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First identification and functional analysis of the human xylosyltransferase II promoter

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Abstract Recently, we demonstrated that the human xylosyltransferase II (XT-II) has enzymatic activity and is able to catalyze the initial and rate-limiting step in the biosynthesis of glycosaminoglycans (GAGs) like chondroitin and dermatan sulfate, as well as heparan sulfate and heparin. Therefore, this enzyme also very likely assumes a crucial regulatory role in the biosynthesis of proteoglycans (PGs). In this study, we identified and characterized for the first time the *XYLT2* gene promoter region and transcription factors involved in its regulation. Several binding sites for members of the Sp1 family of transcription factors were identified as being necessary for transcriptional regulation of the *XYLT2* gene. This was determined by mithramycin A treatment, electrophoretic mobility shift and supershift assays, as well as numerous site-directed mutagenesis experiments. Different 5′ and 3′ deletion constructs of the predicted GC rich promoter region, which lacks a canonical TATA and CAAT box, revealed that a 177 nts proximal promoter element is sufficient and indispensable to drive the constitutive transcription in full strength in HepG2 hepatoma cells. In addition, we also detected the transcriptional start site using 5′-RACE (rapid amplification of cDNA ends). Our results provide an insight into transcriptional regulation of the *XYLT2* gene and may contribute to understanding the manifold GAG-involving processes in health and disease.

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Abbreviations

AP-1/2	Activator protein 1/2
HNF4α	Hepatocyte nuclear factor 4α
Sp1/3	Specificity protein 1/3
XT-I/II	Xylosyltransferase I/II (mRNA and protein)
XYLT1/2	Xylosyltransferases 1/2 (human gene)
Xylt2	Xylosyltransferases (murine gene)
PGs	Proteoglycans
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
EMSA	Electrophoretic mobility shift assay
RT-PCR	Reverse transcriptase polymerase chain reaction
5′–RACE	5′ rapid amplification of cDNA ends
TFBS	Transcription factor binding sites

Introduction

Proteoglycans (PGs) are a large group of specialized polyanionic glycoproteins, which are expressed on virtually every animal cell surface as well as in the extracellular matrix [1]. They are characterized by the presence of one or more glycosaminoglycan chains. These carbohydrate moieties are covalently linked to particular serine residues of the PG core protein backbone and are responsible for the high degree of structural variety [2, 3], reflecting the broad range of biological functions of PGs. They are involved, amongst other things, in the regulation and maintenance of cell adhesion, cell proliferation and differentiation, extracellular matrix deposition, tumor cell growth, viral infections, as well as neurite outgrowth [1]. Many of these biological properties

are closely related to the presence of GAG chains, which contain binding sites for various growth factors, cytokines and extracellular matrix proteins. The glycosaminoglycans chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin are anionic linear polysaccharides consisting of alternating disaccharide units which are attached to the core protein by a uniform tetrasaccharide linker which is composed of D-GlcA-β1-3-Gal-β1-3-Gal-β1-4-Xyl-Ser. The formation of GAG chains occurs by a subsequent addition of monosaccharides in a complex interaction of different enzymes. However, the initial and rate-limiting transfer of D-xylose from UDP-D-xylose to specific serine residues in the core protein is catalyzed by either xylosyltransferase I (XT-I) [4–7] or, as we and others recently demonstrated, by xylosyltransferase II (XT-II) [8, 9]. Interestingly, all vertebrates express two xylosyltransferase isoforms, but the reason has not been elucidated yet. So far, the only applicable difference between XT-I and XT-II is the divergent expression of the respective enzyme in different tissues. High XT-II mRNA levels were found in the kidney, lung and especially in the liver [4, 8, 10]. Although the XT-II cDNA was already cloned in 2000 [7], very little is known about the transcriptional activation and regulation of the *XYLT2* gene. Therefore, the aim of this study was to investigate the minimal active promoter region of this gene and to identify the responsible regulatory transcription factors. A screening of numerous cell lines by us and others revealed that the hepatoma cell line HepG2 has a very similar xylosyltransferase expression pattern compared to human and murine liver tissue with comparatively high levels of *XYLT2* [7–9, 11]. Consequently, this cell line provides all transcription factors necessary for proper expression of XT-II and is therefore suitable for investigating the *XYLT2* promotor region. In so doing, we detected that a proximal 177 nts promoter element is able to drive the transcription in full strength in this cell line. We also demonstrated that members of the Sp1 transcription factor family, Sp1 and Sp3, are substantially involved in the regulation of the *XYLT2* gene.

This study extends our knowledge of the transcriptional regulation of the *XYLT2* gene. Therefore, it may contribute to a better understanding of extracellular matrix formation and remodeling including the aforementioned processes dependent on glycosaminoglycans.

