equals the length of the coding sequence inferred from our cDNA. Thus the HDAC8 gene is organized into 11 exons over a total length of 242.7 kb (Fig. 3). The predicted open reading frame of the HDAC8 cDNA extends on the genomic DNA sequence for another 38 amino acids upstream to a second potential start codon which is followed by a stop codon 2 amino acids further. Extensive 5′ RACE reactions were performed on cDNA from a variety of tissues but the resulting sequences did not extend up to this new potential start codon. In addition, no EST sequences were found which extend into the further upstream start codon, but several cover

the predicted start codon. The additional 38 amino acids show no significant similarity to any other HDACs or any other known proteins. We conclude that HDAC8 has two potential start codons with the further 3′ start codon most probably utilized over the more 5′ start codon.

Interestingly, the phosphorylase kinase α1 gene is located only 7889 bp further downstream from exon 1 of HDAC8 (Fig. 3). This allows to map the HDAC8 gene to position Xq13.1. This region has previously been characterized [28,29] and comprises a highly complex DNA sequence. It contains the brain-specific NAP1L2 gene, two MAGE-like pseudo gene clusters and several other unidentified ESTs [29]. In addition, this region has an unusual high number of L1 repeat sequences. Approximately 350 kb telomeric of the HDAC8 gene lies a 100 kb region which contains isodicentric Xq13 breakpoints which are associated with a subset of acute myeloid leukemias (AML) and preleukemias in elderly females [30,31]. Although the HDAC8 coding sequence lies outside of the mapped breakpoint region, it might be possible that the transcriptional regulation of the gene is modulated by the mutation and thus may be potentially implicated in the generation of AML and hereditary sideroblastic anemia [30–32]. Furthermore, the isodicentric Xq13 breakpoints were mapped 450 kb centromeric to the XIST gene. The untranslated XIST RNA is involved in initiation of X chromosome inactivation, which correlates with histone deacetylation and DNA hypermethylation of the inactive X chromosome [33]. It is intriguing to find a gene for a HDAC in the chromosomal region involved in X chromosome inactivation and future efforts will focus on elucidating any involvement of HDAC8 in this process.

3.3. Expression analysis of HDAC8

In order to establish the gene expression profile of HDAC8, a series of Northern blot hybridizations was carried out. A major RNA species of approximately 2 kb was identified with a second RNA band of approximately 2.2 kb in some tissues like prostate, ovary and testis as well as in most cancer cell lines (Fig. 4A,B). The two different RNA species detected by Northern blot analysis might represent tissue-specific splice variants of HDAC8. Expression levels for HDAC8 were highest in brain, kidney and prostate which is in contrast to HDAC1, 2 and 3, which are not expressed in brain but are highly expressed in heart and placenta [14]. Interestingly, expression levels of HDAC8 are elevated approximately 10-fold compared to normal tissue in seven out of eight cancer cell lines (Fig. 4C). This was also observed for HDAC2, but not for HDAC1 and HDAC3 [14], which is consistent with the proposed role for histone deacetylation in some types of cancer [21,22]. We conclude that the expression profile of HDAC8 is distinctly different from other class I HDACs which might imply a different biological function for HDAC8.

3.4. Overexpression of HDAC8 in mammalian cells

Cell lines were created which constitutively overexpress HDAC8 and HDAC1 approximately 5–6-fold above untransfected cells (Fig. 5A), as established using an antibody specific for HDAC1 (data not shown). Using immunocytofluorescence, recombinant HDAC1 protein was detected and appears evenly distributed in the nucleus of HEK293 cells (Fig. 5B). In contrast, HDAC8 was primarily localized in the nucleus, but appeared to be concentrated to sub-nuclear regions rather than distributed evenly throughout the nucleus (Fig. 5B). This differential nuclear distribution was observed for two different HDAC1- and HDAC8-overexpressing cell clones and was unaffected by the presence or absence of the

