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SP-transcription factors are involved in basal MVP promoter activity and its stimulation by HDAC inhibitors☆

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Abstract

The major vault protein (MVP) has been implicated in multidrug resistance, cellular transport, and malignant transformation. In this study we aimed to identify crucial MVP promoter elements that regulate MVP expression. By mutation as well as deletion analysis a conserved proximal GC-box element was demonstrated to be essential for basal human MVP promoter transactivation. Binding of Sp-family transcription factors but not AP2 to this element in vitro and in vivo was shown by EMSA and ChIP assays, respectively. Inhibition of GC-box binding by a dominant-negative Sp1-variant and by mithramycin A distinctly attenuated MVP promoter activity. In Sp-null *Drosophila* cells, the silent human MVP promoter was transactivated by several human Sp-family members. In human cells the MVP promoter was potently stimulated by the histone deacetylase (HDAC) inhibitors butyrate (NaB) and trichostatin A (TSA), resulting in enhanced MVP expression. This stimulation was substantially decreased by mutation of the single GC-box and by application of mithramycin A. Treatment with HDAC inhibitors led to a distinct decrease of Sp1 but increase of Sp3 binding in vivo to the respective promoter sequence as demonstrated by ChIP assays. Summarising, this study identifies variations in Sp-transcription factor binding to a single proximal GC-box element as critical for basal MVP promoter activation and its stimulation by HDAC inhibitors.

Keywords: Major vault protein; Lung resistance-related protein; Sp1 transcriptional regulation; Promoter transactivation; Sodium butyrate; Histone deacetylase inhibitor

The vault complex represents the largest ribonucleoprotein particle known to date. Vaults constitute evolutionary conserved, hollow structures composed of three different proteins and small, untranslated vault RNAs [1–3]. The 110 kDa major vault protein (MVP) accounts for 70% of the total particle mass. Both the conservation among species and the wide distribution in normal tissues suggest important roles of vaults in eukaryotic cells [1]. Vaults have been implicated in several cellular aspects including transport processes within the cell [3,4], as well as cell shape and motility [5]. A potential role of vaults in malignant transformation has been proposed based on the upregulation of MVP during tumorigenesis [6,7] and an interaction with the tumor suppressor protein PTEN [8]. The finding that MVP and the lung resistance-related protein (LRP), which is overexpressed in toxin-exposed tissues and various multidrug resistant cells, are identical proteins linked vaults to a complex multidrug resistance phenotype (MDR) [6,9–11]. Exposure to sodium butyrate (NaB), a histone deacetylase (HDAC) inhibitor, led to stimulation of MVP expression and enhanced multidrug resistance in human colon carcinoma cells [12].

☆ *Abbreviations:* AP-2, activator protein 2; BCRP, breast cancer resistance protein; ChIP, chromatin immunoprecipitation; DPE, downstream promoter element; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; Inr, initiator element; LRP, lung resistance-related protein; MDR, multidrug resistance; MRP, multidrug resistance protein; MVP, major vault protein; NaB, sodium butyrate; Sp1, specificity protein 1; TSA, trichostatin A; TSP, transcription start point.

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Currently only sparse information exists about activation and regulation of the human MVP gene. The identification of a ubiquitously expressed but translationally inhibited MVP mRNA splice variant suggested regulation of MVP expression by alternative splicing [13]. The exon/intron organisations of the human [14] and the murine [15] MVP genes have been reported and an initial analysis of the 5′-flanking regions has been performed. In the present study, we identify Sp-family transcription factors as essential for basal MVP expression via binding to a single, conserved GC-rich element within the proximal MVP promoter sequence. The stimulation of MVP expression by HDAC inhibitors was shown to involve the identical unique GC-box element as basal promoter activation.

Materials and methods

Cell culture. Tumor cell lines Hep3B (hepatocellular carcinoma), A549 (lung carcinoma), and U937 (histiocytic lymphoma) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). All cell lines were grown in RPMI-1640 Medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum. Schneider's *Drosophila* Line 2 (SL2) cells were cultured in Schneider's *Drosophila* Medium (Invitrogen, Groningen, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum at 27 °C. Cell cultures were frequently checked for *Mycoplasma* contamination.

Cloning of the MVP promoter and construction of luciferase reporter gene vectors. The human MVP promoter was amplified with the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany) using genomic DNA derived from MR-1 glioblastoma cells [6] as template. For promoter construct pMVP1670 primers MVP1 (5′-GATTCTCCTACCTCAGTCTC-3′) and MVP3 (5′-GAGAAGCTTCTAGAAGTGCAGGTAGC-3′) were used for amplification, for promoter construct pMVP336 primers MVP2 (5′-GAGGAGCTCTGTAGCTCAACAGTTAGG-3′) and MVP3 were employed. PCR products were cloned into the pCR2.1-TOPO vector with the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands). pCR2.1-TOPO-pMVP1670 and pCR2.1-TOPO-pMVP336 were digested with *Hin*dIII resulting in a 1716 bp (for pMVP1670) and a 383 bp (for pMVP336) MVP promoter fragment, respectively. Inserts were gel-purified and ligated into the *Hin*dIII-linearised pGL3-Basic vector (Promega, Madison, WI, USA). The full-length promoter construct pMVP1670 was cloned both in sense (pMVP1670) and anti-sense (pMVP1670-as) directions into the luciferase vector. 5′-End deletion constructs (Fig. 1A) were generated by restriction digest of pMVP1670 with *Sma*I (for pMVP1381), *Stu*I/*Sma*I (for pMVP1022), and *Pst*I/*Sac*I (for pMVP124). To generate pMVPΔ−125/−9, a recognition site for *Pst*I was introduced at position −9 relative to the transcription start point (TSP) [14] by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). The region −125 to −9 was then removed using *Pst*I. For construction of pMVPΔ−129/+43, plasmid pMVP1670 was digested with *Pst*I and *Nco*I, thus deleting the region from position −129 to the *Nco*I site in the pGL3-Basic vector. Purification of plasmids was carried out using the EndoFree Plasmid Maxi Kit (QIAGEN, Hilden, Germany). Vector-insert junctions of all constructs were confirmed by restriction analysis and sequencing.