Results

Identification of transcription start site

Agarose gel electrophoresis resolved the 5′–RACE-nested reaction products into two distinct bands which were cloned thereafter. Sequence analysis of eight clones revealed that the longest insert terminated 13 bp 5′ of the translation initiation site of the XT-II mRNA (GenBank accession no. NM_022167) (Fig. 1, TSS).

Identification of *XYLT2* promoter region and activity analysis of promoter luciferase constructs

The putative *XYLT2* gene promoter region was identified using Genomatix ModelInspector software [12]. A genomic fragment spanning 2568 nts 5′ up to 2 nts 3′ of the translation initiation start site was cloned upstream of a firefly luciferase reporter gene in a pGL4.10 vector, as described in the experimental section. Different 5′ and 3′ truncations of this putative promoter fragment were generated and transfected into HepG2 hepatoma cells. The results of the dual luciferase assays are shown in Figs. 2a–c. The highest activity was observed for a fragment spanning 177 nts of the 5′-flanking region. A truncation of 37 nts significantly decreases the promoter activity to 46.5 %±4.2 % (mean± SEM). Only less than one third of the original promoter activity was detected for a further 13 nts 5′-truncated construct. Finally, a truncation to 43 nts completely abolished the promoter activity (Fig. 2a). To further examine the importance of the 177 nts immediately upstream of the

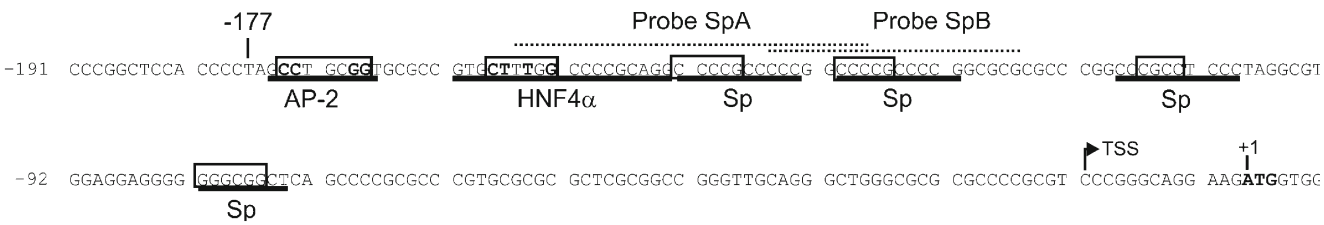

Fig. 1 Nucleotide sequence of the 5′ flanking region of the *XYLT2* gene. Nucleotides ranging from −191 nts upstream to +45 nts downstream of the translation initiation site (ATG). The sequence corresponds to nts 48,423,311 to 48,423,547 of human chromosome 17 (GenBank accession no. NC_000017.10). Numbers to the left indicate nucleotides upstream and downstream of the translation initiation site, which is printed in bold typed letters and indicated by +1. The transcription start site (TSS) is marked by an angeled arrow. Selected putative transcription factor binding sites are labeled below the sequence. The oligonucleotides used as probes in EMSA experiments in Fig. 3 are indicated by dashed lines. The boxed nucleotides representing parts of Sp1 family binding sites were mutated to an *EcoRI* restriction site (GAATTC) in site-directed mutagenesis experiments (see also Fig. 2d). In case of AP-2 and HNF4α the sequences were mutated to **AG**TGC**TC** and **TC**TA**GA**, respectively (mutated nucleotides in bold typed letters)

