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Modulation of Splicing Events in Histone Deacetylase 3 by Various Extracellular and Signal Transduction Pathways

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Within the context of the chromatin environment histone deacetylases are important transcriptional regulators. Three classes of human histone deacetylases have currently been identified on the basis of their similarity to yeast proteins. The class I enzymes contain four members: HDACs 1–3 and HDAC8. Of these, HDAC3 is known to generate transcript variants with altered amino-terminal regions. Here we describe the identification of a novel splice variant of HDAC3, in which exon 3 is alternatively spliced from the messenger RNA transcript. We show that this human HDAC3 splice transcript is upregulated by treatments with histone deacetylase inhibitors. We also demonstrate evidence of splicing events in murine HDAC3 as a response to various signals, including switching between splice transcript isoforms following treatments with kinase inhibitors or by osmotic shock. In contrast, such switching events were not observed in human cells. These results indicate that differential pathways in mouse and human may control the regulation of HDAC3, and that splice variants may play important roles in responding to exogenous stimuli that act via signal transduction pathways.

Alternative splicing Histone deacetylase Signaling

THE appropriate regulation of gene expression in mammalian cells is affected by the constraints of chromatin structure. Modifications of the nucleosomal histone tails cause gene expression changes, and the elucidation of such mechanisms has prompted investigators to hypothesize that a "histone code" exists (23,29). Of these modifications, histone acetylation is probably the best characterized with the acetylation event occurring through the actions of histone acetyltransferases and histone deacetylases (16,20). While histone acetyltransferases are a rather diverse series of proteins with multiple functions, three distinct subfamilies of histone deacetylases have been isolated. Each class has been separated on the basis of their similarity to yeast proteins, all of which have histone deacetylase activity. These are the RPD3-like (class I), Hda1-like (class II), and Sir2-like (class III) histone deacetylases (16,20).

The complete genomic sequences of human histone deacetylase 3 (*HDAC3*) and histone deacetylase 5 (*HDAC5*) have been determined (25,27). The gene for *HDAC3* spans a region of approximately 13 kilobases (kb) and consists of 15 exons ranging in size from 56 to 657 bases (25). The murine equivalent has

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a similar exon structure spanning 14 kb (26). The 2-kb full-length cDNA for *mHdac3* encodes a protein the same size as that for human HDAC3 (428 amino acids) but differs in five amino acid residues, while Northern blot analysis of *mHdac3* expression in murine IMCD cells has revealed the presence of an additional 2.5-kb transcript (3).

With the estimate that the human genome contains approximately 35,000 genes (9), the question of how this number of genes codes for the much higher estimated number of proteins arises. The answer to this discrepancy appears to lie in RNA processing events such as RNA splicing. In this process, precursor mRNAs are spliced in various ways that can lead to exon skipping, intron retention, or variable exon/intron skipping (13,14).

As such, alternative splicing represents a means by which cells can generate novel protein isoforms from a single transcriptional unit. Such alternative splicing may have a very specific tissue distribution, or may have a specialized function within the cell. It has been estimated that alternative transcripts from some single genes can generate more alternatively spliced transcripts than the number of genes within the entire genome. In the drosophila *Dscam* gene its pre-mRNA can be alternatively spliced to 38,000 different mRNA isoforms. Considering that the predicted number of genes for the entire drosophila genome is 14,000, this is a staggering number. In humans the calcium-activated potassium channel gene, *slo*, is estimated to have the capability of synthesizing >500 different mRNAs (14). Alternative splicing exists in many cancer-associated genes including *WT1* and *IGF2* (1,18) and has been suggested to play a major role in tumorigenesis (1).

Alternative transcripts for histone deacetylase mRNA species have also been described. Putative alternative mRNA transcripts of *HDAC3* were originally described in the isolation of human *HDAC3* involving the NH_2-terminus (32), and a second transcript for *mHdac3* was identified in the murine brain (26). A novel testis specific form of the class II mouse *HDA1* (*mHdac4*) has also been described (31). In the human, class II *HDAC9* and *HDAC10* contain splice variants (10,22,30,33), and splice isofoms for *HDAC5* exist in the Entrez nucleotide database.

