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Promoter paper

Characterization of the human zinc finger protein 267 promoter: Essential role of nuclear factor Y

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Abstract

Liver fibrosis results from an excessive deposition of extracellular matrix proteins secreted by activated hepatic stellate cells (HSCs). The activation process is accompanied by an increased activity of various transcription factors, including zinc finger protein 267 (ZNF267). Recently, ZNF267 has been shown to modulate gene expression and to function as a transcriptional repressor. MMP-10 was identified as a target gene; its gene expression and promoter activity are inhibited by ZNF267, which might promote liver fibrogenesis through diminished matrix degradation. However, the transcriptional regulation of the ZNF267 gene is unknown. In the present study, we have cloned and characterized the human ZNF267 promoter containing a 1.5 kb fragment of the 5′-flanking region (−1414/+173). The ZNF267 gene has a TATA-less promoter with multiple transcription initiation sites. Analysis of serial 5′-deletions of luciferase reporter constructs revealed a minimal promoter between −72 and +173 bp. Mutational analysis of putative regulatory elements indicated that a CCAAT box within this region was essential for ZNF267 promoter activity. Electrophoretic mobility shift assays demonstrated that transcription factor nuclear factor Y (NF-Y) bound to the CCAAT box. In co-transfection experiments, NF-YA increased the promoter activity of ZNF267. In conclusion, our results suggest that the binding site for NF-Y is critical for ZNF267 gene regulation and, herewith, the activation of this transcriptional factor may play an important role in the activation process of HSCs and in liver fibrosis.

Keywords: Kruppel-like zinc finger protein; Hepatic stellate cell; Promoter; Gene regulation; Nuclear factor Y; CCAAT box

1. Introduction

Liver cirrhosis is the end stage of chronic liver disease and is characterized by an accumulation of extracellular matrix (ECM) proteins, including collagens type I and type III [1,2]. The activation process of hepatic stellate cells (HSCs) is the central event leading to hepatic fibrosis [1,3]. Activated HSCs are the major source of ECM proteins that are deposited in liver fibrosis and cirrhosis [1]. The activation process is accompanied by an extensive reprogramming in gene expression, which is governed at least in part by transcription factors acting as transcriptional activators or repressors. Several transcription factors are involved in the activation process of HSCs including AP-1 [4,5], NFκB [6,7], CCAAT/enhancer-binding proteins (C/EBP) [8,9], PPARγ [10,11], nuclear factor Y (NF-Y, also named CBF for CCAAT binding factor) [12], and members of the Kruppel-like zinc finger family, such as Kruppel-like factor (KLF) 6 [13], KLF9 [14] and SP1 [12].

Kruppel-like factors contain multiple zinc fingers, which represent one of the most common DNA binding

Abbreviations: HSCs, hepatic stellate cells; ZNF267, zinc finger protein 267; ECM, extracellular matrix; C/EBP, CCAAT/enhancer-binding proteins; NF-Y, nuclear factor Y; KLF, Kruppel-like factor; KRAB, Kruppel associated box; MMP, matrix metalloproteinase; HEK, human embryonic kidney; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; IL, interleukin; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; INFγ, interferon γ; SNAP, *S*-Nitroso-*N*-acetylpenicillamine; RACE, rapid amplification of cDNA ends; CTF, CCAAT transcription factor

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domains. About one third of zinc finger genes contain a conserved domain in the amino terminal part known as KRAB (Kruppel associated box) domain, which mediates transcriptional repression [15–17]. One member of the Kruppel-like zinc finger family is zinc finger protein 267 (ZNF267; also named human zinc finger 2 or HZF2), whose KRAB domain is separated through a linker region from a clustered zinc finger domain [18]. The first zinc finger is followed by three degenerated fingers, and then continues with 13 zinc fingers at the carboxy terminus [18].

Previously we have demonstrated that ZNF267 mRNA is up-regulated during the activation process of human HSCs and in cirrhotic human liver in vivo [19]. ZNF267 protein is constitutively localized in the nucleus and its KRAB domain is functionally associated with transcriptional repression. Using gene array technology we identified genes that are regulated by ZNF267. We found that matrix metalloproteinase-10 (MMP-10) gene expression and promoter activity are inhibited by ZNF267 [19]. The inhibition of MMP-10 expression in activated HSCs might result in an increased accumulation of ECM and promote liver fibrogenesis in vivo.

Nitric oxide treatment reportedly up-regulates ZNF267 mRNA in venous endothelial cells [20]. However, the mechanism regulating ZNF267 gene expression is largely unknown. As an initial step towards understanding the factors controlling the expression of ZNF267 and towards gaining new insights into mechanism of liver fibrosis, we have isolated a 1.5 kb genomic fragment corresponding to the 5′-flanking region of ZNF267 and investigated functional properties and transcription factors involved in the ability of this region to drive expression of a reporter gene. In this study, we report that −72 to +173 bp upstream fragment of the ZNF267 gene is sufficient for basal transcription of ZNF267. Within this region, a CCAAT box as transcription factor binding site of NF-Y is required for ZNF267 gene expression.

2. Materials and methods

2.1. Cloning of the 5′-flanking region of ZNF267 and plasmid constructs

To obtain a ZNF267 reporter gene plasmid, a 1.5-kb fragment (−1414 to +173; +1 corresponding to the major transcription start site) consisting of the 5′-flanking region of the human ZNF267 gene was PCR amplified from genomic DNA, which was isolated from human HSCs using the DNeasy kit (Qiagen, Valencia, CA). The PCR primers used to obtain the ZNF267 5′-flanking region were 5′-GTGAGAGGGTGAGAGGCAGCCAT GC-3′ and 5′-TTCCCAGCTTCCGGGATGCCCTGGCGT-3′. The fragment was inserted into pCR2.1-TOPO (pCR2.1TOPO-ZNF267 (−1414/+173)). The insert was sequenced to confirm the identity to published sequence of genomic DNA (Gene bank accession number AC034105).