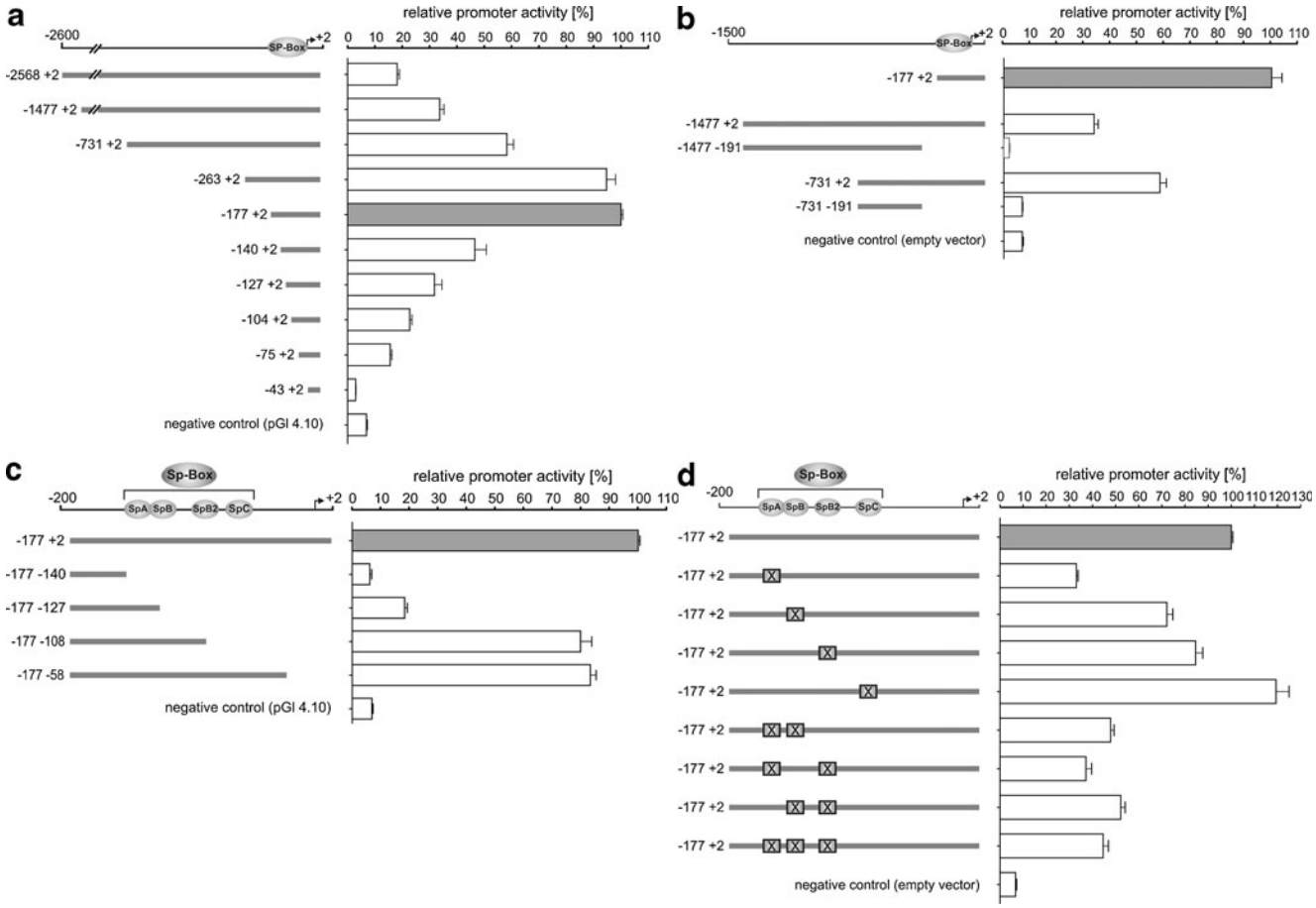

Fig. 2 Functional analysis of human *XYLT2* promoter constructs. **a** Functional analysis of human *XYLT2* promoter activity using 5′ deletion constructs transfected in HepG2 cells. Bars show relative luciferase activity for *XYLT2* promoter constructs cloned into pGL4.10 vector compared to the highest detected activity of the −177 +2 construct, which was set to 100 % (*grey bar*). As a negative control for vector backbone activity the promoterless pGL4.10 vector was used (negative control). The numbers on the left indicate the 5′-ends of the constructs relative to the translation initiation site. The values are the mean±SEM of triplicates from at least three independent experiments. **b** Effect of 191 nts 3′ deletions on *XYLT2* promoter activity for selected promoter constructs. **c** Detection of the minimal active *XYLT2* promoter that provides full constitutive transcriptional activity analyzed by different 3′-truncations of the −177 +2 construct. **d** Analysis of mutated Sp transcription factor binding sites within the −177 +2 proximal promoter construct. All predicted binding sites for Sp1 family transcription factors, termed SpA, SpB, SpB2, and SpC were mutated individually to an *EcoRI* restriction site indicated by an **X**. To elucidate putative cooperative interactions also multiple mutations were inserted

translation initiation site for transcriptional activity, 3′ truncation constructs lacking these nucleotides were generated and the promoter activity was compared to the respective full-length ones. Here, a complete loss of promoter activity for the 3′ truncated fragments was detected (Fig. 2b). To identify the minimal active promoter, we generated several 3′ truncations of the 177 nts fragment. Two constructs, spanning 177 up to 108 nts and 177 up to 58 nts upstream of the translation initiation start nearly exhibited full promoter activity (Fig. 2c). Based upon these findings, the sequence between −177 and −108 was investigated in more detail. MatInspector analysis [12] highlighted several binding sites for transcription factors of the AP-2, HNF4α, and Sp1 family of transcription factors (Fig. 1). Therefore, the core sequences of all binding sites denoted in Fig. 1 were mutated individually to prevent the binding of the putative transcription factors. For the AP-2 and the HNF4α transcription factor binding sites (TFBSs) no significant alterations in the promoter activity were observed (data not shown). In contrast, the mutation of several putative TFBSs for members of the Sp1 family caused considerable changes in the transcriptional level. A mutation of the TFBS termed SpA (in Figs. 1 and 2c–d), decreases the promoter activity by 66.8 %± 0.8 %, whereas a mutation of the nearby SpB and SpB2 binding sites results only in a reduction of 27.8 %±2.6 % and 15.4 %±3.1 %, respectively. Interestingly, a mutation of the further 3′ located putative SpC TFBS leads to a slight increase in promoter activity (Fig. 2d).