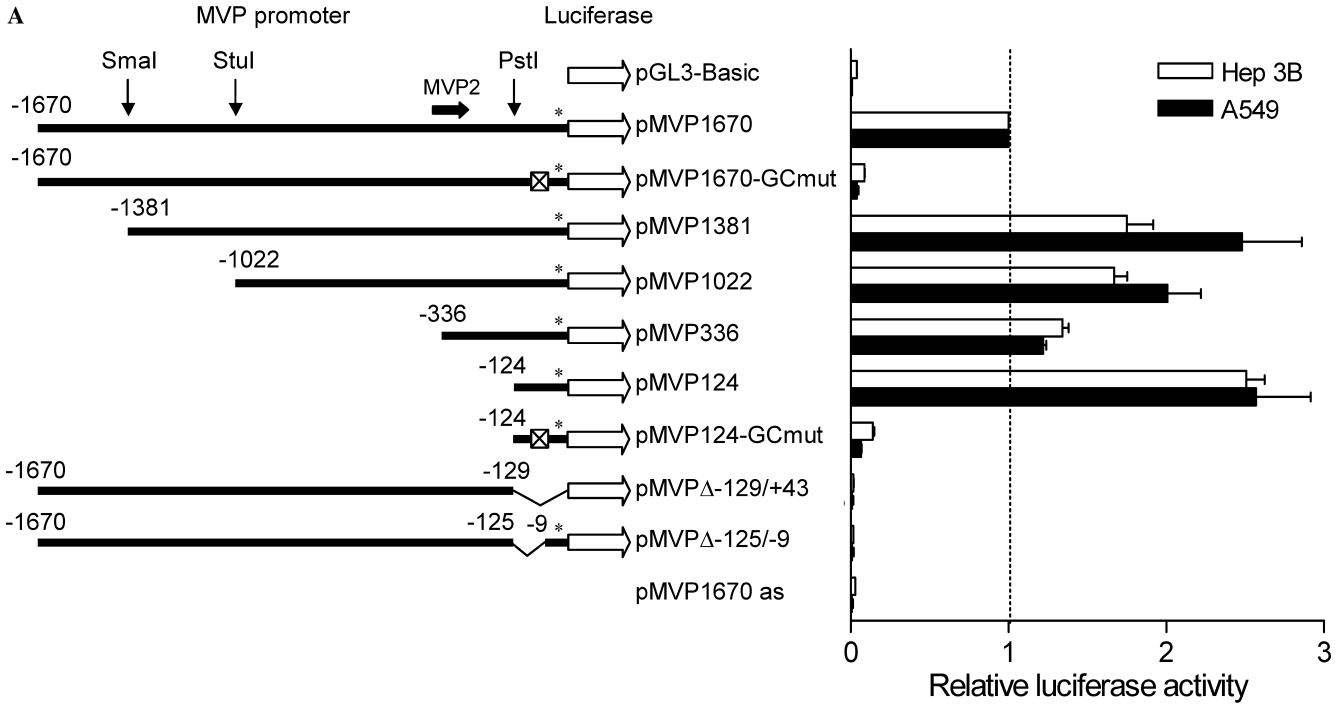
Site-directed mutagenesis. To generate pMVP1670-GCmut, the wild-type promoter construct pMVP1670 was mutated applying the QuickChange Site-Directed Mutagenesis Kit (Stratagene). A 2 bp mutation was set within the putative inverted GC-box (−53 to −66 relative to the TSP) changing the sequence from CAGGGGTGGG GCTT to CAGGGGTaaGGCTT. The mutation was confirmed by sequencing. The 5′-end deletion construct pMVP124-GCmut was generated from pMVP1670-GCmut as described above.

Transfection and luciferase assays. Transient human cell transfection with 0.4 μg reporter plasmid was performed using the Lipofectamin Plus Reagent (Invitrogen) according to the manufacturer's protocol. SL2 cells were transiently transfected using SAINT-MIX (Synvolux Therapeutics B.V., Groningen, The Netherlands) according to the manufacturer's protocol with 0.4 μg reporter gene construct and 0.1 μg Sp-family expression plasmids (pPacSp1, pPacUSp3, and pPacSp4) or control vector (pPacHD) [16]. Luciferase assays were performed 48 h after transfection. Protein concentration was determined with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The protein content was used for normalisation of luciferase activity. Mithramycin A, NaB, and trichostatin A (TSA) were from Sigma. The Sp-family member *Drosophila* expression vectors and the dominant-negative Sp1 construct (SP1-DN; pClneo-HA-Sp1(622–788)) [17] were generous gifts from Hans Rotheneder, Institute of Molecular Biology, University of Vienna, Austria and Guntram Suske, Institut fuer Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Germany.