The ZNF267 luciferase reporter gene plasmid pGL3E-ZNF267(−1414/+173) was constructed by subcloning a *Kpn*I/*Xho*I fragment of pCR2.1TOPO-ZNF267(−1414/+173) into pGL3-Enhancer Vector (pGL3E; Promega, Madison, WI). Various 5′-serially deleted ZNF267 luciferase constructs were prepared. For pGL3E-ZNF267(−683/+173) and pGL3E-ZNF267(−308/+173), pCR2.1TOPO-ZNF267(−1414/+173) was cut with *Sty*I or *Dra*I, respectively, and blunt ends were generated using Klenow polymerase. After phenol/chloroform extraction and ethanol precipitation, vectors were cut with *Xho*I, and the released fragment was recloned into pGL3-Enhancer Vector, which was cut with *Kpn*I (blunt) and *Xho*I. All other constructs, pGL3E-ZNF267(−986/+173), pGL3E-ZNF267(−890/+173), pGL3E-ZNF267(−790/+173), pGL3E-ZNF267(−556/+173), pGL3E-ZNF267(−416/+173), pGL3E-ZNF267(−215/+173), pGL3E-ZNF267(−72/+173), pGL3E-ZNF267(+1/+173), were PCR generated using pGL3E-ZNF267(−1414/+173) as template, a forward primer as indicated in Table 1 and a common reverse primer 5′- TAGATCGCAGATCTCGAGCGG-3′. The two oligonucleotides introduced a *Kpn*I and *Xho*I site at the 5′- and 3′-end, respectively. PCR products were gel purified, digested with *Kpn*I and *Xho*I and the fragments cloned into pGL3E.

A series of plasmids with nucleotide substitutions, which alter consensus sequences for transcription factor binding, was constructed by site directed mutagenesis using Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primers indicated in Table 1 to obtain pGL3E-ZNF267(−72/+173)ΔNFκB, pGL3E-ZNF267(−72/+173)ΔEts-1/ΔStat-1, and pGL3E-ZNF267(−72/+173)ΔNF-Y. All constructs were sequenced to confirm inserts or mutations.

In order to generate reporter genes with the backbone pGL3-Basic Vector (pGL3B; Promega), pGL3E-ZNF267(−1414/+173) and pGL3E-ZNF267(−72/+173), pGL3E-ZNF267(−72/+173)ΔEts-1/ΔStat-1, pGL3E-ZNF267(−72/+173)ΔNF-Y were digested with *Xho*I/*Kpn*I and the released fragments were recloned into pGL3B.

The plasmids pCAG and pCAG-Stat-1 [21,22] were kindly provided by Dr. T. Hirano (Osaka University, Osaka, Japan); pSG5 and pSG5-Ets-1 by Dr. J. H. Chen (Tzu Chi University, Hualien, Taiwan); and pΔNF-YA13 by Dr. R. Mantovani (University of Milan, Milan, Italy).

2.2. Cell culture

The isolation and culture of human HSCs have been described previously [23,24]. Human embryonic kidney (HEK) 293 cells were cultured on plastic tissue culture dishes in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf

Table 1
Oligonucleotides used for plasmid constructs and oligonucleotides for EMSA

Name	Sequence
Primer for plasmid constructs	
pGL3E-ZNF267(−986/+173)	5′-GGGGTACCCCTCAGGAGATGGAGACCAGC-3′
pGL3E-ZNF267(−890/+173)	5′-GGGGTACCCCAGCTACTCAGGAGGCT-3′
pGL3E-ZNF267(−790/173)	5′-GGGGTACCCCCTGTCTCAAAACAAAAGC-3′
pGL3E-ZNF267(−556/+173)	5′-GGGGTACCCCAGCCCCTCTTCCACGT-3′
pGL3E-ZNF267(−416/+173)	5′-GGGGTACCCCGGCCTTCTGTTCCCAGCT-3′
pGL3E-ZNF267(−215/+173)	5′-GGGGTACCCCAAGTCGCAGCTCACCCCT-3′
pGL3E-ZNF267(−72/+173)	5′-GGGGTACCCCGCTGGAGTGGAAACCGTCC-3′
pGL3E-ZNF267(+1/+173)	5′-GGGGTACCCCTTCGTCGGCTCCAGTTAGAGC-3′
pGL3E-ZNF267(−72/+173)ΔNFY	5′-GGCGCTGGAGTGGAAACCGTAGTCGCAGGAGTGAAGCCGG-3′
pGL3E-ZNF267(−72/+173)ΔEts1/ΔStat1	5′-CGGAGAGCAGGGGCGGCGGGAGGGATTTGGCGG-3′
pGL3E-ZNF267(−72/+173)ΔNFκB	5′-CGGAGAGCAGGGGCGGCTTCCTTTGCGCAGCGGGGGCCTTCGTCG-3′
Oligonucleotides for EMSA	
ZNF267(−74/−29)NFY	5′-GGCGCTGGAGTGGAAACCGTCCAATCAGGAGTGAAGCCGG-3′
ZNF267(−74/−29)ΔNFY	5′-GGCGCTGGAGTGGAAACCGTAGTCGCAGGAGTGAAGCCGG-3′
ZNF267(−37/−5)Ets1/Stat1	5′-CGGAGAGCAGGGGCGGCTTCCGGGATTTGGCGG-3′
ZNF267(−37/−5)ΔEts1/ΔStat1	5′-CGGAGAGCAGGGGCGGCGGGAGGGATTTGGCGG-3′

serum (FCS) and standard antibiotics in 95% air–5% CO_2 humidified atmosphere at 37 °C.