To elucidate possible synergistic or antagonistic interactions of the TFBSs, several combinations of mutations were

generated. Promoter constructs containing multiple mutations including a mutated SpA TFBS, only exhibited marginal changes in their transcriptional activity compared to the single mutated SpA binding site. In contrast, a mutation of both, SpB and SpB2, decreases the promoter activity to 52.2 %±1.9 %.

EMSA and supershift analysis

To clearly identify the species binding to the TFBS SpA, which exhibited the highest activity in the luciferase experiments, electromobility shift and supershift assays using a 5′-biotinylated double stranded probe containing the SpA binding site of the *XYLT2* promoter were used (probe is also indicated in Fig. 1 by a dotted line). The obtained results are presented in Fig. 3. The probe was either incubated with HepG2 nuclear extracts or recombinant Sp1 protein. For the probe incubated with the nuclear extract, two specific bands were observed that were supershifted either by an antibody against Sp1 or Sp3. The specificity of the transcription factor binding was proven by competition experiments, in which a molar excess ranging from 40 to 200 fold of the respective unlabeled probe was added. The lowest concentration of unlabeled probe was sufficient to compete for the binding of the transcription factor to the biotin-labeled probe demonstrating the specificity of protein binding. In experiments using a specific antibody against Sp1 and Sp3 protein or both, supershifted bands were observed accompanied by a considerable reduction in the shifted band. A very distinct shift and supershift were also observed for the recombinant Sp1 protein.

A similar but considerable weaker binding was observed using a probe containing the sequence of the TFBS SpB (data not shown; probe indicated in Fig. 1 by a dashed line). To semiquantify the affinity of Sp1 protein binding, both probes were incubated with the same amount of recombinant Sp1 protein and resolved on the same gel. Afterwards, the band intensities were subjected to densitometric

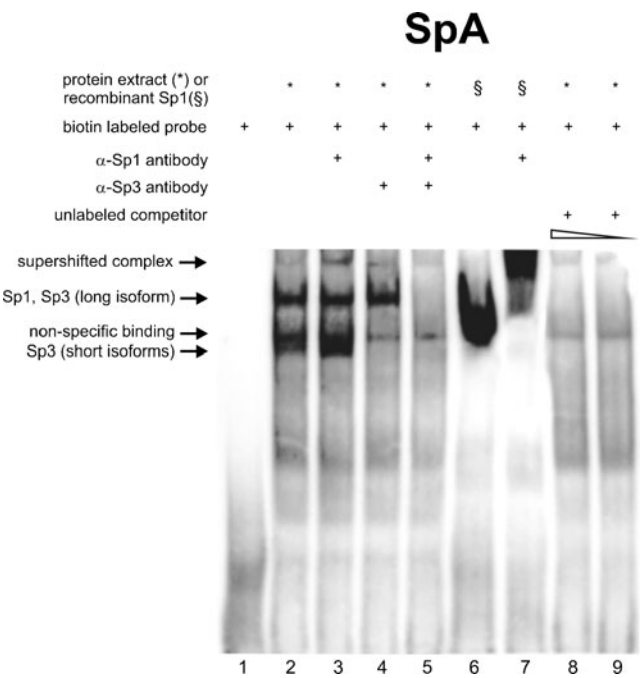

Fig. 3 Binding of HepG2 nuclear proteins to probe SpA of the *XYLT2* promoter. HepG2 nuclear protein extracts (*) were incubated with biotin-labeled probes and subjected to an electromobility shift assay. Two distinct shifted DNA-protein complexes (lane 2) were obtained (Sp3 long and Sp3 short denote the different Sp3 isoforms). Addition of an anti-Sp1 antibody resulted in a faint supershift (lane 3) whereas anti-Sp3 (lane 4) antibody or both antibodies combined (lane 5) resulted in a specific elimination of the DNA-protein complexes and supershifts. Using recombinant Sp1 protein with or without an anti-Sp1 antibody (lanes 6 and 7) shifted and supershifted the probe in a strong and specific manner. Addition of an excess amount of unlabeled probe (200 fold and 40 fold) resulted in the elimination of the specific DNA-protein complexes (lane 8 and 9)