Here we identify a novel splice variant of the class I histone deacetylase *HDAC3*. As originally described for the full-length transcript, the expression of this splice form was shown to be responsive to histone deacetylase inhibitors (4). We also demonstrate alternative splicing events in murine cells. In the latter, several inhibitors of signaling pathways and osmotic stress conditions resulted in modulation of the expression of alternatively spliced *mHdac3*. Finally, we show that the expression of this splice variant is elevated in human kidney tumors.

MATERIALS AND METHODS

Cell Culture and Treatments

A human T-cell clone was grown under standard conditions, as described previously (5). Mouse IMCD cells were grown in DMEM/Ham's F12 (1:1) and were supplemented with 2% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (JRH Biosciences). Hep3B and CRL-1611 cell lines were maintained in Dulbecco's modified Eagle medium (low glucose) with L-glutamine, 1000 mg/L D-glucose containing sodium pyruvate (Invitrogen), supplemented with penicillin/streptomycin (Invitrogen) at a concentration of 50 U (penicillin), 50 μg (streptomycin)/ml, and in the presence of 10% fetal bovine serum (Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C.

For each treatment cells were trypsinized, counted, and 1.5×10^6 cells were seeded into 10-cm plates. Following a 24-h recovery period the cells were then treated as indicated for an additional 16 h following which total RNA was extracted for subsequent analysis.

Trichostatin A (TSA) was purchased from Upstate Biochemicals, dissolved in DMSO, and cells were treated at a concentration of 240 ng/ml as described previously (21).

Suberoylanilide hydroxamic acid (SAHA) was purchased from Upstate Biochemicals and dissolved in PBS. Cells were treated at a final concentration of 2.5 μM.

Sodium phenylbutyrate (Tributyrate®) was obtained from TripleCrown and dissolved in water. Cells were treated at a final concentration of 10 mM.

Cycloheximide was purchased from Sigma and dissolved in water. Cells were treated at a final concentration of 10 μg/ml. Cells were treated with cycloheximide for 2 h and then treated with either Tributyrate®, SAHA, or TSA.

Mannitol was purchased from Sigma and dissolved in water, and cell treatments were made at a final concentration of 50 mM. Calphostin C (Calbiochem) was dissolved in DMSO, and cells were treated at a final concentration of 200 nM. SB203580 (Calbiochem) was dissolved in water and cell treatments were at a concentration of 2.5 μM (human) or 50 μM (mouse). PD98059 (Calbiochem) was dissolved in DMSO. Cells were treated at a final concentration of 25 μM (human) or 50 μM (mouse). Genistein (Calbiochem) was dissolved in DMSO and cells were treated at a final concentration of 200 μM. H-7 dihy-

drochloride (Calbiochem) was dissolved in water and cells were treated at a final concentration of 50 μM.

Tumor Specimens

Ten clear cell renal cell carcinomas with matched normal counterpart tissue from the same individual were provided by the Cooperative Human Tissue Network. The full details of each sample are provided in Table 1.

Nucleic Acid Isolation

Total RNA was prepared by the method described previously (2), or by the RNAzol B method according to the manufacturer's instructions (Tel-Test).

Northern Blot Analysis

A Northern blot of mouse IMCD cells treated under the conditions described was obtained as a gift from Dr. Steven Gullans (Harvard Medical School). Separation of total RNA (20 μg/lane) was performed using standard formaldehyde agarose gel electrophoresis. Transfer to a nylon filter was followed by probing of the filter with a ^{32}P-labeled EST AA102998 probe encoding *mHDAC3* obtained from Genome Systems. The blots were washed at room temperature twice (2× SSC, 0.1% SDS, 25 min), once at 60°C (0.2× SSC, 0.1% SDS, 20 min), with a final wash at room temperature (2× SSC, 0.1% SDS, 5 min) and autoradiographed. Equivalent loading was verified by identification of ribosomal bands in the agarose gel.