2.3. Transient transfections and luciferase reporter assays

Transient transfections were performed using 1×10^6 HEK 293 cells in 60-mm dishes transfected with 0.125 μg of the various pGL3 reporter plasmids using LipofectAMINE Plus (Invitrogen) in a medium containing 10% FCS. Cell lysates were collected 24 h after transfection and assayed for luciferase activity using the Luciferase Assay System (Promega). To normalize transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega) was transfected additionally and renilla luciferase activity was measured by a luminometric assay (Promega). For co-transfection experiments, 0.2 μg of the reporter gene plasmids and 0.1 μg of the effector plasmids were incubated for 5 h in a medium containing 10% FCS. The medium was then replaced by serum-free medium and cells were incubated for 19 h before harvesting. For cytokine studies, cells were transfected in medium without serum. Cells were stimulated with interleukin (IL) 1β, transforming growth factor (TGF) β1, tumor necrosis factor (TNF) α, interferon (INF) γ (all from R&D Systems, Minneapolis, MN) or *S*-Nitroso-*N*-acetylpenicillamine (SNAP; Merck, Germany) 1 h after transfection and analyzed 24 h after transfection.

2.4. RNA isolation and rapid amplification of cDNA ends (RACE)

Total RNA from human HSCs was isolated by use of an RNA extraction kit (Qiagen).

The 5′-terminus of ZNF267 mRNA was determined by rapid amplification of cDNA ends (5′-RACE) using First Choice 5′ RLM RACE (Ambion, Austin, Texas) as described previously [19]. The sequence for the gene specific outer primer was 5′-TTCCCAGCTTCCGGGATGCCCTGGCGT-3′ (+147 to +173; +1 corresponding to the major transcription start site) and for the gene specific inner primer 5′-CTTAGCTACGAATCTCCCAGTGCCTGCA-3′ (+119 to +146). PCR products were analyzed on a 2% agarose gel, isolated, subcloned into pCR2.1-TOPO, and 18 clones were sequenced.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HEK 293 cells were prepared as described [25,26]. EMSA for NF-Y, Ets-1 or Stat-1 was performed as described [26–28]. Double-strand wild-type and mutant oligonucleotide probes, which harbor nucleotide substitutions altering consensus sequences for transcription factor binding, are described in Table 1. For supershift analysis or competition experiments, prior to adding the ^{32}P-labeled oligonucleotides, extracts were incubated on ice with 1–4 μl of antibody or an equal amount of H_2O, or with the cold non-radiolabeled probe, respectively. The antibodies used for supershift assays were anti-NF-YA (Rockland, Gilbertsville, PA), anti-Stat-1 (E23; Santa Cruz, Santa Cruz, CA), anti-Spi-B (N-16; Santa Cruz), anti-Elk-1 (L-20; Santa Cruz), anti-Fli-1 (C19; Santa Cruz), anti-Elf-1(c-20; Santa Cruz) and anti-Ets-1 (N-276; Santa Cruz) antibody. Complexes were assessed by autoradiography and phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA).

2.6. Statistics

The results were analyzed for statistical significance according to the Mann–Whitney *U*-statistic test. Statistical values of $P \leq 0.05$ were considered to be significant.

3. Results

3.1. The 5′-flanking region of the human ZNF267 gene shows promoter activity

To characterize the potential sequences and transcription factors involved in ZNF267 gene expression, we first determined the transcription start site of the human ZNF267 gene. A template was generated by reverse transcription of human HSC mRNA. 5′-RACE with that template and gene specific primers produced a prominent single DNA fragment with an approximate size of 200 bp (Fig. 1A). After subcloning, eighteen 5′-RACE products were sequenced, and eight different transcription initiation start sites were found in a region between 145 and 186 bp upstream of the start codon ATG (indicated by bold letters in Fig. 2) suggesting the existence of multiple transcriptional start sites. The most frequent one was found 173 bp upstream of ATG and was designated as +1. There was no difference between 5′-RACE products and the published short ZNF267 proximal promoter sequence (+46 to +173; Gene bank accession number NM_003414).

To determine whether an approximate 1.5 kb genomic DNA fragment of the 5′-upstream region contains a functional promoter, we constructed plasmids containing different 5′-flanking regions of the ZNF267 gene inserted upstream of the promoter-less luciferase reporter gene pGL3E. The entire isolated genomic sequence (−1414/+173) is in agreement with a human genomic clone

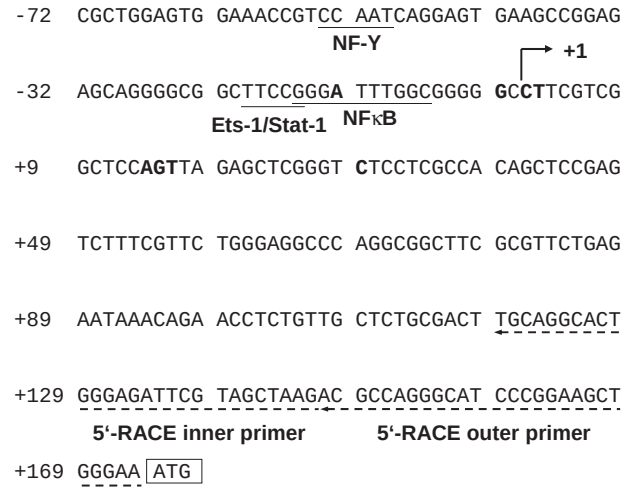

Fig. 2. 5′-flanking region of the ZNF267 gene and putative transcription factor binding sites. Potential transcription factor binding sites between −72 and +173 bp are underlined (MatInspector). The transcription start sites are in bold letters, the major one is marked with an arrow (+1). Boxing denotes the translational initiation code. The gene specific inner and outer primers for 5′-RACE are underlined with dotted arrows.