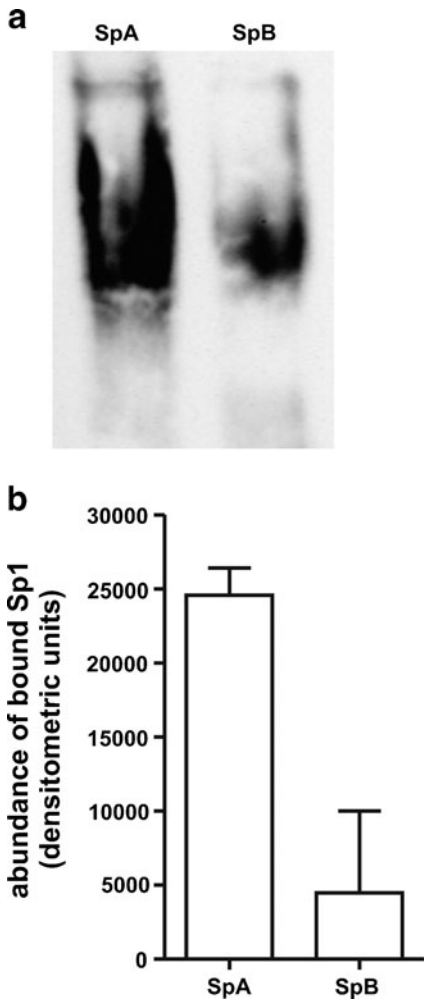

Fig. 4 Binding of recombinant Sp1 protein to biotin-labeled EMSA probes SpA and SpB. Exemplified presentation of three independent binding reactions to probe SpA and SpB (**a**) and their densitometric examination (**b**)

measurement. Here, we detected that Sp1 binds with a more than 5-fold greater affinity to SpA than to SpB (Fig. 4).

Mithramycin A treatment

To analyze the role of Sp1 family members in the regulation of the native *XYLT2* promoter, HepG2 cells were treated with different concentrations (10 nM–1 μM) of mithramycin A, which prevents the binding of transcription factors to GC-rich sequences and is a well known inhibitor of Sp transcription factor binding [13]. Even 100 nM mithramycin A in the cell culture supernatant were sufficient to reduce the XT-II mRNA levels to 27.2 %±1.7 % compared to untreated controls (Fig. 5, $p<0.0005$).

Discussion

XT-II is the only xylosyltransferase expressed in the liver of humans and mice [4, 8, 10] and very likely plays an important role in liver PG homeostasis [10]. The particular importance of this enzyme was demonstrated by *Xylt2* gene knockout experiments. Mice lacking the *Xylt2* gene exhibit a substantial reduction in PGs and a phenotype characteristic of many aspects of polycystic liver and kidney disease resulting from alterations in the PG and GAG concentrations [10]. Furthermore, the murine liver was identified as a significant source of circulating xylosyltransferase activity in serum. This suggests that XT-II is also the predominant xylosyltransferase in human serum [14]. Additionally, we provided further evidence for the biological importance of XT-II in humans: mutations in the *XYLT2* gene are genetic risk factors for diseases that are characterized by an altered

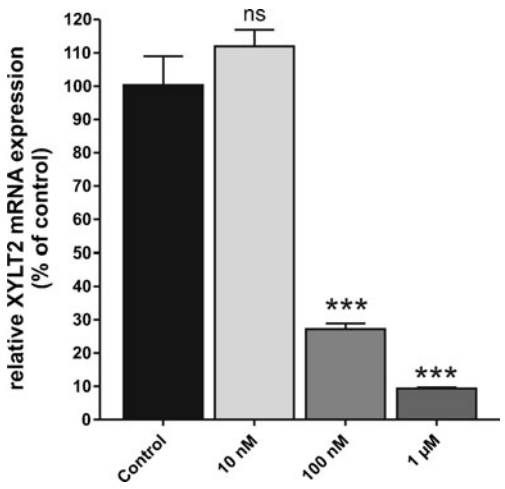

Fig. 5 Real-time quantitative RT-PCR analysis of the XT-II mRNA content of HepG2 cells after mithramycin A treatment. Cells were treated with indicated concentrations of mithramycin A for 24 h. Values are displayed as mean±SD. ***$p<0.0005$; ns, non significant