Multiple human and mouse tissue blots were obtained from Clontech. ^{32}P-Labeled probes corresponding to the *HDAC3* cDNA splice product were prepared using the Multiprime DNA labeling system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The blots were hybridized overnight at 68°C, and washed under high stringency conditions as above. MTN blots were analyzed using a Fuji BAS1000 phosphorimager.

Isolation and Cloning of the Murine and Human HDAC3 *Splice Variant*

Total RNA (10 μg) was digested with RQ1 DNase (Promega) for 1.5 h at 37°C, phenol extracted, and ethanol precipitated. The RNA was redissolved in 15 μl of sterile water, and 5 μl was removed for use as a negative DNA control in the PCR amplifications after cDNA generation. Random hexamer primer was added to the remainder of the RNA at a final concentration of 0.75 μM; the sample was then heated to 85°C for 5 min, cooled rapidly on ice, and then reverse transcribed with MMuLV-RTase according to the manufacturer's instructions (Invitrogen).

The primers used to amplify HDAC from the resulting cDNA were: forward, 5′-CGCCGGCACCATGGCCAAGA-3′; reverse, 5′-GCTGGGTTGCTCCTTGCAGA-3′.

The conditions for the amplification of the *HDAC3* splice were approximately 100 ng of template (or 1 μl of cDNA as appropriate) and 0.48 μM of each primer in the presence of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 unit of Taq DNA polymerase with supplied buffer in a total volume of 50 μl. Cycling conditions were 95°C for 5 min followed by 3 cycles (of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C) followed by 32 cycles (of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C) with a final extension at 72°C for 10 min.

The splice product was run out in 1% agarose and isolated using a QIAEX II Gel Extraction Kit (Qiagen). Following purification the fragment was subsequently ligated into pCRII using TOPO TA cloning (Invitrogen). Confirmation of the cloned splice product sequence was carried out using an ABI3000 capillary sequencer according to the manufacturer's instructions.

TABLE 1
RCC TUMOR SAMPLES USED IN THIS STUDY

Case No.	RCC Type	% Tumor	Fuhrman Grade	Additional Information
1	clear cell	100%	?	
2	clear cell	90%	II	
3	clear cell	100%	III	5% fibrosis
4	clear cell	N/A	II	
5	clear cell	100%	II	25% necrosis
6	clear cell	100%	III	10–15% necrosis
7	clear cell	100%	I	
8	clear cell	100%	I	
9	clear cell	N/A	II	
10	clear cell	N/A	II	

N/A, not available.

Preparation of Probe and RNase Protection Analysis

Sp6 and T7 RNA polymerases (Life Technologies) were used to prepare RNA probes from appropriate templates according to the protocol provided in the RPA II Kit (Ambion). When incorporating radioactivity into the probe, radioactive [^{32}P]UTP with a specific activity of 800 Ci/mmol was used. Cold UTP was added such that the final UTP specific activity was 80 Ci/mmol for the β-actin and cyclophilin probes and 400 Ci/mmol for the *HDAC3* and *Hsp70* probes.

The probes used in this study were generated as follows: The *HDAC3* splice probe was a 220-bp PCR product cloned into pCRII as described above. If the resulting plasmid was subsequently digested with *Eco*RV, a 319-bp probe could be generated using Sp6 RNA polymerase of which 220 bases hybridize specifically to HDAC3 mRNA transcripts.

To make the *Hsp70* probe a 284-bp fragment was generated by PCR (forward primer, 5′-GGTGTGTA ACCCCATCATCAG-3′; reverse primer, 5′-TTCAT CTCTGCATGTAGAAAC-3′) and cloned into pCRII using a TOPO TA cloning kit (Invitrogen). Following linearization with *Hin*dIII a 412-bp probe could be generated of which 284 bases are protected from RNase digestion.