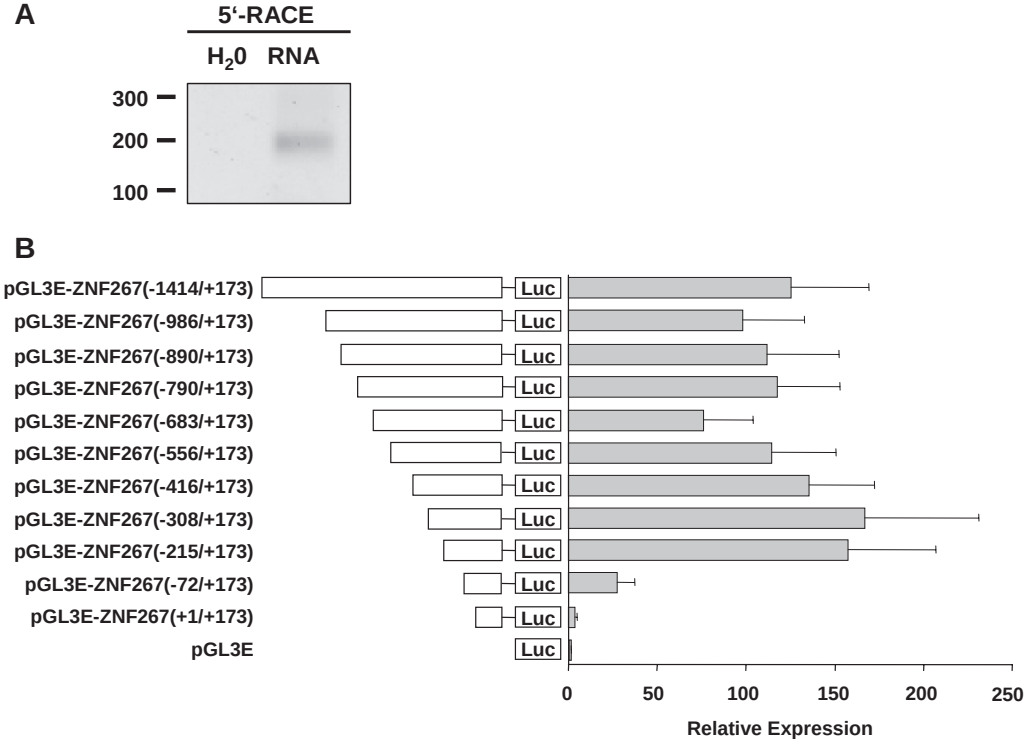

Fig. 1. Transcriptional initiation site and promoter activity of serial 5′-deletion constructs of the ZNF267 promoter. (A) The transcriptional initiation site of the human ZNF267 gene was determined by 5′-RACE, and a DNA fragment was amplified. A negative control (H_2O) was included with PCR reactions. The molecular weight marker is indicated on the left. (B) HEK 293 cells were transfected with the indicated (left side) ZNF267 promoter constructs (0.125 μg per 60-mm dish) and assayed for luciferase activity after 24 h. Values were corrected for transfection efficiency by co-transfection with the renilla expression plasmid. Data represent the mean ± standard error (S.E.) of three experiments performed in duplicate and are expressed relative to the luciferase activity of the promoter-less reporter gene pGL3E (right side).

deposited in the Gene bank under accession number AC034105. All constructs had a common 3′-end. The luciferase activities of these constructs were assessed by transient transfections into HEK 293 cells. The plasmid pGL3E-ZNF267(−1414/+173) containing the entire 1.5 kb length of the 5′-flanking region showed a level of luciferase activity, which was approximately 125-fold of the luciferase activity obtained with the empty vector pGL3E alone (Fig. 1B). The plasmid pGL3E-ZNF267(−72/+173) showed a 27-fold higher luciferase activity as compared to the empty vector pGL3E alone, but only a 5.8-fold lower activity as compared to the next longer construct pGL3E-ZNF267 (−215/+173) (Fig. 1B). These findings indicate that the minimal 5′-fragment required for the expression of human ZNF267 was −72 bp to +173 bp and is therefore defined as minimal promoter of ZNF267.

3.2. Mutational analysis of putative regulatory elements in the ZNF267 minimal promoter

Computer assisted analysis using MatInspector [29] revealed various consensus elements for transcription factors in the 5′-flanking region of the human ZNF267 gene. There was no TATA box observed within the upstream sequence of the transcription start sites. A high confidence binding site for NFκB occurred adjacent to the major transcription start site (Fig. 2). Additional binding sites for Ets-1/Stat-1 and NF-Y were observed farther upstream (Fig. 2).

To determine whether these putative binding sites are necessary for ZNF267 gene expression, reporter constructs that had mutations for transcription factor binding of NFκB, Ets-1/Stat-1 and NF-Y were constructed. Wild-type pGL3E-ZNF267(−72/+173) and different mutant promoter constructs were separately transfected in HEK 293 cells, and luciferase activity was measured. The mutation of the NF-Y or Ets-1/Stat-1 binding site strongly decreased ZNF267 promoter activity as compared to cells transfected with wild-type pGL3E-ZNF267(−72/+173) (Fig. 3). However, the mutation of the NFκB binding site slightly increased promoter activity (Fig. 3). Taken together, these results suggest that binding of Ets-1/Stat-1 and NF-Y might be important for the basal promoter activity of the human ZNF267 gene.

3.3. Transient over-expression of NF-YA increases ZNF267 promoter activity

Having demonstrated that NF-Y and Ets-1/Stat-1 binding sites upstream of the ZNF267 gene are necessary for ZNF267 promoter activity, transient co-transfection experiments were performed in HEK 293 cells. The minimal promoter pGL3B-ZNF267(−72/+173) was co-transfected with wild-type expression vectors for Ets-1 (pSG5-Ets-1), Stat-1 (pCAG-Stat-1) or NF-YA (pΔNF-YA13). Luciferase activities were compared with those obtained with the corresponding empty expression vector as control. When pΔNF-YA13 was co-transfected with the minimal promoter construct pGL3B-ZNF267(−72/+173) into HEK 293 cells, ZNF267 promoter activity was significantly increased as compared to control cells transfected with the empty vector pSG5 (Fig. 4A). In contrast, the co-transfection of pGL3B-ZNF267(−72/+173) with pCAG-Stat-1 did not affect the minimal promoter activity of ZNF267 as compared to cells transfected with pCAG as control (Fig. 4B). Cells co-transfected with pGL3B-ZNF267(−72/+173) and pSG5-Ets-1 showed a slightly higher, but statistically not significant minimal promoter activity of ZNF267 as compared to cells co-transfected with pSG5 as control (Fig. 4C).

