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Nuclear factor Y activates the human xanthine oxidoreductase gene promoter

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Abstract To study the regulation of the human xanthine oxidoreductase (XOR) gene, we cloned 1.9 kb of the promoter region. In reporter gene assays, a construct encompassing nucleotides between −142 to +42 conferred maximal basal activity of the XOR promoter in 293T cells, in comparison with shorter (−92 to +42) or longer (up to −1937 to +42) constructs. The promoter activity was low in NIH-3T3 cells. The most active construct contained a putative CCAAT motif at −119 to −123. Electrophoretic mobility shift assays showed that this sequence binds the ubiquitous nuclear factor Y (NF-Y). Mutation of the CCAAT motif (CTGAT) abolished the NF-Y binding and considerably reduced the promoter activity. Our data suggest an important functional role for NF-Y in the transcriptional activation of the human XOR gene.

Key words: Xanthine oxidase; Xanthine dehydrogenase; Gene regulation; Promoter region; Transcription factor; Nuclear factor Y

1. Introduction

Xanthine oxidoreductase (XOR) oxidizes hypoxanthine to xanthine and further to urate. In this process electrons are passed to either NAD^+ (xanthine dehydrogenase form, EC 1.1.1.204) or molecular oxygen (xanthine oxidase form, EC 1.1.3.22), leading to the formation of NADH or superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively [1]. The enzyme in vivo is predominantly in the dehydrogenase form, but can be converted into the oxidase form reversibly by sulfhydryl oxidation or irreversibly by proteolysis [2]. Based on its ability to produce reactive oxygen species, XOR has been ascribed a role in ischemia-reperfusion injury [3].

Because of its proposed pathogenic role, the regulation of XOR by a number of effectors has been studied. Hypoxia elevates XOR activity in animal cells, by a mechanism that is controversial, since both transcriptional and posttranslational regulation have been implicated [4–6]. Cytokines (IFN-γ, IL-1, IL-6, TNF-α) and dexamethasone induce both XOR enzyme activity and mRNA expression in cultured cells [7–9], which is in line with the proposed role of XOR in inflammatory conditions. During lactation there is a clear increase of XOR protein levels in the mammary gland [10] and the enzyme is present in human milk [11]. In a cell line derived from mouse mammary gland, prolactin with dexamethasone or cortisol increases both XOR protein and mRNA levels [12,13].

Most of the data supporting the pathophysiological role of XOR come from studies using experimental animals or non-human cell lines. However, in order to assess the role of XOR in human pathophysiology it is mandatory to study the human enzyme for two main reasons. Firstly, since urate is the end product of purine catabolism only in man and other primates, the regulation of its production may differ from other species. Secondly, the expression of the human XOR gene, at the level of enzymatic activity, immunoreactive protein, and mRNA is confined to relatively few organs, with the highest levels in the liver, intestine, and mammary gland [10,14], whereas in rodents the enzyme is strongly expressed in several organs [15,16].

The cDNA of the human XOR gene has been cloned [17,18], and its structure as well as the sequence of 2 kb of its 5′-untranslated region have been published [19]. The gene has been localized to chromosome 2p22 [20,21]. In the only reported functional study of the human XOR promoter, repressing elements, specifically an E-box and a TATA-like element, were identified and a considerably lower level of transcription of the human gene compared to the mouse XOR gene was shown [22]. Activating transcription factors of the human XOR promoter have not been described.

The aim of this study was to identify promoter regions with potential regulatory importance, and to analyze, which protein(s) may play a role in transcriptional regulation of the human XOR gene.

2. Materials and methods

2.1. Plasmids

Five human XOR gene promoter fragments were isolated by PCR either from human genomic DNA (XOR1 and XOR2) or by using XOR2 as the template (XOR4, XOR5, and XOR6). The primers were designed on the basis of the sequence of the human XOR gene promoter [19] and included restriction enzyme sites for *Bgl*II or *Mlu*I in their 5′-ends and for *Hin*dIII in 3′-ends. The 5′-primer sequences were 5′-GGGAAGATCTTGTGGTTTGTAGGATGTTTAGT-3′ (complementary to nucleotides −1937 to −1913 in the human sequence, underlined), 5′-GGGGACGCGTCTTACTTAAGGAAGGCTGGC-3′ (−1174 to −1153), 5′-GTGGAGATCTTAATTTGCTGTGTGT-