The β-actin clone (pTRI-β-Actin-Human) used in these experiments was purchased from Ambion. When hybridized to mRNA this probe protects 245 bases from digestion.

The cyclophilin clone (pTRI-Cyclophilin-Human) used in these experiments was purchased from Ambion. When hybridized to mRNA this probe protects 103 bases from digestion with RNAse.

RESULTS

Isolation of a Novel HDAC3 *Splice Product*

While analyzing the effects of various drug treatments on *HDAC3* expression in human T-cell clones a second band approximately 100 bases smaller than expected was consistently observed in each PCR amplification from cDNA, a representative of which is shown in Figure 1A. To determine whether or not this PCR product was a splicing product of *HDAC3*, the smaller PCR product was subsequently isolated and subcloned. Sequence analysis revealed that this was a bona fide *HDAC3* sequence. Comparison of this sequence with the available genomic data revealed that exon 3 was exactly spliced out of this PCR product (Fig. 1B). Experiments in the human cell lines Hep3B (liver) and CRL-1611 (kidney) also demonstrated the presence of this splice product by RT-PCR (data not shown).

Identification of Modulatable Alternative Hdac3 Isoforms in Murine Tissues

Because the DNA sequence identity for the region amplified is so highly conserved between human and mouse (>90%), we used the cloned splice product as a probe to examine the expression of the *HDAC3* splice transcript in different mouse tissues by Northern blot (Fig. 1C). A single transcript of approximate size to the main *mHdac3* transcript was observed in all tissues (long exposure; data not shown). However, even after a short exposure, a second larger additional band was observed for *mHdac3* in kidney, indicating that this splice product may be tissue specific (Fig. 1C). The presence of a second transcript confirms our

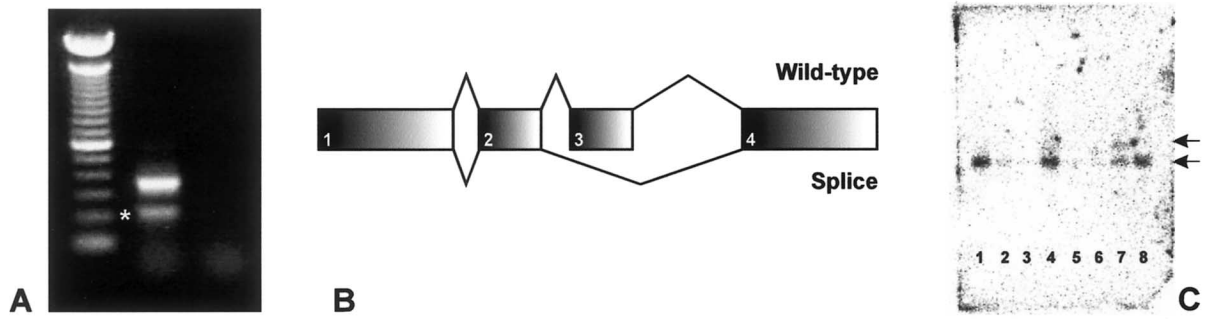

Figure 1. Identification of a splice variant of *HDAC3*. (A) PCR from cDNA showing the presence of a second PCR product (*) in addition to that of the expected size. (B) Diagrammatic representation of the first four exons of *HDAC3* showing the splice pattern determined for the second PCR product. (C) Northern blot analysis of a mouse multiple tissue blot using the cloned *hHDAC3* splice product as probe. Lane 1: heart; lane 2: brain; lane 3: spleen; lane 4: lung; lane 5: liver; lane 6: skeletal muscle; lane 7: kidney; lane 8: testis. The presence of positive hybridizations is indicated by arrows.

previous observations in IMCD cells (3). A similar larger transcript of mouse Hdac3 has also been reported in brain tissues (26). While we have identified that exon 3 is missing from our splice variant, we do not know the exact size of the alternatively spliced transcript, and as such this may in fact be larger than the originally described mRNA. The results obtained from the Northern blot fit with our previously observations and those from other groups (3,26).