Since positive synergistic effects of NF-Y and other transcription factors on promoter activity in co-transfection experiments have been reported [30], we performed co-

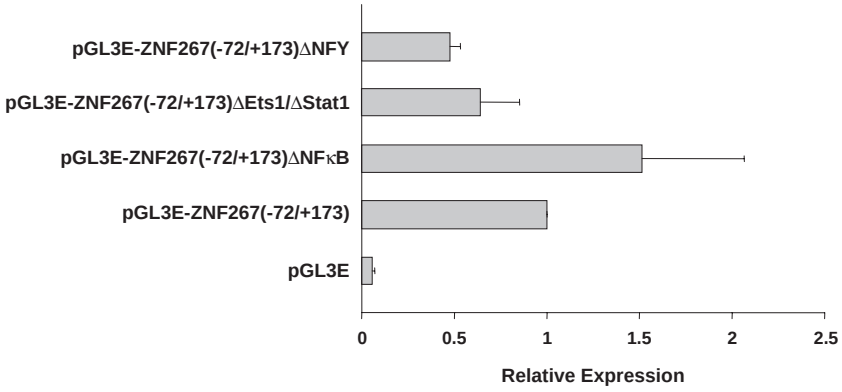

Fig. 3. Mutational analysis of potential regulatory elements in the ZNF267 promoter. The wild-type minimal ZNF267 promoter construct pGL3-ZNF267(−72/+173) or reporter constructs containing individual mutant transcription factor binding sites for NF-Y (pGL3E-ZNF267(−72/+173)ΔNF-Y), Ets-1/Stat-1 (pGL3E-ZNF267(−72/+173)ΔEts-1/ΔStat-1), or NFκB (pGL3E-ZNF267(−72/+173)ΔNFκB) were transiently transfected into HEK 293 cells (0.125 μg per 60-mm dish) and assayed for luciferase activity after 24 h. Luciferase activities were normalized for transfection efficiency by co-transfection with the renilla expression plasmid. Data represent the mean ± standard error (S.E.) of three experiments performed in duplicate and are expressed relative to the luciferase activity of the promoter-less reporter gene pGL3E.

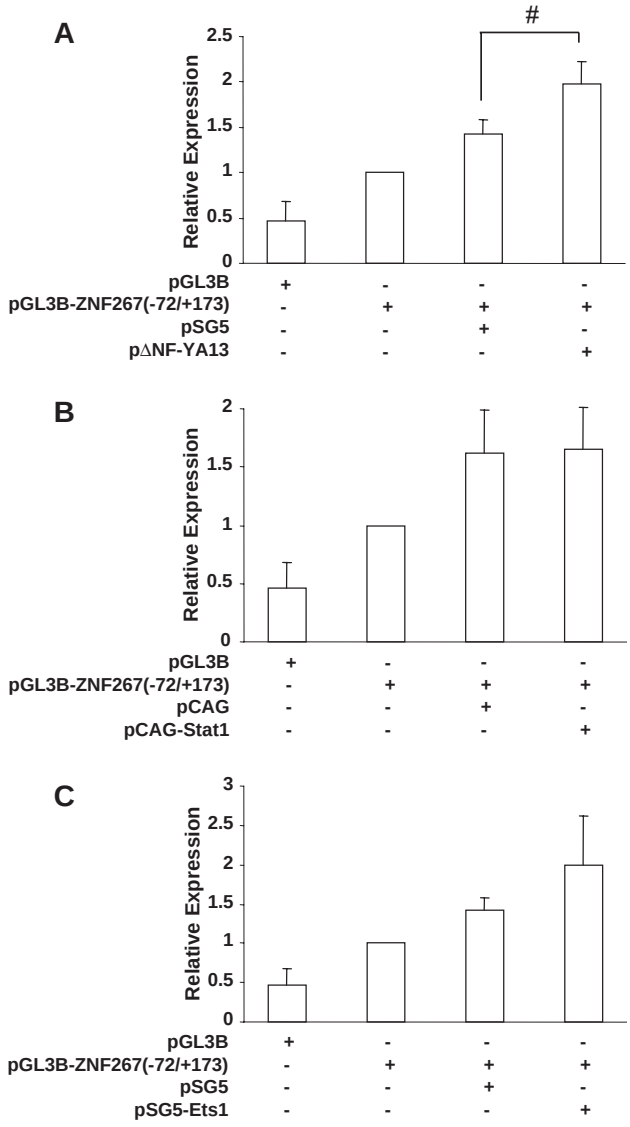

Fig. 4. Effect of NF-YA, Ets-1 or Stat-1 expression on ZNF267 promoter luciferase activities. HEK 293 cells were transfected with the reporter plasmid pGL3B-ZNF267(−72/+173) (0.2 μg per 60-mm dish), together with the plasmids (A) pΔNF-YA13 expressing NF-YA, (B) pCAG-Stat-1 expressing Stat-1, (C) pSG5-Ets-1 expressing Ets-1, or the corresponding empty vectors pSG5 or pCAG (0.1 μg of each expression plasmid per 60-mm dish). Background measurements were performed in cells transfected with the empty luciferase reporter plasmid pGL3B alone. Luciferase activity was measured 48 h after transfection and normalized for transfection efficiency to renilla luciferase activity. Data represent the mean ± standard error (S.E.) of four experiments performed in duplicate and are expressed relative to the luciferase activity of the reporter gene pGL3B-ZNF267(−72/+173). $^{\#}P$=0.04.

transfection experiments of pΔNF-YA13 with pSG5-Ets-1 or pCAG-Stat-1, and determined the effect on the minimal promoter construct pGL3B-ZNF267(−72/+173). However, we did not observe a positive synergism between NF-YA and Ets-1 or Stat-1 on the minimal promoter activity of ZNF267 (data not shown).