Electrophoretic mobility shift assay and Western blot. Electrophoretic mobility shift assays (EMSAs) were performed using the Gel Shift Assay System (Promega) according to instructions. The sequences of the oligonucleotides were: Sp1 consensus (Sp1-cons), 5′-ATTCGATCGGGGCGGGGCGAGC-3′; GC wild-type (GC-wt), 5′-TTAACTCCCAAGCCCCACCCCTGGGCTT-3′; GC mutated (GC-mut), 5′-TTAACTCCCAAGCCTTACCCCTGGGCTT-3′; and AP-2 consensus (AP2-cons), 5′-GATCGAACTGACCGCCCGCG GCCC GT-3′. Eight micrograms of U937 nuclear extract [18], 100 ng human recombinant Sp1 (rhSP1, Promega), or 0.3 μl AP-2 extract (Promega) was used. For competition experiments a 100-fold molar excess of unlabelled DNA was added to the binding reaction. For supershift experiments 2 μg of polyclonal Sp1 (PEP2), Sp3 (D-20) or AP-2α (C-18) gel shift antibody (all Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was used. Western blots and cell fractionations were performed as described [6] using the identical antibodies as in EMSA.

Northern blot. Total RNA was extracted from control and HDAC inhibitor-treated cells by the Trizol extraction method (Invitrogen). Northern blots were performed as described previously [19]. LRP mRNA was detected using a ^{32}P-labelled probe corresponding to a 1225 bp *Xba*I fragment from DKFZp434L1720 [13]. GAPDH mRNA was detected as a reference using a cloned PCR-product as probe prepared by the oligonucleotide primers described previously [11].

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit from Upstate (Lake Placid, NY) according to the manufacturer's instructions. Conditions for the preparation of sheared chromatin of appropriate length were optimised for a Bandelin sonoplus sonicator (Berlin, Germany) with a 2 mm tip. Chromatin of 10^6 A549 or Hep3B cells, treated with NaB or TSA for 18 h as described above, was crosslinked by addition of formaldehyde to a final concentration of 1% (10 min, 37 °C) and resuspended in 200 μl SDS lysis buffer. Each sample was sonicated at 30% intensity for 5×10 s bursts with a gap of 60 s between each burst. Cells were maintained on ice throughout. Five micrograms of polyclonal Sp1 (PEP2) and Sp3 (D-20) was employed for immunoprecipitation. PCR was performed using primers 5′-GAAGCAGCCCGGAGAATAAAATGA GAAC-3′ and MVP3 to produce a product of 172 bp. PCR conditions included one cycle (5 min at 95 °C) and 37 cycles (30 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C) with a final extension step (8 min at 72 °C).



B

mouse MVP	-12	GGC**AGGGGCGG**A**GC**T**T**GTGTATT -34
mouse MVP	-162	AGT**A**A**GGG**T**GGGG**GT**G**GGGGATG -184
mouse MVP	-168	GGT**GGGGG**T**GGGG**GA**T**GGTGGCT -190
rat MVP	-49	GGC**AGGGGCGG**A**GC**T**T**GGGTATT -71
rat MVP	-209	CTG**GT**G**GGG**G**GGGGC**A**G**GGGGGTT -231
human MVP	-51	CCC**AGGGG**T**GGGGC**T**T**GGGAGTT -73

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Fig. 1. Characterisation of the human MVP promoter. (A) Deletion and mutation analysis. 5′-ends of the indicated MVP promoter luciferase constructs are given relatively to the TSP (*). Restriction sites and the oligonucleotide primer MVP2 used for cloning are shown. Mutations in the proximal GC-box element are indicated by ⊠. Results from transient transfection experiments of the indicated cell lines are shown. Luciferase values were normalised to the protein content and given relative to the full-length promoter (pMVP1670). Means and SD of at least three experiments are shown. (B) Evolutionary conservation of GC-box elements. A sequence comparison of the GC-box elements in human, rat, and mouse *MVP* genes is shown. The respective Sp1/Ap2-binding site (GC-box) is indicated by asterisks. Bold face indicates accordance with the GC-box consensus of MatInspector software. Mutated bases (GG → AA) in the GC-mut reporter constructs are underlined.

Results

Functional analysis of the human MVP gene promoter

The putative promoter region of the human MVP gene −1670 to +43 relative to the TSP (pMVP1670, "full-length" promoter) showed distinct promoter activity in luciferase assays (Fig. 1A). To further map the MVP promoter regions responsible for basal activity several 5′-truncations were generated and transiently transfected into Hep3B and A549 cells. Results indicated several positive and negative regulatory elements including a repressing element in the region −1670 to −1381 and several weak *cis*-acting enhancer elements within the region −1381 to −336. Further deletion of the 5′-region down to position −124 (pMVP124) led to a strong upregulation of promoter activity. This suggested that the 124 bp region immediately upstream of the TSP is essential for high basal activity. Correspondingly, deletion constructs pMVPΔ−125/−9 and pMVPΔ−129/+43 completely lacked promoter activity. This could not be attributed to deletion of the TSP [14] which was lacking in pMVPΔ−129/+43 but still present in pMVPΔ−125/−9.