PG metabolism, such as pseudoxanthoma elasticum [15], osteoarthritis [16] or diabetic nephropathy [17, 18]. However, little is known about the transcriptional regulation and possibilities of modulating the expression of the *XYLT2* gene. To gain an insight into the transcriptional activation, we identified and characterized the promoter region, as well as the transcription start site of the *XYLT2* gene. We also determined important transcription factors involved in its regulation. A detailed *in silico* analysis of the sequence located immediately upstream of the *XYLT2* gene revealed that this region does not contain a canonical TATA or CAAT box. In contrast, especially the first 200 nts 5′ of the translation initiation start exhibit several GC boxes and an extraordinary high overall GC content of about 84 %. The absence of a TATA box and the presence of CpG islands are often encountered within housekeeping gene promoters [19–21]. This might be an explanation for the wide distribution of *XYLT2* gene transcripts in different tissues [4, 7, 10]. As shown in Fig. 1, among the predicted transcription factor binding sites (TFBSs) several are belonging to the group of Sp1 proteins. Multiple Sp1/Sp3 TFBSs were also found to be active in many other ECM protein coding genes, *i.e.* collagens like Col11a2 [22], proteoglycan core proteins like the human glypican-3 [23] and the murine glypican-4 [24]. Sp1 and Sp3 proteins are also involved in the regulation of other enzymes participating in GAG biosynthesis like hyaluronan synthase 2 [25] or the UDP-glucose dehydrogenase [26, 27]. In contrast to the promoter region of the XT-II isoform XT-I, no binding site for the Activator protein 1 (AP-1) transcription factor, which plays a considerable role in the regulation of the *XYLT1* gene was identified in the regulatory regions of the *XYLT2* gene [28]. This fact might participate in the divergent expression and different regulation of both genes in different tissues and cell lines [11].

Due to the fact that genes expressed in the same functional context, like a metabolic pathway, often share promoter modules or TFBSs [29–31], an important participation of Sp1 family members can be assumed. Although software-calculated predictions of putative *cis* acting elements are a useful starting point for gene regulation analysis, they do not provide evidence for their actual function in the respective cell line [32]. Therefore, we analyzed the activity of 5′ and 3′ truncated *XYLT2* promoter constructs to locate the minimal active promoter. Our results revealed that a 177 nts fragment immediately upstream of the translation initiation site is the smallest promoter insert that mediated full transcriptional activity (Fig. 2a). We also demonstrated that this part of the promoter is indispensable for constitutive transcription of the *XYLT2* gene, since constructs lacking these nucleotides exhibit no transcriptional activity (Fig. 2b). Further 3′ truncations of the 177 nts construct discovered that the TFBSs primarily responsible for the *XYLT2* promoter activity are located between base

pairs −177 and −108. Here, four adjacent Sp TFBSs referred to as Sp-Box in Fig. 2a–d were identified. By individually mutating all of these *cis* acting sites within this region, we analyzed the contribution of each Sp site to the *XYLT2* promoter activity. The most considerable effect was detected for the most 5′ located TFBS (termed SpA in Fig. 2c–d) indicating that this sequence is essentially responsible for driving the *XYLT2* gene expression. The influence of the nearby 3′ located SpB TFBS on the promoter activity was considerably less, although the binding sequence of both, SpA and SpB, is equivalent to the consensus sequence, CCCGCCC, for the binding of Sp1 and Sp3. A similar result was observed for the third putative *cis* acting element (SpB2), indicating that spatial effects, *i.e.* the distance to the TSS, as well as the order of TFBSs, are important for transcriptional regulation [33]. Interestingly, the mutation of the fourth putative Sp binding site has only marginal effects on the reporter gene expression. This possibly arises from the binding of negative regulatory elements in this region. The mutation of multiple binding sites, for elucidating putative cooperative interactions, revealed that the simultaneous mutation of SpB and SpB2 lead to a significant decrease in transcriptional activity, considerably more distinctive than the respective single mutation. This makes a synergistic interaction of the bound transcription factors probable. In contrast, constructs with multiple mutations including the SpA binding site, exhibit only marginal alterations in the reporter gene expression. These results provide evidence that the SpA binding site is primarily responsible for the transcriptional activation. Furthermore, these data are in concordance with the results obtained from EMSA experiments. Both probes used for SpA and SpB gave typical EMSA profiles showing the binding of commonly occurring Sp1 and multiple Sp3 isoforms [22, 34, 35] (Fig. 3a-b). The specificity of binding was demonstrated by the addition of excess unlabeled probe. Supershift analyses further confirmed the specific binding of Sp1 and Sp3, although for Sp1 only a faint but distinct supershifted band was observed. This might indicate that Sp3 is the main binding species in HepG2 cells under the conditions investigated (see below). Furthermore, using a mixture of an anti-Sp1 and anti-Sp3 antibody results in the disappearance of the shifted protein bands again demonstrating the binding of Sp1 and Sp3 to the promoter probe. Notably, the magnitude of protein binding was different between both probes, with enhanced protein binding to probe SpA. These results were further verified using recombinant Sp1 protein to semiquantify the affinity of the specific transcription factor binding to the respective oligonucleotide (Fig. 4). Here, the densitometric examination revealed a more than 5-fold higher binding affinity of recombinant Sp1 to probe SpA compared to SpB (based on the densitometric data, Fig. 4). This additionally emphasizes the results obtained from the site-directed mutagenesis experiments. Results from the mithramycin A treatment further confirmed the participation of Sp1 and Sp3 in the regulation of the *XYLT2* gene expression. The transcriptional activity of Sp1 family proteins is thought to be dependent on the cellular context, the structure and arrangement of the recognition sites, as well as on the Sp1:Sp3 ratio within the cell [35, 36]. Therefore, the other Sp binding sites within the investigated proximal 177 nts construct may function as modulators of the *XYLT2* promoter activity. Changes in the cellular environment and response to external stimuli have the potential to alter the respective levels of Sp1 and Sp3 and in so doing may modulate their function on the *XYLT2* promoter as well [35–38].