These results suggest that NF-YA has the capacity to enhance the activity of the ZNF267 promoter.

3.4. NF-YA binds to the CCAAT box in the minimal promoter of ZNF267

To further explore the role of Ets-1, Stat-1 and NF-Y in ZNF267 promoter activity, EMSA was performed to investigate DNA binding activity in the 5′-flanking region of ZNF267. The incubation of a double stranded oligonucleotide probe (ZNF267(−74/−29)NF-Y) containing the NF-Y binding site with nuclear extracts from HEK 293 cells revealed a complex, which was supershifted after adding an antibody against NF-YA indicating that the complex contained (at least) NF-YA (Fig. 5A, lanes 1 and 2). The specific NF-YA DNA binding complex formation was completely absent using a mutant oligonucleotide probe (ZNF267(−74/−29)ΔNF-Y) with or without the addition of anti-NF-YA antibody (lane 5 and 6). The labeled wild-type or mutant probe alone is shown in lanes 4 and 8, respectively.

Three other protein complexes bind to the labeled probe containing the wild-type NF-Y binding site as well as to the labeled probe containing the mutant NF-Y binding site (lanes 1 and 5). Unlabeled wild-type or mutant oligonucleotide at 200-fold excess competed with the labeled probe demonstrating binding specificity (lanes 3 and 7). Since the difference between the wild-type and mutant oligonucleotide consists of 6 nucleotide substitutions in the core flanked by 14 and 20 unchanged nucleotides on the 5′- and 3′-end, respectively, we interpret that the other three complexes bind to this flanking regions. However, we only can speculate whether the function of these three bound proteins is transcriptional repression or activation.

Next, we investigated whether Ets-1 binds to the putative Ets-1 consensus binding site in the minimal promoter of ZNF267. Nuclear extracts from HEK 293 cells were incubated with a double stranded oligonucleotide probe containing the wild-type Ets-1/Stat-1 binding site (ZNF267(−37/−5)Ets-1/Stat-1), which resulted in the formation of four DNA–protein complexes (Fig. 5B, lane 1). Interestingly, the incubation of the same extracts with the oligonucleotide probe containing the mutant Ets-1/Stat-1 binding site (ZNF267(−37/−5)ΔEts-1/ΔStat-1) revealed that the same four protein complexes bound to DNA (Fig. 5B, lane 5). No signal was detected with the labeled wild-type or mutant probe alone (lanes 4 and 8). Using an anti-Ets-1 antibody, which reportedly supershifts complexes containing Ets-1 [27], no complex was supershifted (Fig. 5B, lanes 2 and 6). We also used antibodies against Stat-1 and Ets-1 family members (Spi-B, Elk-1, Fli-1, and Elf-1) in the EMSA supershift assays. The addition of any of these antibodies did not result in a supershift (data not shown).

Together, these results suggest that neither Ets-1 nor Stat-1 binds to the putative consensus sequence in the minimal promoter of ZNF267. To rule out the possibility that Stat-1 or Ets-1 was not sufficiently present in nuclear extracts to be detectable in EMSA, Stat-1 (pCAG-Stat-1) or Ets-1 (pSG5-Ets-1), or their empty control vectors