A phylogenetic conserved GC-rich region is critical for basal MVP promoter activity

In order to further map essential *cis*-acting elements responsible for the high promoter activity of region −124 to +43 the sequence was searched for consensus binding elements by MatInspector (http://www.genomatix.de) and TESS (http://www.cbil.upenn.edu/tess). In accordance with a previous report [14], no classical core promoter elements including TATA-box, initiator (Inr), or downstream core promoter element (DPE) were found. However, the MVP promoter contained several putative transcription factor binding sites in-

cluding STAT-, p53-, E-box-, MyoD-, GATA-, inverted CCAAT-, and GC-box elements (suggested as combined Sp1/AP-2 binding site). When the human sequence −124 to +43 was aligned with the respective sequences from rat (GenBank entry NW_043396) and mouse [15] (AF466399) a strong conservation of GC-box elements became obvious (Fig. 1B). In contrast to the single human GC-box, the rat promoter harboured two and the murine three (two partially overlapping) GC-box elements. These observations prompted us to hypothesise that the single GC-box element in the human MVP promoter might be essential for basal promoter activity.

Indeed, mutation of the GC-box element (Fig. 1B) in promoter constructs pMVP1670 and pMVP124 (pMVP1670-GCmut, pMVP124-GCmut) distinctly reduced MVP gene promoter activity to <10% in human Hep3B and A549 cells as compared to the wild-type constructs pMVP1670 and pMVP124, respectively (Fig. 1A).

Binding of Sp1 and Sp3 to the −75/−48 region of the human MVP gene promoter

To identify transcription factors binding to the critical GC-box region in vitro and in vivo EMSA and ChIP assays were performed, respectively. Incubation of both the wild-type oligonucleotide (GC-wt) spanning the GC-rich region from −75 to −48 and the Sp1 consensus oligonucleotide (Sp1-cons) with U937 nuclear extracts resulted in the formation of one major specific DNA–protein complex in EMSA (Fig. 2A). When using recombinant human Sp1 (rhSP1) instead of nuclear cell extracts, a comparable DNA–protein complex was observed in case of GC-wt but not GC-mut containing a 2 bp mutation in the core of the GC-box. Dominant presence of Sp1 in the specific complex was proven by supershift experiments. Despite presence of Sp1 and Sp3 in the nuclear extracts used (Western blots are shown as insets), the major DNA/protein complex was supershifted by the Sp1 antibody while minor amounts were