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Abbreviations: CBF, CCAAT binding factor; CDP, CCAAT displacement protein; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility shift assay; NF1, nuclear factor 1; NF-Y, nuclear factor Y; XOR, xanthine oxidoreductase

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GATTGTT-3′ (−224 to −203), 5′-CTGAAGATCTGAGCTGG-TTCCCTCCCATTG-3′ (−142 to −120), and 5′-TGGGACGCG-TAACTTTCAGGTCACAGAGCA-3′ (−92 to −69) corresponding to promoter fragments in XOR1, XOR2, XOR4, XOR5 and XOR6, respectively (Fig. 1A). The 3′-primer sequence for all PCR reactions was 5′-CTACTAAGCTTTCTGCCATTCACAAAGAAAAC-3′ (+42 to +19). XOR3 was obtained by shortening XOR1 by exonuclease III digestion (Erase-a-Base System, Promega) and its sequence corresponds to nucleotides −547 to +42. All promoter constructs were sequenced and the sequence of XOR1 was submitted to GenBank (GenBank AF203979). XOR5mut [23] was created by PCR using XOR2 as the template. The mutagenic primer was 5′-CTGAA-GATCTGAGCTGGTTCCCTCCCATCAGTGGACCT-3′ (mutated base pairs underlined), the 5′-end of the primer including a *Bgl*II site. The 3′-primer was as above. The PCR products and XOR3 were cloned into pGL3-basic (Promega) bearing a luciferase gene.

2.2. Cell culture and transfection

Human embryonic kidney 293T cells (from Dr. Kalle Saksela, University of Tampere, Finland) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. NIH-3T3 mouse fibroblasts (from ATCC) were cultured in DMEM supplied with 10% calf serum and antibiotics as above. 24 h before transfection the 293T cells were seeded on 12-well plates (2×10^5 cells per well), NIH-3T3 cells on 6-well plates (3×10^5 per well), and transfected with 0.5 μg or 1 μg of XOR promoter constructs, respectively, using FuGene6 (Roche Molecular Biochemicals) transfection reagent. In all experiments, 10 ng (293T cells) or 0.5 μg (NIH-3T3 cells) of pCMVβ (Clontech), a β-galactosidase expression vector, were cotransfected to monitor transfection efficiency, and empty pGL3-basic vector was used as a control. Medium was changed 16 h after transfection and the cells were further incubated for 24 h. Luciferase activity was determined with reagents from Promega using a Luminoskan RT reader (Labsystems). β-Galactosidase activity was determined according to Rosenthal [24].

2.3. Nuclear protein extracts

Cells were harvested in ice-cold PBS and nuclear protein extracts were prepared as described [25]. Briefly, after lysis in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM

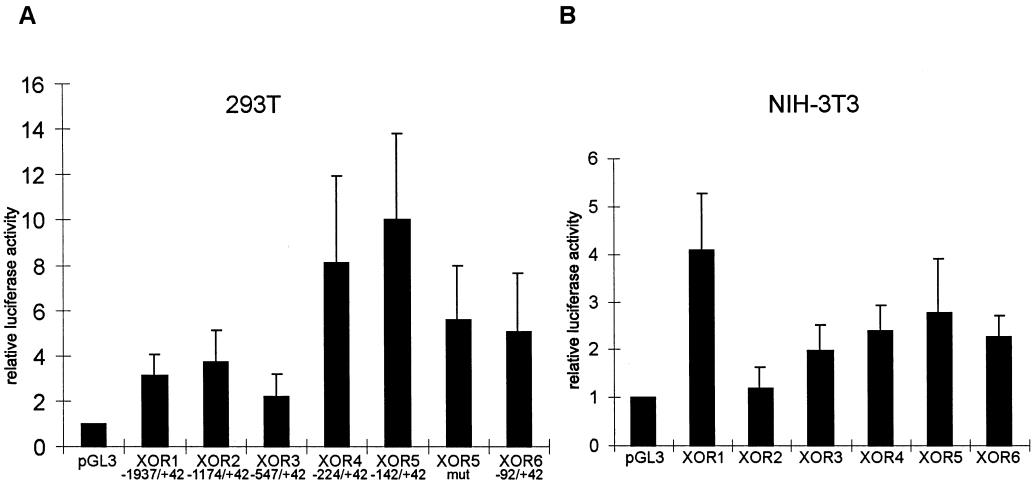

Fig. 1. Activity of the human XOR promoter luciferase constructs. A: Relative reporter activity in transiently transfected 293T cells. The cells were transfected with 0.5 μg of XOR constructs of indicated lengths or with pGL3-basic (pGL3) alone. B: Relative reporter activity in transiently transfected NIH-3T3 cells. The cells were transfected with 1 μg of XOR constructs or pGL3-basic alone. The bars show luciferase activity relative to β-galactosidase activity. The relative values for the empty pGL3-basic were set as 1. The data are the means ± S.D. of at least three independent experiments, each performed in triplicate. C: Human XOR promoter sequence from −224 to +42 aligned with the sequence of the rat XOR promoter. The 5′-ends of XOR4, XOR5, and XOR6 are indicated. The mutated nucleotides in XOR5mut are shown on top of the human sequence in boldface type. The human sequence corresponding to oligo1 is underlined. The inverted CCAAT motifs are indicated with boxes, and the C/EBP binding sites of the rat promoter sequence are underlined. The potential transcriptional initiation sites at −59 and −81 are indicated with arrows. The translational initiation codon is double underlined.

dithiothreitol, 0.5 mM PMSF, 3 μg/ml leupeptin and 5 μg/ml pepstatin, the cells were left on ice for 15 min and then vortexed for 10 s. After centrifugation for 5 s at 14 000 rpm in an Eppendorf centrifuge, proteins from the nuclear pellet were extracted for 20 min on ice in a buffer (20 μl/nuclei from 10^7 cells) containing 20 mM HEPES, pH 7.9, 25% (vol./vol.) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 1 mM dithiothreitol, 0.5 mM PMSF, 3 μg/ml leupeptin and 5 μg/ml pepstatin. Nuclear debris was removed by centrifugation for 5 min at 4°C at 14 000 rpm in an Eppendorf centrifuge. Protein concentrations were measured using Bio-Rad *DC* protein assay (Bio-Rad Laboratories) and the extracts were stored in aliquots at −80°C.

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA probes XOR5E and XOR5Emut were created by digesting the plasmids XOR5 and XOR5mut with *Bgl*II and *Hin*dIII. The restriction fragments were purified on 8% polyacrylamide gel. Oligo1 (Fig. 1C) was prepared by annealing the oligonucleotides 5′-GAGCTGGTTCCCTCCCATTGGTGGACCTTATTT-3′ and 5′-AATGAAATAAGGTCCACCAATGGGAGGGAACCA-3′ and purifying the annealed oligonucleotide on polyacrylamide gel. NF-Y/CBF (CCAAT binding factor) and C/EBP (CCAAT/enhancer binding protein) consensus double-stranded oligomers and the mutated C/EBP oligomer used in competition assays were purchased from Santa Cruz. The antibodies for supershift assays were from Santa Cruz (C/EBP-β, sc-150X; CBF-A/NF-Y-B, sc-7711X, and GATA-6, sc-7244X, used as an unrelated antibody). The probes were 5′-end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Nuclear proteins (10 μg) were incubated on ice for 10 min with 2 μg poly(dI-dC)(dI-dC) (Amersham Pharmacia Biotech) in 20 mM HEPES (pH 7.9), 10% glycerol (vol./vol.), 50 mM KCl, 0.5 mM EDTA, 1 mM DDT, 1 mM $MgCl_2$, 0.5 mM PMSF and 1 μM leupeptin. A 5′-end-labelled probe (5000–10000 cpm) was then added and the incubation was continued at 22°C for 30 min. In the competition experiments, 10–100-fold molar excess of the unlabelled probe was added before the labelled probe as described in the figure legends. Reaction products were separated on 4% polyacrylamide gels run in 22.5 mM Tris-borate, 0.5 mM EDTA for 2–3 h at 200 V at 22°C. After electrophoresis, the gels were dried and visualized by autoradiography. In the supershift assays, the antibodies (2 μg/20 μl incubation vol.) were added after binding reactions and incubation was further continued for 50 min at 22°C.

3. Results

3.1. Functional promoter analysis

The activity of the different XOR promoter luciferase constructs was studied in transient transfection assays in 293T and NIH-3T3 cells. XOR5 corresponding to the promoter region from −142 to +42, relative to the translational initiation site, showed the highest promoter activity in 293T cells, the activity being ∼10-fold higher than that of the promoterless pGL3-basic, whereas XOR6 (from −92 to +42) was less active (Fig. 1A). Interestingly, the promoter activities of the longer constructs, XOR1, XOR2, and XOR3, were approximately one third of the activity of XOR5 (Fig. 1A), suggesting a repressor binding to the promoter. XOR1 gave the highest activity in NIH-3T3 cells (Fig. 1B).