Osmotic Stress and Drug-Specific Responses in IMCD Cells

The IMCD cell line is derived from murine renal inner medullary collecting duct cells and is adjusted to growth conditions under high osmotic pressure. IMCD cells express both the larger *mHdac3* isoform and the previously identified isoform of expected size of ~2 kb (3). To identify some of the potential signaling pathways by which this splice event may be altered in mouse kidney we examined *mHdac3* expression by Northern blot of cells subjected to a variety of kinase inhibitor drug treatments. Under normal growth conditions, both *mHdac3* transcripts can be demonstrated by Northern blot (Fig. 2). When cells were subjected to osmotic shock by treatment with mannitol, the upper splice product remained as the most prominent transcript (Fig. 2). Treatments with genistein (a protein kinase inhibitor) caused alterations to the *mHdac3* expression patterns, switching the predominance of expression to the smaller, previously reported 2-kb transcript (3). This was subsequently reversed by treatment with mannitol in these cells (Fig. 2). Treatment with calphostin C (a PKC inhibitor) also switched transcripts to the unspliced mRNA, which was also reversed by cotreatment with mannitol. If cells were treated with H7 (a broad-based serine/threonine kinase inhibitor) a slight decrease in the larger *mHdac3* transcripts was observed, while cotreatment with mannitol switched the pattern of expression to that of untreated cells. When cells were subjected to treatment with cycloheximide, the 2-kb *mHdac3* transcript was induced, while that for the larger transcript was unaffected, and cotreatment with mannitol was able to reverse this (Fig. 2). These results indicate that the relative abundance of the unspliced (i.e., 2 kb) and spliced (2.5 kb, possibly including intronic or untranslated regions) transcripts are affected by several signaling pathways in these osmotically adapted murine kidney cells in vitro, with a remarkable ability for mannitol to induce a switch of expression to that of the larger 2.5-kb transcript.

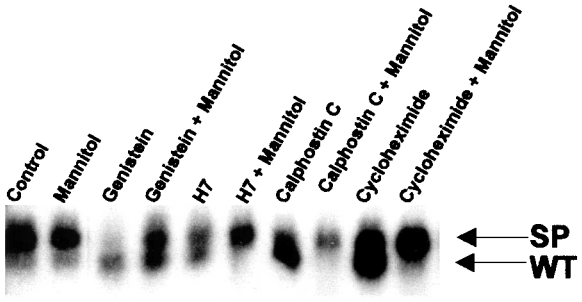

Figure 2. Effects of various signaling inhibitors and osmotic stress on the expression of *mHdac3* in mouse IMCD cells. Cells were treated as indicated above each lane. Wild-type (WT) and splice (SP) variants are indicated.

Effects of Various Inhibitors and Osmotic Stress on HDAC3 *Splice Expression in Human Cells*

Because previous studies have shown that the responses of murine and human histone deacetylases to various stimuli can vary dramatically (6,19), we tested the effects of various inhibitors on *HDAC3* mRNA expression in both Hep3B and CRL-1611 cells. In contrast to the mouse, no changes to expression of the human *HDAC3* splice mRNA were observed for various kinase inhibitors targeting PKC (calphostin C), p38 MAP kinase (SB203580), and MAP kinase kinase (MEK) (PD98059). If the cells were subjected to osmotic stress by treatment with mannitol, no effect was seen in the renal cell carcinoma-derived cell line CRL-1611. In contrast, in the hepatocellular carcinoma-derived cell line Hep3B osmotic stress upregulated expression of the splice transcript (Fig. 3A). To ensure that this effect was due to osmotic stress we examined the expression of *Hsp70*, which has previously been shown by others to be induced in cells undergoing osmotic stress (12,28). In addition, it has recently been demonstrated that class I histone deacetylase complexes coimmunoprecipitate with this protein and have increased catalytic activity when present with this chaperone (24). When *HSP70* was examined in these cells, a similar effect to that of *HDAC3* was observed (Fig. 3B). Thus, in contrast to IMCD cells, we found that in human cell lines which are derived from tissues that commonly encounter situations of osmotic pressure such as CRL-1611, the expression of the *HDAC3* splice transcript is unchanged, whereas in human cells derived from organs not normally subjected to such osmotic fluctuations the expression of the splice transcript responds to such conditions.