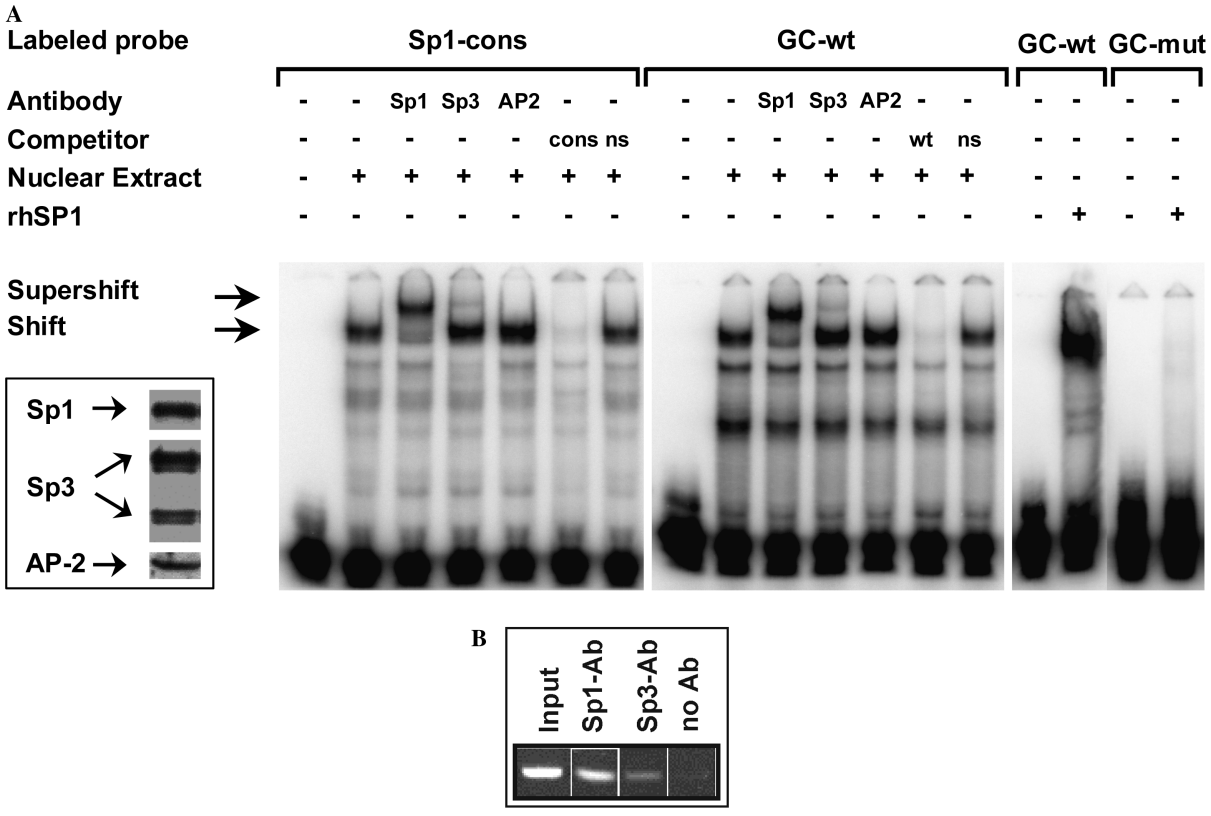


Fig. 2. Binding of transcription factors to the GC-rich element of the human MVP promoter in vitro and in vivo. (A) In vitro binding analysis by EMSA was performed with the indicated oligonucleotides (labelled probe) containing the Sp1-consensus binding site (Sp1-cons), the GC-box sequence of the human MVP promoter (GC-wt), and the mutated GC-box sequence (GC-mut). Labelled probes were incubated with nuclear extract of U937 cells or recombinant human Sp1 (rhSp1). Presence of Sp1, Sp3, and Ap2 in the nuclear extract is shown by Western blot as inset. Competition assays included a 100-fold molar access of specific and non-specific (ns) unlabelled oligonucleotide (competitor). DNA–protein complexes were separated by non-denaturing polyacrylamide gel electrophoresis. Complexes inhibited by an excess of the specific competitors are marked as shift. For supershift experiments the indicated antibodies were included in the binding reaction and supershifted DNA-protein complexes are marked. (B) In vivo binding of Sp1 and Sp3 to the proximal MVP promoter was demonstrated by ChIP assay. Chromatin was isolated from formalin-fixed Hep3B cells and precipitated by antibodies against the indicated Sp-family proteins. PCR primers used to amplify a 172 bp fragment of the proximal MVP promoter including the investigated GC-box element are given in the Materials and methods section.

shifted by the Sp3 antibody. AP-2 did not bind to the GC-wt oligonucleotide as indicated by the absence of any specific DNA–protein complex by incubation with an AP-2 extract (not shown) and the lack of a super-shifted band by incubation with an AP-2 antibody (Fig. 2A). Binding of Sp1 and Sp3 to the respective promoter region in vivo was demonstrated by ChIP assays (Fig. 2B).

GC-box binding of Sp-transcription factors is critical for basal MVP promoter activation

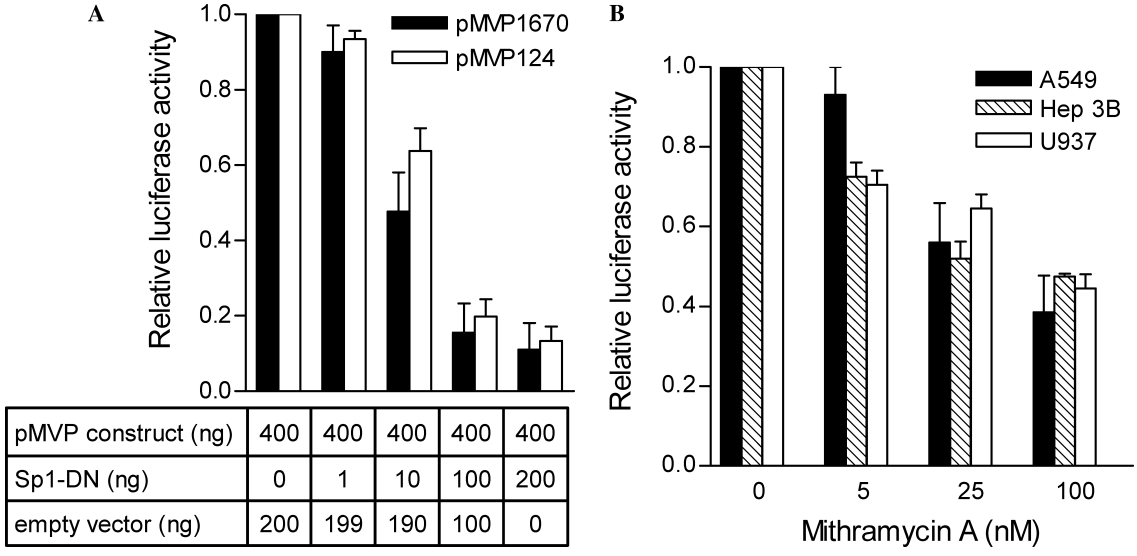
In order to prove the transactivating activity of Sp1 on the human MVP promoter a dominant-negative Sp1 construct (Sp1-DN) was cotransfected with MVP promoter luciferase constructs. Sp1-DN inhibited the transcriptional activity of pMVP1670 and pMVP124 promoter constructs dose-dependently (Fig. 3A). Additionally mithramycin A, a drug that interferes with the binding of Sp-family transcription factors to GC-rich promoter regions [20], suppressed pMVP124 (Fig. 3B) and pMVP1670 (not shown) promoter activity dose-dependently in all tested cell lines (Hep3B, A549, and U937).

Activity of the human MVP promoter in Drosophila SL2 cells

In order to investigate the impact of Sp-family transcription factors on the human MVP gene promoter in a background devoid of GC-box binding activity SL2 cells were used [21]. Transfection of the human MVP promoter construct MVP1670 into SL2 cells resulted in negligible luciferase activity (Fig. 4). However, cotransfection with plasmids carrying human Sp-family members led to potent activation of the human MVP promoter. By far the highest activation (>130-fold) was induced by Sp1. Sp3 and Sp4 activated the human MVP promoter to a significantly lower extent than Sp1.

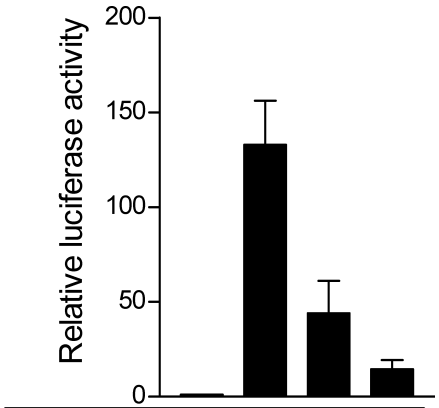
Stimulation of MVP expression by HDAC inhibitors