3.2. DNA–protein interactions of the proximal XOR promoter

A sequence analysis of the promoter region from −142 to −92, which is included in XOR5 but not in XOR6, revealed two inverted CCAAT motifs (Fig. 1C). EMSAs were performed using nuclear extracts from 293T and NIH-3T3 cells and labelled XOR5E and oligo1 as probes. Both nuclear extracts yielded a strong retarded band of the same size with XOR5E, which was competed by a 100-fold molar excess of unlabelled XOR5E (Fig. 2A). When a double-stranded oligonucleotide oligo1, corresponding to the nucleotides from −135 to −107 of the XOR promoter and encompassing one of the putative CCAAT motifs, was used in EMSAs, two (293T cells) or one (NIH-3T3 cells) specific DNA–protein

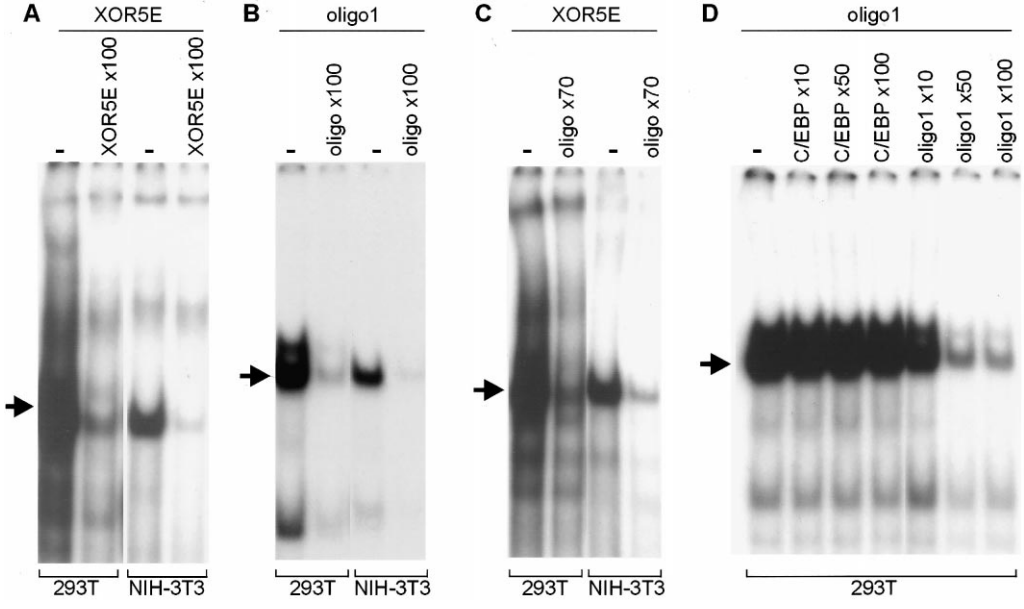

Fig. 2. Characterization of the XOR promoter region interacting with nuclear proteins. A: Nuclear proteins (10 μg) extracted from 293T or NIH-3T3 cells were incubated with ^{32}P-labelled XOR5E, corresponding to region −142 to +42 of the human XOR promoter. The major specific protein–DNA complex formed is indicated with an arrow. Unlabelled XOR5E was used in competition experiments in 100-fold molar excess. B: ^{32}P-labelled oligo1 corresponding to the promoter region −135 to −107 was incubated with nuclear proteins as above. The strong retarded band (indicated with an arrow) formed by both nuclear extracts was efficiently competed with 100-fold molar excess of unlabelled oligo1. C: The binding of proteins to ^{32}P-labelled XOR5E (corresponding to region −142 to +42 of the XOR promoter) was competed with 70-fold molar excess of oligo1. The arrow indicates the band formed by both, 293T and NIH-3T3, nuclear extracts. D: The binding of nuclear proteins extracted from 293T cells to ^{32}P-labelled oligo1 was competed with 10-, 50- or 100-fold molar excess of unlabelled consensus C/EBP oligomer or 10-, 50- or 100-fold molar excess of unlabelled oligo1 as depicted.