Effects of HDAC Inhibitors and Cycloheximide on HDAC3 *Splice Expression*

The expression of members of the class I histone deacetylases have been shown to be upregulated fol-

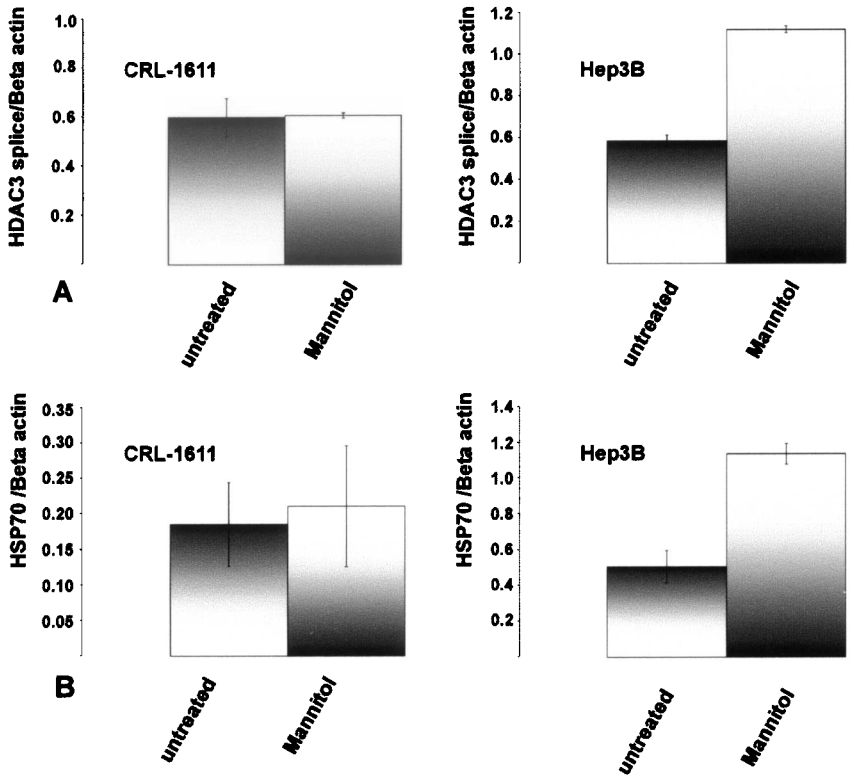

Figure 3. Effect of mannitol on expression of the *HDAC3* splice and *HSP70* in human tissue-derived cell lines. Expression of (A) the *HDAC3* splice and (B) *HSP70* in the human cell lines CRL-1611 and Hep3B. Results are presented as the mean ± SEM for three independent experiments.

lowing treatments with histone deacetylase inhibitors (4,5,15). We sought to examine if these inhibitors would also affect the expression of the splice transcript, using RPAs. The results are shown in Figure 4. In both CRL-1611 and Hep3B cells the expression of the splice transcript was upregulated by all three histone deacetylase inhibitors tested. Cycloheximide is an inhibitor of general protein synthesis. We examined the effect of this drug on the expression of the splice transcript in both the murine and human settings. The levels of the 2-kb murine *Hdac3* were elevated following treatment with cycloheximide, while the 2.5-kb transcript levels appeared unchanged (Fig. 2), but this pattern was also reversed by mannitol. In human cells the levels of the exon 3 skipping transcript were increased following treatment with cycloheximide (Fig. 4). When cycloheximide was added to the histone deacetylase inhibitors, the effects were enhanced for all three drugs (Fig. 4).