Incubation of cells with two substances known to inhibit HDAC activity, namely NaB [12] and TSA, led to a significant upregulation of MVP expression at the mRNA and protein levels in A549 (Figs. 5A and B, respectively) and Hep3B cells (data not shown). This corresponded to a distinct stimulation of MVP promoter activity (up to around 11-fold) of the full-length (not shown) and the minimal pMVP124 reporter construct (Fig. 5C) by both inhibitors. Stimulation of the minimal pMVP124 promoter construct indicated that the decisive elements for HDAC inhibitor-stimulated promoter activation resided within the proximal core promoter sequence. While the promoter stimulation was unaltered or even enhanced by mutations in several other transcription factor binding sites residing in this region (data not shown) it was significantly reduced to around 5-fold by mutation of the single GC-box (Fig. 5C; compare Fig. 1). The stimulation of pMVP1670 and pMVP124 promoter constructs by NaB



pMVP construct (ng)	400	400	400	400	400
Sp1-DN (ng)	0	1	10	100	200
empty vector (ng)	200	199	190	100	0

Fig. 3. Inhibition of basal human MVP promoter activity by blocking Sp-family transcription factor interaction. (A) Human Hep3B cells were transiently transfected with pMVP1670 or pMVP124 constructs as well as increasing amounts of a dominant-negative human Sp1 expression plasmid (Sp1-DN). Luciferase values were normalised to the protein content and are represented relative to the control group without Sp1-DN. Total amounts of transfected DNA were kept constant by addition of the empty vector. Means and SD of three independent experiments are shown. (B) The indicated cells were transiently transfected with pMVP124 and incubated in increasing concentrations of the GC-box binding inhibitor mithramycin A for 24 h before luciferase detection. Luciferase values were normalised to the protein content and are represented relative to the solvent control group without mithramycin A. Means of two comparable experiments are shown.



p1670	+	+	+	+
pPacHD	+	-	-	-
pPacSp1	-	+	-	-
pPacUSp3	-	-	+	-
pPacSp4	-	-	-	+

Fig. 4. Transactivation of the human MVP promoter by human Sp-family members in *Drosophila* SL2 cells. SL2 cells were transiently co-transfected with the full-length human MVP promoter construct pMVP1670 and the indicated Sp-family expression constructs or the empty vector (pPacHD) and processed for luciferase assays. Values were normalised to the protein content and are represented relative to the empty vector control. Means and SD of three experiments are shown.

and TSA was comparably and severely reduced (Hep3B) or completely blocked (A549) by application of mithramycin A (Fig. 5D). In order to investigate the influence of HDAC inhibitors on the in vivo binding of Sp-transcription factors to the single GC-box element of the human MVP promoter ChIP assays were performed. Both Sp1 and Sp3 proteins were present on the MVP core promoter sequence in HDAC inhibitor treated and untreated Hep3B (Fig. 6A) and A549 cells (data not shown). HDAC inhibitors, however, led to a significant reduction of Sp1 and a simultaneous increase in Sp3 binding to the respective MVP promoter region. The influence of HDAC inhibitors on Sp-transcription factor binding properties was neither based on a change in the expression levels of Sp1 or Sp3 nor on a change in their intracellular localisation as analysed by cell fractionation and Western blot (Fig. 6B). This was also confirmed on the single cell level by immunofluorescence staining (data not shown).

Discussion

Vaults and their main constituent, the major vault protein MVP, are highly conserved in evolution and widely expressed in human tissues [1]. They have been implicated in several cellular processes including nucleocytoplasmic and vesicular transport [4], cell motility [5], chemoresistance (for review: [22]), as well as malignant transformation, and tumor aggressiveness [6,7,23]. Vaults are abundant with over 10^4 particles per differentiated cell [10]. However, distinct variations in MVP expression have been observed in different tissues and cell types [24], and MVP expression may change distinctly during malignant transformation [6], following cytotoxic impacts [11], and by application of the HDAC inhibitor NaB [12]. Activation and control of MVP expression, however, have not been analysed extensively so far. In the present study we have assigned the major MVP promoter activity to a single GC-box element activated in trans by binding transcription factors of the Sp-transcription factor family. Interestingly, not only basal promoter activation but also stimulation of MVP promoter activity by NaB and another HDAC-inhibitor TSA involved the unique GC-box element essential for basal promoter activation. Stimulation by HDAC inhibitors was accompanied by a distinct shift from Sp1 towards Sp3 binding to the respective MVP promoter element in vivo.

Sequencing of the human MVP promoter sequence revealed, in agreement with published data [14], that the promoter is TATA-less and lacks a distinct CpG island as well as other core promoter elements including an initiator (Inr) and a downstream core promoter element (DPE). The absence of a TATA-box is a notable feature of many housekeeping genes, oncogenes, growth factors, and transcription factors [25]. TATA-less and Inr-less promoters have been described for genes characterised by multiple TSPs and an Sp-binding GC-box element located −40 to −80 bp upstream of the TSPs [25]. Despite a comparable situation in case of the human MVP promoter, indications for multiple TSPs have neither been reported [14] nor found in our experiments ([13] and data not shown). The MVP proximal promoter sequence harbours binding sites for several sequence-specific transcription factors including p53, STAT1, MyoD, an E-box, a GATA-box, an inverted CCAAT-box, and a GC-box element.