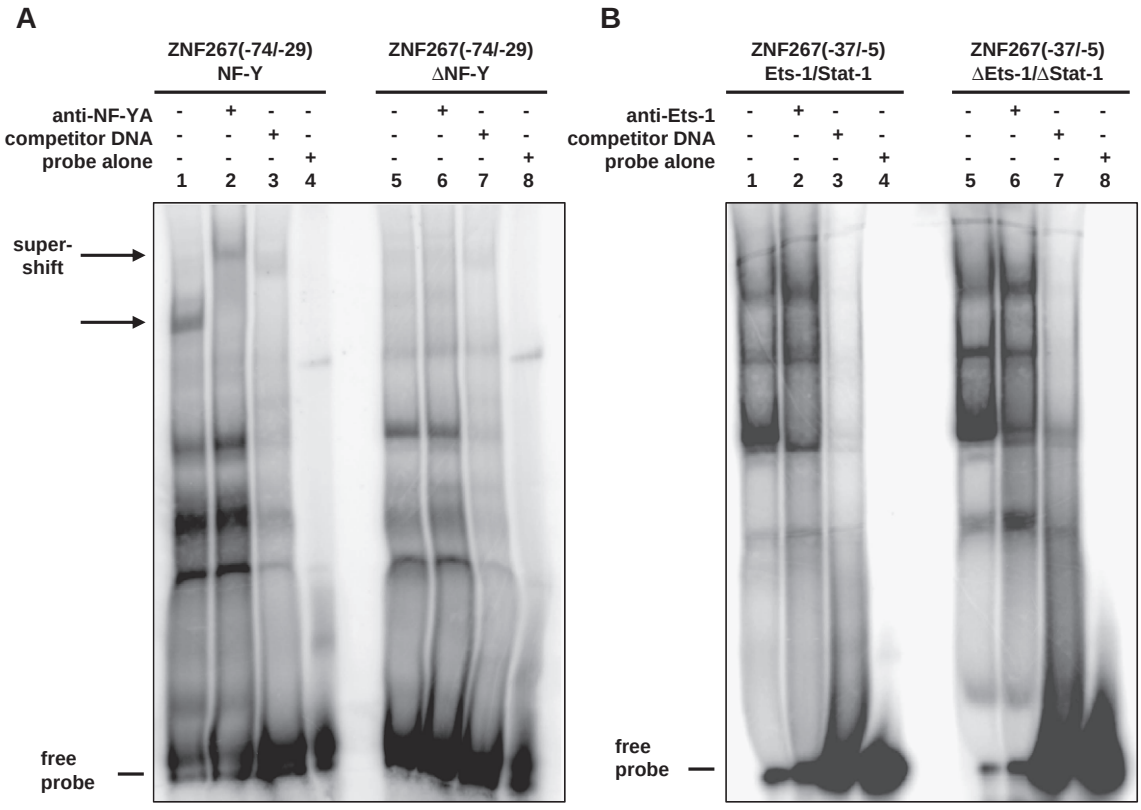

Fig. 5. EMSA for NF-Y and Ets-1 binding sites in the ZNF267 promoter. (A) The DNA binding activity of NF-Y was assessed using EMSA. Nuclear extracts (10 μg) were incubated for 20 min with a radiolabeled oligonucleotide probe containing a NF-Y binding site (ZNF267(−74/−29)NF-Y; lanes 1–3) or mutant NF-Y binding site (ZNF267(−74/−29)ΔNF-Y; lanes 5–7), followed by 4% non-denaturing gel electrophoresis. For supershift analysis, extracts were incubated with anti-NF-YA antibody for 30 min on ice prior to the addition of the radiolabeled probe (lanes 2 and 6). The NF-YA DNA binding complex and the supershifted complex are indicated by arrows. In lanes 3 and 7, a competition with 200-fold excess unlabeled probe is shown. The labeled wild-type or mutant probes alone are shown in lanes 4 and 8, respectively. Shown is a representative experiment, which was performed in triplicate. (B) EMSA was performed using nuclear extracts (10 μg) and an oligonucleotide probe containing a Ets-1/Stat-1 (ZNF267(−37/−5)Ets-1/Stat-1; lanes 1–3) or a mutant Ets-1/Stat-1 binding site (ZNF267(−37/−5)ΔEts-1/ΔStat-1; lanes 5–7). Supershift assays were performed by preincubation with an anti-Ets-1 antibody (lanes 2 and 6). In lanes 3 and 7, a competition with 200-fold excess unlabeled probe is shown. The radiolabeled wild-type and mutant oligonucleotides are shown in lanes 4 and 8, respectively. Shown is a representative experiment, which was performed in duplicate.

(pCAG or pSG5), was transiently over-expressed in HEK 293 cells, and the nuclear extracts were incubated with the double stranded oligonucleotide probe containing the wild-type or mutant Ets-1/Stat-1 oligonucleotide probe. Similar to the results obtained with endogenous nuclear extracts, there was no difference in the DNA–protein complexes comparing wild-type or mutant Ets-1/Stat-1 oligonucleotide probe (data not shown). In supershift assays, no specific Ets-1 or Stat-1 containing DNA binding complex was observed (data not shown).

Taken together, endogenous NF-Y, but not Stat-1 or Ets-1 family members bind to the ZNF267 promoter in the region containing the putative transcription factor binding sites.

3.5. Modulation of the ZNF267 promoter activity through various mediators

To further characterize the promoter activity of the ZNF267, the potential of various mediators and cytokines was screened to modulate its activity. Therefore, 1 h after the transfection of HEK 293 cells with the empty reporter gene pGL3B as control (Fig. 6A) or the entire 1.5 kb ZNF267 promoter construct (pGL3B-ZNF267(−1414/+173) (Fig. 6B), cells were stimulated with TGFβ1, IL1β, TNFα, SNAP, a nitric oxide donor, and IFNγ for an additional 23 h. No difference was observed in the luciferase activity of the empty reporter gene pGL3B (Fig. 6A) as compared to the luciferase activity driven by the entire 1.5 kb promoter construct (Fig. 6B).

4. Discussion

Following activation as a central element in hepatic fibrogenesis, HSCs are the main producers of ECM proteins. The activation process of HSCs is governed by the modulation in gene expression through the altered activity of transcription factors. We have previously demonstrated that the expression of ZNF267 is up-regulated in activated HSCs [19]. ZNF267 as transcriptional repressor inhibits MMP-10 gene expression [19]. Since chronic liver injury is characterized by excessive matrix deposition and

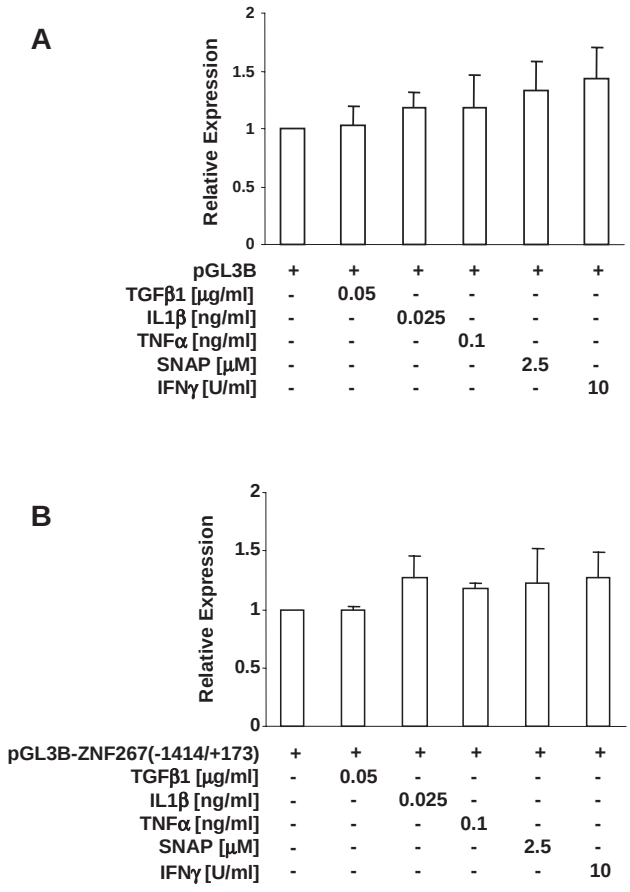

Fig. 6. Influence of various mediators on the promoter activity of ZNF267. The promoter-less luciferase reporter plasmid pGL3B (A) or the luciferase promoter construct pGL3B-ZNF267(−1414/+173) containing the entire 1.5 kb ZNF267 promoter (B) (0.2 μg of each plasmid per 60-mm dish) was transiently transfected into HEK 293 cells. 1 h after transfection, cells were stimulated with TGFβ1, IL1β, TNFα, SNAP, a nitric oxide donor, and IFNγ in medium without serum. Luciferase activity was measured 24 h after transfection and was corrected for transfection efficiency by co-transfection with the renilla expression plasmid. Data represent the mean ± standard error (S.E.) of three experiments performed in duplicate and are expressed relative to the luciferase activity of the reporter gene pGL3B (A) or pGL3B-ZNF267(−1414/+173) (B).

insufficient matrix degradation and remodeling, the decreased expression of MMP-10 might increase this uncoordinated response to chronic liver injury. Extracellular matrix degradation is further inhibited, and liver fibrogenesis progresses.