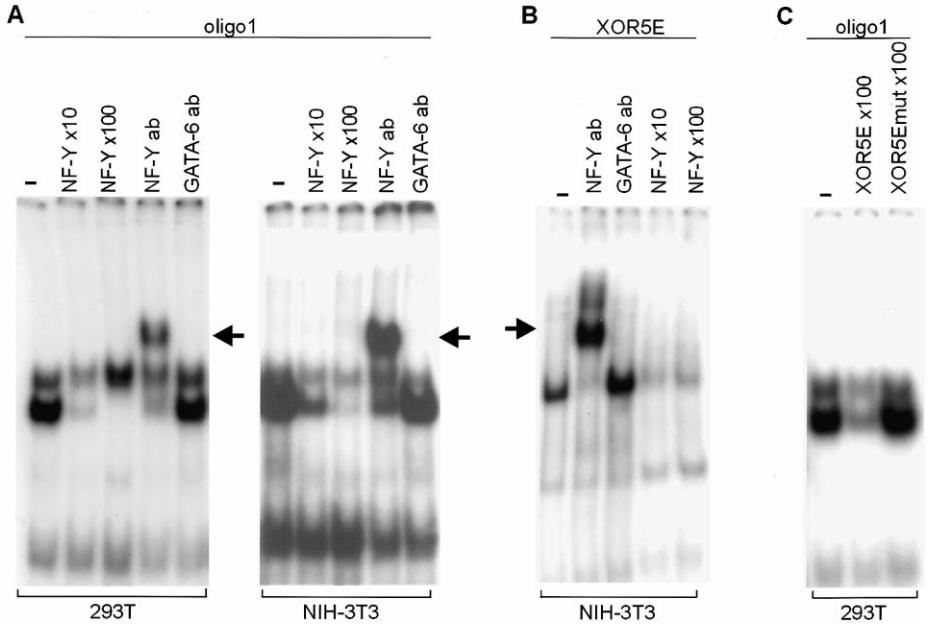

Fig. 3. Identification of NF-Y as the nuclear protein binding to the XOR promoter CCAAT motif. A: ^{32}P-labelled oligo1 (−135 to −107) was incubated with nuclear proteins (10 μg) extracted from 293T or 3T3 cells. Unlabelled NF-Y consensus oligomer was used in 10- (NF-Y ×10) or 100-fold (NF-Y ×100) molar excess in competition experiments. Antibodies against NF-Y (NF-Y ab) or GATA-6 (GATA-6 ab, used as a control) were used in supershift experiments. Arrows indicate supershifted complexes seen with NF-Y antibody. B: Nuclear proteins from NIH-3T3 cells were incubated with ^{32}P-labelled XOR5E (−142 to +42). NF-Y or GATA-6 antibodies were used in supershift experiments. Supershift seen on lane 2 is indicated with arrow. Unlabelled NF-Y oligomer was used in 10- or 100-fold molar excess in competition experiments. C: ^{32}P-labelled oligo1 was incubated with nuclear proteins (10 μg) extracted from 293T cells. The binding of proteins was competed with 100-fold molar excess of unlabelled XOR5E (−142 to +42) or XOR5Emut containing a mutated CCAAT motif.

complexes were formed (Fig. 2B,D). In the presence of unlabelled oligo1 in molar excess, the major complex formed by protein binding to XOR5E was markedly attenuated (Fig. 2C), demonstrating that the region −135 to −107 is important for the protein binding.

The transcription factors NF-Y, C/EBP, nuclear factor 1 (NF1), and CCAAT displacement protein (CDP) can all potentially bind to the CCAAT motif [26]. In order to identify the protein specifically binding to the promoter region −135 to −107, competitive binding reactions were performed. Neither the consensus (Fig. 2D) nor the mutated (data not shown) C/EBP oligonucleotides competed out the binding to oligo1 even in 100-fold molar excess. The oligo1–protein complex was not influenced by C/EBP-β antibody (data not shown). Neither did C/EBP oligonucleotide or C/EBP-β antibody change the protein binding pattern to XOR5E (data not shown), indicating that C/EBP is not capable of binding to this region of the proximal XOR promoter in vitro. NF-Y consensus oligonucleotide, on the other hand, competed out the protein binding to oligo1 and XOR5E, and CBF-A/NF-Y-B antibody recognized the bound protein in supershift experiments (Fig. 3A,B). Together these results indicate that the protein interacting with the XOR promoter DNA is indeed NF-Y.

