Repressed Expression of the Human Xanthine Oxidoreductase Gene

E-BOX AND TATA-LIKE ELEMENTS RESTRICT GROUND STATE TRANSCRIPTIONAL ACTIVITY

Ping Xu, Patricia LaVallee, and John R. Hoidal‡

From the Department of Internal Medicine, Division of Respiratory, Critical Care and Occupational Medicine, University of Utah Health Sciences Center and Veterans Affairs Medical Center, Salt Lake City, Utah 84132

Studies were initiated to address the basis for the low xanthine oxidoreductase (XOR) activity in humans relative to nonprimate mammalian species. The expression of the XOR in humans is strikingly lower than in mice, and both transcription rates and core promoter activity of the gene are repressed. Analysis of human XOR promoter activity in hepatocytes and vascular endothelial cells showed that the region from −258 to −1 contains both repressor and activator binding regions regulating core promoter activity. The region between −138 and −1 is necessary and sufficient for initiating, and the region between −258 and −228 is critical for restricting core promoter activity. Within the latter region, site-directed mutations identified a consensus sequence “acacaggtgtgg” (−242 to −230) that contains an E-box that binds a repressor. In addition, the TATA-like element is also required to restrict promoter activity and TFIID binds to this site. The results demonstrate that both an E-box and TATA-like element are required to restrict gene activity. A model is proposed to account for human XOR regulation.

Xanthine oxidoreductase (XOR; EC 1.1.3.22) is a molybdoflavoprotein hydroxylase that is widely distributed in nature. It is a homodimer with each subunit being about 150,000 Da, and containing four redox active centers: two iron-sulfur, one FAD, and one molybdopterin (1–3). In mammals XOR exists in two interconvertible forms, xanthine dehydrogenase and xanthine oxidase. Xanthine dehydrogenase transfers the reducing equivalents generated by the oxidation of substrates to NAD+ whereas xanthine oxidase transfers them to oxygen with resultant production of superoxide anion and hydrogen peroxide. As the rate-limiting enzyme in nucleic acid degradation, XOR catalyzes the final two reactions of purine catabolism with resultant production of urate. Urate, which scavenges hydroxyl radical, singlet oxygen, hypochlorous acid, oxoheme oxidants, and hydroperoxyl radicals, and is a potent iron chelator (4–7), has been proposed as a major antioxidant in plasma (4, 5, 8) and hydroperoxyl radicals, and is a potent iron chelator (4–7), has been proposed as a major antioxidant in plasma (4, 5, 8) and is necessary and sufficient for initiating, and the region between −258 and −228 is critical for restricting core promoter activity. Within the latter region, site-directed mutations identified a consensus sequence “acacaggtgtgg” (−242 to −230) that contains an E-box that binds a repressor. In addition, the TATA-like element is also required to restrict promoter activity and TFIID binds to this site. The results demonstrate that both an E-box and TATA-like element are required to restrict gene activity. A model is proposed to account for human XOR regulation.

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Juxtaposed to the role of urate as a protective antioxidant is that XOR-derived superoxide anion and hydrogen peroxide lead to oxidative tissue injury in a variety of animal models simulating several clinical disorders including renal failure, endotoxin-induced mucosal injury, viral pneumonia, ischemia-reperfusion injury, and cutaneous photosensitivity to hematoporphyrins (11–18). Recent studies demonstrate that XOR is a highly regulated enzyme. XOR activity and gene expression are increased by cytokines in a profile consistent with an acute phase response and during ischemia/reperfusion (19–22).

Somewhat surprising in view of the high urate concentrations, XOR enzyme activity in humans is 100 times lower than that in nonprimate species including rats and mice (23–25). Post-translational mechanisms appear to only partially explain the low activity (26, 27). We hypothesized that the low activity in humans represents an adaptive mechanism to control the potentially deleterious formation of urate crystals and that repression of XOR expression is partially responsible. To begin to pursue this hypothesis, we cloned the human XOR (hXOR) cDNA and characterized the chromosomal location and genomic organization of the gene (28–30). In the present study, we determined that expression of XOR in human tissues is strikingly restricted relative to that in mouse. Analysis of XOR transcription rates and core promoter function of the 5′-untranslated region of the human and mouse genes indicates that transcription and core promoter activity of hXOR are repressed. An E-box with a consensus GTTTC and a TATA-like element present in the human, but not in the mouse 5′-untranslated region are required for the repression. A model is proposed to account for human XOR regulation.