Another interesting aspect is the existence of an HNF4α binding site within the proximal *XYLT2* 177 nts fragment. Although no significant changes in promoter activity were observed after mutating this *cis* acting element, a possible role in the transcriptional regulation of the *XYLT2* gene in hepatocytes cannot be excluded. HNF4α is a liver-enriched transcription factor that is also expressed in HepG2 cells. Notably, its activity in these cells and other hepatoma cell lines was described as very low or absent due to the underexpression of coactivators necessary for its activity [39].

In summary, we have identified the transcription factors Sp1 and Sp3 as principal mediators of constitutive *XYLT2* transcription in the hepatoma cell line HepG2. Four adjacent recognition sites upstream of *XYLT2* exon 1 were demonstrated to be functional, with SpA as the most active one. We have also shown that the putative AP-2 and HNF4α binding sites are not active in HepG2 cells. In contrast to the recently described XT-I promoter [40], no putative AP-1 binding site within the *XYLT2* promoter was identified. This might be a first step towards explaining the different regulation of XT-I and XT-II as described in previous studies [41–43]. Furthermore, it might help to elucidate the reason for the divergent expressions and activities of these enzymes in different tissues. This aspect will be the subject of further studies.

Experimental procedures

Cell culture

HepG2 (ATCC, human, hepatoma cells) were grown in Dulbecco's modified eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % (v/v) fetal calf serum (Biowest, Nuaillé, France), L-glutamine (PAA Laboratories, Pasching, Austria) and antibiotic-antimycotic (Biowest). The cells were maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C. Culture medium was changed twice a week. Where appropriate, cells were incubated with different concentrations of mithramycin A (10 nM–1 μM) (Sigma, Hamburg, Germany) for 24 h. Before being used for

promoter analysis or RNA lysis, cells were washed twice in phosphate buffered saline (PBS).

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was extracted from cells using the NucleoSpin RNA II Kit (Macherey & Nagel) according to the manufacturer's instructions. cDNA synthesis was performed with oligo (dT) primers using SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). The mRNA expression was analyzed using a SYBR green Taq-DNA polymerase mixture (Platinum SYBR Green qPCR SuperMix UDG, Invitrogen) on an Eppendorf Real-Plex MasterCycler-System (Eppendorf, Germany). Thermal cycling conditions included enzymatic degradation of uracil containing DNA at 50 °C for 2 min, activation of the DNA polymerase at 95 °C for 2 min followed by 45 cycles at 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. The transcriptional level of XT-II was normalized to constant mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following specific oligonucleotide primers were used: XT-II (forward: 5′-CCT TGT GCT GCC CTT GAC-3′; reverse: 5′-GCC CTG GAA ACT CTG CTC-3′), GAPDH (forward: 5′-AGG TCG GAG TCA ACG GAT-3′; reverse: 5′-TCC TGG AAG ATG GTG ATG-3′).