Presence of the HDAC3 *Splice Transcript in Human Renal Cell Carcinomas*

Previously we have demonstrated that the expression of the class I histone deacetylases is affected in liver primary tumors (17). As such we examined the expression of the splice variant in a series of clear cell renal cell carcinomas using RPA. The result of this analysis is shown in Figure 5. When compared with their matched counterpart tissue the expression of the exon 3 skipping variant was clearly elevated in 4 out of 10 (40%) of the tumors examined, and most other samples exhibited a tendency for upregulation.

DISCUSSION

The tight confines of chromatin have necessitated the evolution of specialized regulatory mechanisms for the appropriate regulation of gene expression within the mammalian cell. One such mechanism that has recently been elucidated involves the acetylation of the histone tails within the nucleosome. Histone deacetylases are involved with removing such modifications and have been subclassified into three classes depending on their similarity to yeast proteins (16, 20). The number of members identified within this family has grown dramatically since the isolation of

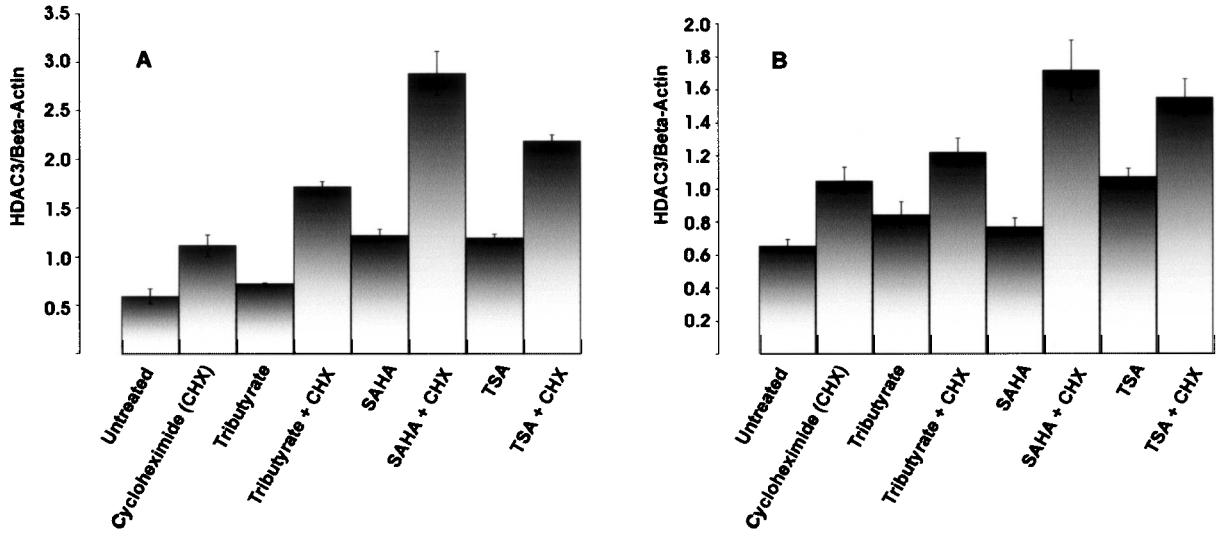

Figure 4. Effect of various HDAC inhibitors and cycloheximide on the expression of the HDAC3 splice variant. Treatments and abbreviations used: cycloheximide (CHX); Tributyrate®; suberoylanilide hydroxamic acid (SAHA); trichostatin A (TSA). Expression of the splice variant in (A) CRL-1611 cells and (B) Hep3B cells. Results are graphed as the mean ± SEM for three separate experiments.

the first HDAC and now this family consists of at least 20 members (7,11,16,20).