MATERIALS AND METHODS

Measurement of XOR Transcripts—XOR transcript levels in human or mouse tissues were analyzed using reverse transcription (RT)-PCR. Total RNA (tRNA) was isolated from tissue using RNAgent Total RNA Isolation System (Promega) following the supplier’s protocol. The RT reaction mixture (20 µl volume) contained 2 µg of tRNA, 200 units of Superscript II RT (Life Technologies, Inc.), 4 units of RNase inhibitor, 5 mM dithiothreitol, 0.5 mM each deoxyribonucleotide (dNTP), 2.5 mM MgCl2, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.5 µg of oligo(dT). Samples were incubated for 1 h at 42 °C and then heated for 5 min at 94 °C to stop the reaction. For PCR amplification of XOR transcripts, the primers GCAGAAGGATAAGTTCTT (forward) and CTCCAG-GTAAGTTGTCTTTT (reverse) were used. The primers ATGGTGG-GAAATGGTGTAGAAG (forward) and GCAGTCTAGTAAAGGG (reverse) were used to amplify β-actin transcripts.
Two PCR-based methods were used to quantify XOR transcripts. In the first, XOR products were detected by β-actin. Each reaction mixture (25 μl volume) contained 1 μl of RT product, 1 μl of each primer (25 pmol/μl), 2.5 μl of 10 × PCR buffer (Perkin Elmer), 0.5 μl of each dNTP, and 1.25 units of Taq polymerase. The reaction conditions were denatured at 94 °C for 2 min followed by 30 cycles of amplification at 94 °C for 30 s, annealing at 65 °C (15 s), and extension at 72 °C (10 s). The reactions were completed by a final 2-min extension at 72 °C. The PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with EtBr. The image was scanned and analyzed using Bio-Rad Gel Doc-1000 with Multi-Analysis software. The ratio of image intensities of β-actin bands to the sample was determined and used to normalize the XOR transcript level.

The second method for quantifying transcripts used a fluorescence temperature cycler (LightCycler™ LC24, Idaho Technology, Idaho Falls, ID) for continuous fluorescent monitoring of PCR (31). Each PCR reaction mixture (10 μl volume) contained 1.2 μl of diluted (1:10) RT product, 3 mM MgCl2, 50 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 1:30,000 dilution of SYBR Green I, 5 μM of each primer, 0.05 unit/μl Taq polymerase, and 11 ng/μl TaqStart™ antibody (CLONTECH Laboratories, Palo Alto, CA). The reaction conditions were heating at 20 °C to 95 °C with a 0-s hold, cooling at 20 °C to 60 °C with a 0-s hold, heating at 5 °C to 82 °C (2 °C below the product melting temperature, Tm) with a 2-s hold to detect the fluorescent product at the last step of each cycle. Under these conditions, the product was denatured at 94 °C, heated at 20 °C to 95 °C, cooled at 20 °C to 50 °C, and slowly heated at 0.2 °C to 94 °C with fluorescence data collection at 0.2 °C intervals. The PCR cycle number at which the fluorescent signal of the product reached the fluorescence threshold was analyzed automatically by computer software (LightCycler™ LC24, Idaho Technology). The XOR transcript level is presented as the PCR cycle number at the fluorescence threshold.