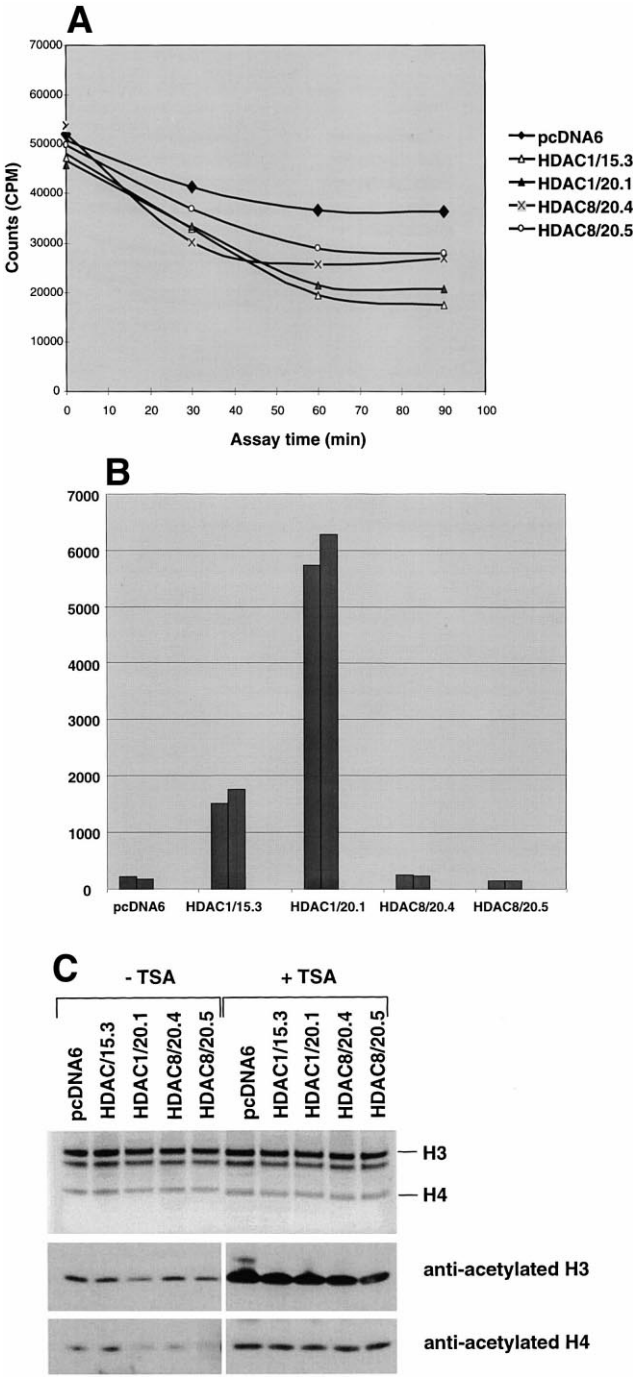

Fig. 6. A: Histone deacetylation assay using total cell extracts of HEK293 cell lines overexpressing HDAC1 (clone 15.3 and clone 20.1), HDAC8 (clones 20.4 and 20.5) and control cell line (pcDNA6). The average of duplicate assays is shown. B: Histone deacetylation assay using immunoprecipitated fractions from HDAC-overexpressing cell lines (see above). An anti-V5 antibody was used for immunoprecipitation of V5-tagged HDAC1 and HDAC8. C: Western blot analysis of histones isolated from HDAC-overexpressing cells (see above). Top panel: Coomassie-stained histones; middle panel: anti-acetyl histone H3; bottom panel: anti-acetyl histone H4.

HDAC inhibitor trichostatin A (data not shown). The significance of this difference in distribution is not clear but one might speculate about the targeting of HDAC8 to particular sites in the nucleus.

Cell extracts from these recombinant cell lines were used to assay for HDAC activity utilizing as a substrate a histone H4 peptide acetylated at lysine residue 16 (see Section 2). In a time course experiments, total cell extracts showed increased deacetylation activity for both HDAC1- and HDAC8-overexpressing cell lines, as compared to the control cell line (pcDNA6) which expresses only endogenous HDACs (Fig. 6A). HDAC1-overexpressing cell lines showed consistently higher activities (Fig. 6A). When overexpressed HDACs were immunoprecipitated using an antibody specific for the C-terminal V5-tag, only HDAC1-overexpressing cells showed significant HDAC activity as compared to cells transfected with an empty vector (Fig. 6B). Furthermore, histones were isolated from the cell lines and the acetylation status was assessed using antibodies specific for acetylated histone H3 and H4. No significant changes in the level of histone acetylation were observed in the cell lines overexpressing HDAC1 and HDAC8 as compared to vector-transfected cells (Fig. 6C). HDAC1 has previously been shown to be active when immunoprecipitated from cell extracts [14,17], however, HDAC8 shows no significant activity when immunoprecipitated (Fig. 6B). This might be due to the loss of protein factors during the enrichment, which are required for enzymatic activity of the HDAC8 protein, but might also reflect the fact that the acetylated H4 peptide is not a good substrate for deacetylation by HDAC8. In fact, there is mounting evidence that acetylation/deacetylation is not only utilized as a means to alter chromatin states but is also involved in the modification of a variety of proteins from transcription factors to proteins involved in nuclear transport [34,35]. Thus HDAC8 may act on non-histone proteins, and may have only limited deacetylation activity on histone proteins. The failure to detect deacetylation of histones in the cells overexpressing HDACs might be a consequence of the presence of opposing HAT activities in the cells and thus, small changes might not be detected due to the low sensitivity of the Western blot assay.

In conclusion, we have cloned and characterized a novel human HDAC cDNA which we designate HDAC8. HDAC activity of HDAC8 is low compared to HDAC1 and the precise deacetylation target remains to be identified. The HDAC8 gene is located on the X chromosome at position q13 which contains chromosomal breakpoints leading to some form of leukemia and furthermore is involved in X chromosome inactivation.

4. Note added in proof

Erding et al. [J. Biol. Chem. 275 (2000) 15254–15264] also reported on the cloning and characterization of HDAC8.

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