As reported previously [14], deletion of the 5′-part of the promoter led to a significant increase in promoter activity. This localises a repressing activity at the distal part of the promoter. Further deletions performed in this study localised the highest promoting activity to the proximal promoter region close to the TSP (−124 to +43). Out of the putative binding sequences located within that region site-directed mutagenesis experiments disclosed the single GC-box as an essential element for activation of the human MVP promoter. GC-boxes play an important role in transcription from TATA-less promoters, by binding members of the Sp-family and other transcription factors [26]. By several methodical approaches we showed that transactivation of the unique GC-box element in the human MVP gene promoter

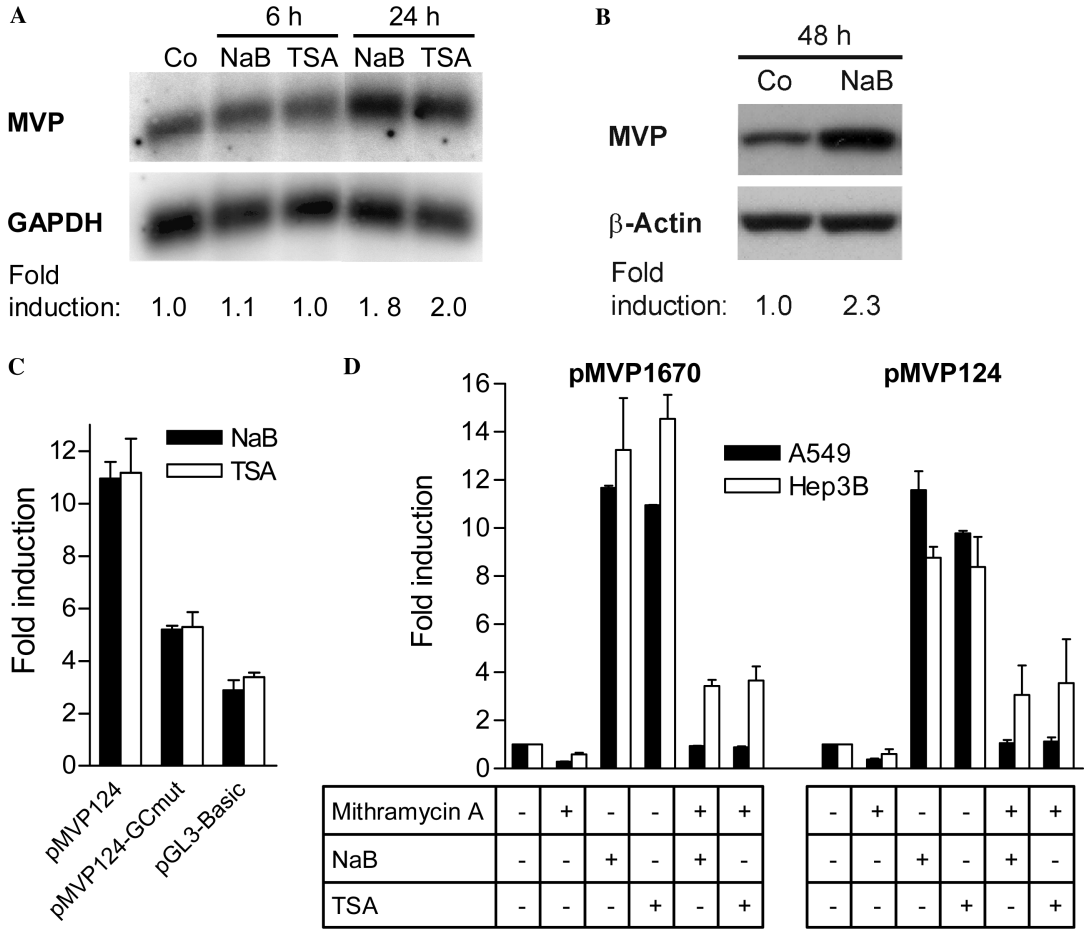


Fig. 5. Stimulation of MVP expression and promoter activity by HDAC inhibitors NaB (2 mM) and TSA (100 ng/ml) and role of GC-box binding factors. (A,B) A549 cells were treated for the indicated times with HDAC inhibitors NaB and TSA and MVP mRNA (A) as well as protein (B) were detected by Northern blot and Western blot, respectively. (C) Hep3B cells were transiently transfected with pMVP124, pMVP124-GCmut, or empty vector (pGL3-Basic) and after 24 h exposed to NaB or TSA for additional 24 h. Luciferase data are given relatively to the value for untreated cells. (D) Cells were transfected with the indicated MVP promoter constructs and treated for 24 h before luciferase detection with HDAC inhibitors alone or in combination with 100 nM mithramycin A as indicated. Means and SD of three independent experiments are given as fold induction as compared to the untreated controls.

by Sp-transcription factor family members is critical for basal and stimulated promoter activity. A TATA-less promoter activated by Sp1 is also characteristic for many multidrug resistance genes including human MDR1 [27], MRP1 [28], MRP3 [29], and BCRP [30]. This goes well together with co-expression of MVP and several of these transporter proteins in potentially toxin-exposed tissues [24] and again indicates a role of MVP in protection from xenobiotics [22].