In this article we have described the identification of a novel splice variant of the class I histone deacetylase, *HDAC3*. This histone deacetylase was first independently identified by three research groups (5,8,32). The demonstration of alternative splicing of *HDAC3* was first shown by Seto and colleagues in their isolation of this gene. Two variants with alternative NH_2-terminal ends were subcloned (32). The complete genomic clone of the human *HDAC3* has since been isolated and sequenced. The gene is encoded by 15 exons ranging in size from 56 to 657 bp, spanning a region of at least 13 kb, and is present as a single copy (25). We have identified a splice variant of this gene involving exon 3 of the deduced genomic clone. This splicing event was not limited to human cells but was also observed in murine cells.

The regulation of this splicing event was examined through the use of various signaling pathway inhibitors. The analysis of these experiments demonstrated that in the mouse certain signaling pathways altered the expression of this splice. In contrast to the mouse, similar treatments in human cells did not result in

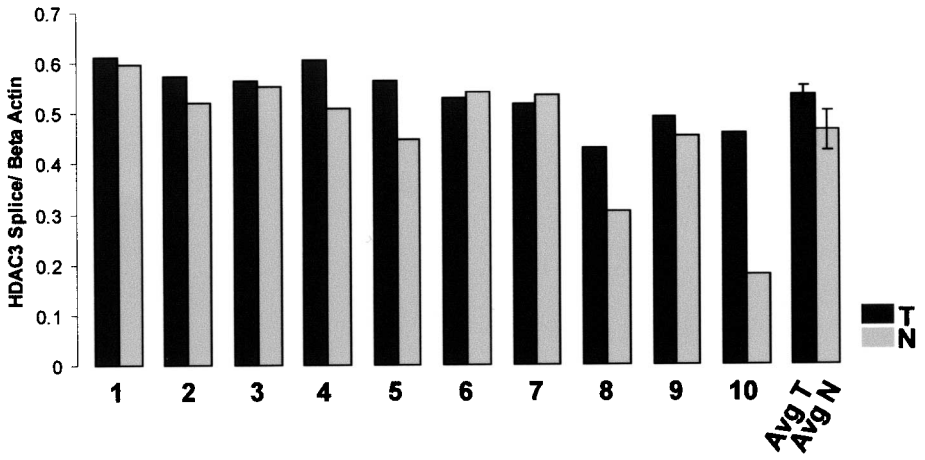

Figure 5. Presence of the HDAC3 splice variant in renal cell carcinomas. RNAse protection analysis (RPA) for the splice variant in a series of matched tumor/normal paired samples. The average ± SEM for both the tumor and normal samples is included.

splice variant modulation. This is in agreement with our previous data where we have shown that the expression pattern of the human and mouse class I histone deacetylase *HDAC1* is comparatively different (6). In addition, exposure of murine cells to hyperosmotic conditions using mannitol reversed the effects of these drugs. No response to mannitol could be seen in human cells used to hyperosmotic conditions, but the expression of the splice variant was responsive to treatment with mannitol in liver-derived human cells. Similar responses could be observed for *HSP70*, another gene shown to be responsive to mannitol treatments.

In agreement with several other reports showing that treatment with histone deacetylase inhibitors upregulates the expression of histone deacetylases (4,15), we showed that such treatments induced the expression of the splice variant in human cells. In addition, enhancement of inhibitor-induced upregulation of histone deacetylases has also previously been shown by us following PHA stimulation in immune cells (4).

Interestingly, we report here that this HDAC inhibitor-induced upregulation can also be enhanced by treating the cells with cycloheximide, indicating that a) new protein synthesis is not required for this effect, or b) cycloheximide may block the synthesis of proteins that actually counteract the expression of HDAC3 or its splice variants.

Finally, we have shown that in human renal cell carcinomas expression of this splice variant is elevated in 40% of tumors when compared with their matched normal kidney counterparts from the same individual. Similar increases in *HDAC* mRNA expression have previously been observed for hepatoblastoma samples (17).

The results presented here demonstrate that alternative splicing of *HDAC3* yields transcripts that may have specific regulatory activity in response to extracellular signals. Our studies provide a rationale for isolating the complete set of human and murine HDAC3 mRNA variants, and further explore their role in the regulation of gene expression.

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