However, the promoter region of the ZNF267 gene and its transcriptional regulation remained unknown. In this study, we cloned and characterized the human ZNF267 promoter and the transcriptional elements that are responsible for regulating ZNF267 expression. The study was undertaken to test the hypothesis that differential regulation of the ZNF267 gene might have functional consequences for fibrogenesis and the progression of liver fibrosis to cirrhosis.

Various transfection reagents are not able to mediate gene delivery in activated rat HSCs (myofibroblasts) [31]. In addition, the transfection efficiency using cationic liposome reagents in primary human HSCs or the human hTERT hepatic stellate cell line [23] is very low, and results obtained of transfection experiments in these cells are inconsistent (unpublished data). Therefore we used highly transfectable HEK 293 cells to study the promoter and transcriptional regulation of the human ZNF267 gene.

Promoter deletion and transient transfection experiments showed that the core promoter of human ZNF267 gene resides between −72 and +173 bp. The human ZNF267 gene has multiple transcription initiation start sites, and its minimal promoter lacks a TATA box and an initiator sequence. This is in accordance with reports that some TATA-less promoters retain the ability to direct transcription initiation from a specific nucleotide, while others possess multiple start sites [32]. Genes that are abundantly expressed usually contain a consensus TATA box [32], which is consistent with the expression pattern of human ZNF267. Although ZNF267 mRNA is detected in all major human organs tested, its expression is highest in the pancreas, placenta and kidney, but hardly detectable in the liver, lung and skeletal muscle [20].

By introducing specific mutations into the putative regulatory elements, a CCAAT box located 54 bp upstream of the major transcription initiation site was found to be critical for human ZNF267 promoter activity. The CCAAT box is present in about 30% of eukaryotic promoters and has been found in close proximity to the transcription initiation site in TATA-less promoters [33]. Several CCAAT binding proteins have been identified, including C/EBP, the CCAAT transcription factor (CTF) and NF-Y [34]. The latter one has been identified to be the major CCAAT binding protein in various promoters [35]. NF-Y is a ubiquitously expressed transcription factor and is a heterotrimeric complex composed of three subunits, NF-YA, NF-YB and NF-YC [35]. All three subunits are required for DNA binding [35]. NF-YB and NF-YC form a tight dimer prior to the association with NF-YA and binding to the CCAAT box [35]. Using a specific anti-NF-YA antibody, the CCAAT box was shown to bind NF-Y in EMSA. The specificity of NF-Y to the CCAAT box is further demonstrated by competition experiments. Additional evidence for the involvement of NF-Y in the regulation of ZNF267 results from transient NF-YA over-expression experiments that significantly increased the minimal promoter activity of ZNF267. Perhaps we did not observe a stronger induction of ZNF267 promoter activity by NF-YA, since the other two subunits necessary for DNA binding, NF-YB and NF-YC, are needed in addition to the over-expression of NF-YA.

The mechanism by which NF-Y regulates gene expression and the regulation of NF-Y itself is complex and different in various cell systems [35]. One recognized regulatory pathway is the modification of NF-Y on the post-transcriptional level, including differences in binding

activity and protein levels [36,37]. In line with these findings are results from in vitro studies regarding hepatic fibrosis. The binding activity of NF-Y increased in activated HSCs [12]. Therefore, an increased binding activity of NF-Y might be responsible for the up-regulation of ZNF267 during the activation process of HSCs.

An interesting finding of our study is the decrease in ZNF267 promoter activity upon the disruption of a putative Ets-1/Stat-1 binding site, while the mutation of a directly adjacent NFκB site revealed no effect on promoter activity. Since we did not observe any difference in proteins bound to the oligonucleotide containing the putative Ets-1/Stat-1 binding site as compared to the oligonucleotide with the mutant binding site, we conclude that actually no detectable endogenous protein binds to the putative Ets-1/Stat-1 site in the ZNF267 promoter. Additionally, we ruled out the possibility that endogenous Ets-1 or Stat-1 is not sufficiently expressed to be detectable in EMSA. Using nuclear extracts from cells transiently over-expressing these transcription factors, no difference in DNA–protein interaction was observed as compared to endogenous nuclear extracts. In addition, we did not see any supershift using Ets-1 and Stat-1 specific antibodies. These results suggest that Stat-1 and Ets-1 do not bind to the putative transcription factor binding site in the minimal promoter of ZNF267. One explanation for the contribution of this site to ZNF267 promoter activity could be that the change in the nucleotide sequence might modify the structure of the DNA. Consequently, the basal transcription machinery, which is in close proximity to the nucleotide mutations, might not be able to bind efficiently DNA and to initiate transcription. Another possibility could be that physical interactions of transcription factors might be interrupted through an altered nucleotide sequence. However, considerable future research is required to investigate the exact mechanism of the contribution of these nucleotides to basal ZNF267 promoter activity.

In summary, we have identified and characterized the promoter region of the human ZNF267 gene. Our results indicate that the binding site for NF-Y is critical for ZNF267 transcription. Since the binding activity of NF-Y increases in activated HSCs [12], this might be the molecular mechanism that increases the expression of ZNF267 during the activation process of HSCs.

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