3.3. Functional analysis of the XOR promoter CCAAT motif

We mutated the putative NF-Y binding site to evaluate its functional role in human XOR gene transcriptional regulation. In EMSA, XOR5Emut with the mutated CCAAT motif did not compete with the binding of the protein recognized by NF-Y antibody on XOR5E (data not shown). Neither did XOR5Emut compete with the protein binding to oligo1, whereas XOR5E did (Fig. 3C), demonstrating the importance of the intact CCAAT motif in the binding of the transcription factor NF-Y. In transiently transfected 293T cells, the activity of the promoter construct with the mutated CCAAT site (XOR5mut) was reduced by 45% compared to the intact XOR5 (Fig. 1A), indicating that the motif is indeed mediating transcriptional activation of XOR gene promoter in intact cells.

4. Discussion

We have characterized the transcriptional regulation of the human XOR gene by studying the promoter activity in transient transfections with a 5′ deletion series. A fragment corresponding to the human XOR promoter from −142 to +42 (XOR5) yielded the highest promoter activity of the series in human 293T cells. Comparison of the activities provided by both shorter and longer fragments suggests the presence of positive regulatory elements between −92 and −142, and negative regulatory elements in the region upstream of −224. Xu et al. recently reported that the region from −258 to −228, harboring an E-box, represses basal promoter activity in HepG2 cells and in HUVECs [22], which is in accordance with our findings. In contrast with the report by Xu et al. [22], we did not detect an increase in the promoter activity by extending the 5′-end of the promoter fragment from −142 to −224. Different cell lines used for transfection experiments could account for the differences in promoter activity. Also the fact that our promoter constructs included the translated nucleotides of the first exon (up to +42) may have contributed to the differences. Interestingly, the coding region of the first exon of the rat XOR gene harbors transcription factor bind-

ing sites that have a functional role in promoter regulation [27].

The region of the human XOR promoter that was crucial for maximal activity contains two putative CCAAT motifs. The transcription factors NF-Y, NF1, C/EBP, and CDP can all potentially bind to the consensus CCAAT motif [26], which is one of the most common elements in the proximal promoter of many mammalian genes transcribed by RNA polymerase II [28]. C/EBP regulates many liver-specific genes and is involved in inflammatory responses [29]. C/EBP-β and C/EBP-α were shown to bind to the proximal promoter of the TATA-less rat XOR promoter [30], whereas enhancer binding factor I, closely resembling NF-Y did not bind to this promoter [31]. C/EBP also regulated the basal transcription of the rat gene together with downstream elements located in the coding region [27]. However, the rat C/EBP binding site is not conserved in the human promoter (Fig. 1C), which is in line with our finding that C/EBP does not bind to human XOR promoter.

Our data indicate that NF-Y binds to the CCAAT motif, located between nucleotides −119 and00000 −123 from the translational start site of the proximal human XOR gene promoter. The CCAAT motif is conserved in rat and man and partly overlaps the reported rat C/EBP binding site. In the human XOR promoter, two putative transcription start sites located 59 and 81 nucleotides upstream of the start codon have been identified [19]. Our finding that NF-Y binds to a CCAAT motif located at −60 from the first transcriptional initiation site is in accordance with the typical location of the CCAAT motif [28]. Furthermore, mutation of the NF-Y site severely compromised the promoter activity, indicating that the site is functional in intact cells, thus suggesting an important functional role for NF-Y in the transcriptional regulation of the human XOR gene.

The reason for the differences in the tissue expression of XOR between man and rodents is unclear. Xu et al. reported a considerably lower transcriptional activity of the human gene compared to that of the mouse gene [22]. The proximal rat and mouse promoters are very similar [32,33], whereas the promoters of the human and rat genes show only limited sequence similarities in (Fig. 1C). Taken together, this suggests that transcriptional regulation may account for the species specific expression of the XOR gene.

NF-Y is a ubiquitous transcription factor that binds to DNA as a heterotrimer. It has been shown to interact with the TATA-binding protein [34], with different coactivators, such as p300 [35], and to promote the binding of other transcription factors to nearby sequences [28]. For instance, in the promoter of the liver specific albumin gene, the precise positioning of C/EBP next to NF-Y is required for optimal activation [36]. However, the *trans*-acting factor or factors cooperating with NF-Y in the human XOR gene regulation are not known. Identifying these factors will be crucial in order to understand the regulation of the human XOR gene in response to various extracellular signals.

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