Nuclear extracts and gel retardation assays

Nuclear extracts were obtained from HepG2 hepatoma cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, USA) containing a proteinase inhibitor cocktail (Sigma) according to the manufacturer's protocol. Protein concentration in the nuclear fraction was determined by BCA assay (Sigma). Two 5′-biotinylated oligonucleotides containing putative active transcription factor binding sites (TFBS) of the *XYLT2* promoter were used (SpA: 5′-TTG GCC CCG CAG GCC CCG CCC CCG GCC C-3′, SpB: 5′-CCC GGC CCC GCC CCG GCG CG-3′, and the respective complementary strand). Detection of the Sp oligonucleotide-protein complexes was performed using the LightShift chemiluminescent EMSA kit (Pierce). Briefly, nuclear protein extract (6 μg) or 1 μg recombinant Sp1 protein (Promega, Mannheim, Germany) was incubated in a buffer containing 10 mM Tris, 50 mM KCl, 1 mM DTT (pH 7.5), 5 % glycerol, 75 ng/μl poly(dI·dC), 0.5 mg/ml BSA and 0.025 % NP-40 for 1 h on ice. Afterwards, 25 fmol of the biotinylated oligonucleotide was added and the reaction mixture was allowed to incubate for another 20 min at room temperature. Finally, the DNA-protein complexes were resolved on a 6 % polyacrylamide gel (Invitrogen) in 0.5x TBE buffer for 1.5 h at 100 V. Following the transfer on a positive charged nylon membrane (380 mA, 30 min) in 0.5x TBE, the protein DNA complexes were crosslinked to the membrane by UV-light (312 nm) for 15 min and finally detected in a chemiluminescent reaction using a streptavidin horseradish peroxidase conjugate. For competition and supershift experiments, unlabeled probes or antibodies were added to the reaction mixture 1 h before addition of the labeled probe. The anti-Sp1 and Sp3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Synthesis of promoter construct inserts

All promoter fragments were obtained by PCR amplification from genomic DNA by standard methods and subcloned into the pBlue TOPO vector (Invitrogen). Afterwards, the respective pBlue TOPO vectors were digested with *SacI* and *HindIII* (New England Biolabs, Hertfordshire, UK). The promoter fragments were analyzed by 1.5 % agarose gel electrophoresis, gel-purified using the NucleoSpin Extract II kit (Macherey & Nagel), and subsequently ligated into a promoterless pGL4.10 luciferase reporter vector. To ensure the fidelity of the cloned promoter fragments, all final constructs were sequenced using the vector specific primers pGL4F (5′-CTA GCA AAA TAG GCT GTC CC-3′) and pGL4R (5′-CTT AAT GTT TTT GGC ATC TTC CA-3′).

Transient plasmid transfections

Plasmids were purified from bacterial cultures using the GeneElute (HP) Plasmid Midiprep kit (Sigma). HepG2 cells were seeded on 4.0 cm^2 12 well plates. After 24 h, the cells were transiently transfected with a mixture of 1 μg of the particular promoter fragment containing pGL4.10 plasmid and 10 ng of the pGL4.74 vector (Promega) using Lipofectamine 2000 transfection reagent (Invitrogen) in accordance with the manufacturer's instructions. The pGL4.74 vector, which contains the thymidine kinase promoter from herpes simplex virus and the Renilla luciferase reporter gene, was used as an internal control to normalize for the efficiency of the transient transfection. After 48 h, the luciferase activity was assayed with the Dual Luciferase Reporter assay system (Promega) on a Lumat LB9705 luminometer (EG&G, Berthold, Bad Wilbad, Germany). As a control for vector backbone-based luciferase expression, the promoterless pGL4.10 vector was used.

Statistical analysis of promoter activity assay data and real-time quantitative RT-PCR data

Promoter activity was calculated as the ratio of the luminescence values for each *XYLT2* promoter firefly-luciferase reporter construct and the corresponding value for the cotransfected pGL4.74 vector for normalization of transfection efficiency. All assays were performed in triplicate and each experiment was repeated at least three times. Relative expression values and fold changes in expression of real-

time quantitative RT-PCR data were calculated using the equation published by Pfaffl *et al.* [44], which considers the PCR efficiency. Activity and expression levels between the two groups were compared using the Mann–Whitney-*U* test. *P*-values were considered significant below 0.05. All tests were calculated using GraphPad Prism 4.0 (GraphPad Prism Software, San Diego, CA, USA).

Densitometric detection

ImageJ 1.40 g software (Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij) was used for the semiquantitative determination of band intensities.

5′ rapid amplification of cDNA ends (5′-RACE)

Total RNA from human dermal fibroblasts was isolated using the mirVana RNA isolation kit (Ambion) as recommended by the manufacturer prior to 5′-RACE (Vertis Biotechnologie, Freising, Germany). The obtained full length cDNA was amplified in PCR and nested PCR reactions, analyzed by 1.5 % agarose gel electrophoresis, cloned in a pCR2.1 TOPO vector (Invitrogen) and finally sequenced using Big Dye terminator chemistry (Applied Biosystems, Warrington, Cheshire, UK) using vector-specific M13 primer (Invitrogen).

Site-directed mutagenesis

Site-directed mutagenesis experiments were performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. The respective oligonucleotide primers were purchased from biomers (biomers.net, Ulm, Germany).

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