Sp1 [31] is a transcriptional activator interacting with several subunits of the human TFIID complex, thus facilitating assembly of the basal transcription machinery especially to TATA-less promoters [32]. Corresponding to its function in the activation of many house-keeping genes, Sp1 is widely expressed in human tissues. An increasing amount of data, however, accumulates that Sp1-dependent transcription might also be involved in expression regulation [26]. This raises the question whether Sp-transcription factors may also contribute to tissue-specific regulation and/or stimulation of MVP gene expression [24]. We show that several members of the Sp-family are able to transactivate the human MVP promoter in SL2 cells at different potencies. Besides Sp1 also Sp3, however, with lower affinity, interacted with the critical GC-box in EMSA and ChIP experiments. Thus, the ratio of Sp-family members, differing in their activating potential [26], might have an influence on tissue-specific MVP transcription. AP-2 has been shown to increase or repress Sp1-dependent activity of promoters containing combined AP-2/Sp1 sites [32]. However, the GC-rich element in the human MVP promoter lacked any interaction with AP-2 making a major regulatory function of AP-2 rather unlikely. Also NF-Y, often cooperating with Sp1 [33], is unlikely to be involved in the MVP promoter activation since deletion of the inverted CCAAT-box-containing extended region of the promoter (CCAAT-box at position −270 to −285) did not result in an inhibition of MVP promoter

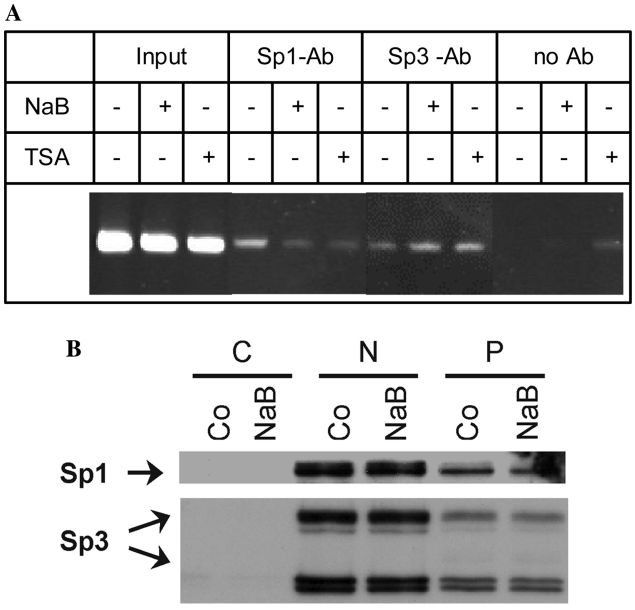


Fig. 6. Impact of HDAC inhibitors on MVP promoter binding, expression level, and subcellular localisation of the Sp-transcription factors Sp1 and Sp3. (A) ChIP assays were performed as described under Fig. 2B using chromatin from Hep3B control cells and cells treated with NaB (2 mM) or TSA (100 ng/ml) as indicated. (B) Western blot analysis of cytosolic (C), nuclear (N), and particle (P) fractions prepared from Hep3B control cells and cells treated with NaB is shown.

activity. The binding sequences for Sp1 and p53 in the human MVP promoter overlap which suggests possible competition for the promoter binding site, as known for the MRP1 promoter [28]. Accordingly, preliminary data generated in our laboratory suggest inhibition also of the human MVP promoter by wild-type p53 (unpublished observation).

NaB, a fermentation short fatty acid product, induces differentiation and apoptosis in several cell types and can affect transcription in a positive and negative manner [34]. Only around 2% of all genes are regulated by NaB. It exerts its main effects by inhibition of HDAC and involves besides histone hyperacetylation and chromatin remodelling, also acetylation of non-histone proteins and binding to non-HDAC proteins [34]. NaB potently activates MVP expression in colon cancer cells leading to enhanced drug resistance and nuclear doxorubicin export [12]. We demonstrate here that the stimulation of MVP expression by NaB is mediated by a transcriptional effect. This effect is believed to involve the HDAC-inhibitory activity of NaB, since also TSA, another HDAC inhibitor [35], comparably activates MVP transcription. Interestingly, the MVP promoter stimulation by both compounds was partly dependent on the intact conserved GC-box within the proximal promoter sequence. This indicates that MVP belongs to the short list of genes (including p21/WAF1) activated by NaB involving a GC-box-dependent mechanism [34]. Correspondingly, MVP promoter stimulation by HDAC inhibitors was severely attenuated or even blocked by mithramycin A which interferes with binding of Sp-family members to GC-boxes. The stronger inhibitory effect of mithramycin A (up to 100%) as compared to the mutation in the single GC-box (~50%) suggests that possibly other GC-rich promoter regions might support the investigated GC-box element in promoter activation by HDAC inhibitors. It has been reported that HDAC inhibitors might severely change the transactivating activity of Sp family transcription factors [36]. By ChIP assays we demonstrated a distinct decrease in the association of Sp1 and in parallel an increase of Sp3 with the respective MVP promoter element. These observations were not based on changes in Sp1 or Sp3 expression or their subcellular localisation by HDAC inhibitors. This suggests a regulation of Sp transcription factor recruitment to the MVP promoter by posttranslational modifications. Sp1 has been shown to be phosphorylated by HDAC inhibitors and Sp1 phosphorylation may result in enhanced, unaltered, but also decreased DNA-binding activity [37,38]. The TSA-induced upregulation of p21/WAF1/Cip1 was shown to depend on Sp3 and not Sp1 transactivation despite the presence of both proteins at the respective binding site [36]. Our ChIP assay data suggest a distinct influence of HDAC inhibitors on the protein complexes associated with the respective GC-box in the human MVP promoter. The precise influence of different post-transcriptional modifications of Sp-family members [37,39] on MVP expression has to be further analysed.

Taken together our results suggest an essential function of the ubiquitous transcription factor Sp1 and probably other Sp-family members in basal human MVP gene expression via binding to a single GC-box element within the proximal MVP promoter. The identical GC-box element is also involved in the stimulation of MVP expression by HDAC inhibitors. To what extent interactions of Sp-family members and other transcription factors regulate tissue-specific MVP expression needs to be established in further studies.

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