The continuous fluorescent monitoring was also used to determine nascent transcribed XOR transcripts from nuclei. To obtain nuclei human or mouse livers were homogenized, filtered through a mesh, and centrifuged at 1000 × g for 5 min to collect the individual cells. The cells were incubated in lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris-HCl, 0.5 mM EDTA, 1% SDS, 100 mM NaCl, 30 mM EDTA, and 1% SDS at room temperature for 30 min. Then 50 μl of 5 M KCl, 0.5 mM EDTA, 0.5 μM dithiothreitol, 0.01% Nonidet P-40 at room temperature for 30 min. The cells were centrifuged at 1000 × g for 15 min to collect the supernatant. The reaction buffer (10 mM Tris-HCl, 10 mM NaCl, 0.5 mM MgCl2, 0.5 mM dithiothreitol, 1 mM MgCl2, 0.5 mM EDTA, 0.05% Nonidet P-40, and 25 μg of bovine serum albumin plus 3 μg of poly(dI-dC) or poly(dA-dT) to reduce nonspecific binding. For competition studies, the samples were preincubated with unlabeled oligonucleotides for 10 min at room temperature. The samples were then employed with nuclear extract and labeled probes. To determine whether TFIID binds to the TATA box sequences, incubation of the EMSA reaction mixtures for 15 min at room temperature was followed by addition of the TFIID (or irrelevant) antibodies (Santa Cruz Biotech, Inc.) and incubation for an additional 30 min at room temperature. The reaction samples were electrophoresed on 4% acrylamide, 10% glycerol gels. After electrophoresis the gels were transferred to nylon membranes by electroblotting. The oligonucleotides were fixed onto the membranes by baking at 100 °C for 30 min. The chemiluminescent signals were detected following the DIG Gel Shift Kit protocol (Roche Molecular Biochemicals).

Electrophoretic Mobility Shift Assays (EMSAs)—EMSA were employed to study nuclear protein or human recombinant hTFIID (hrTFI- FID) binding to DNA fragments (37). Briefly, fragments of approximately 100 bp were generated from the cloning vector containing the -5′- untranslated region of XOR using specific primers and PCR. The fragments were designed based on “hot-spots” suggested by mutational analysis using the luciferase reporter constructs. The PCR fragments were purified using QIAquick PCR Purification Kit (Qiagen) and then labeled with digoxigenin-11-ddUTP using terminal transferase following the DIG Gel Shift Kit protocol (Roche Molecular Biochemicals). The gel shift assay using nuclear extract, 15–30 fmol of labeled probe was incubated for 30 min at room temperature with 5–10 μg of nuclear protein in a 50-μl reaction mixture containing 4% glycerol, 10 mM Tris (pH 7.5), 10 mM NaCl, 0.5 mM dithiothreitol, 1 mM MgCl2, 0.05% Nonidet P-40, and 25 μg of bovine serum albumin plus 3 μg of poly(dI-dC) or poly(dA-dT) to reduce nonspecific binding. For competition studies, the samples were preincubated with unlabeled oligonucleotides for 10 min at room temperature. The samples were then electrophoresed on 4% acrylamide, 10% glycerol gels. After electrophoresis the gels were transferred to nylon membranes by electroblotting. The oligonucleotides were fixed onto the membranes by baking at 100 °C for 30 min. The chemiluminescent signals were detected following the DIG Gel Shift Kit protocol.

DNase I Footprinting Analysis—Probes for DNase I footprinting were generated from genomic DNA templates by PCR using specific primers. Probes of 140–210 bp were then single-end-labeled with digoxigenin-11-ddUTP. The labeled probes were polyacrylamide gel purified, confirmed by dot blot, and quantitated by EtBr staining. The single end-labeled probes (25 fmol) were incubated with 33 ng of hrTFIID in a 50-μl reaction mixture containing 25 mM Tris (pH 8.0), 0.25 mM MgCl2, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 0.01% Nonidet P-40 at room temperature for 30 min. Then 50 μl of 5 mM MgCl2, 50 mM Tris (pH 8.0), 200 μM each nucleotide, and 15 μM of each primer was added and incubated at room temperature. Following incubation, DNase I was added to the reaction mixture and incubated for 1 min at room temperature. The reactions were terminated by adding 90 μl of stop solution containing 200 mM NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml yeast RNA. The samples were precipitated with ethanol and loaded onto a pre-run 6% acrylamide, 7 M urea sequencing gel. The chemiluminescent signals were detected as described above.
To examine this possibility, we determined XOR transcripts in mouse and human livers, using RT-PCR. The top panel shows the XOR transcripts and the lower panel the β-actin transcripts that are constitutively expressed and used to normalize the transcript levels of XOR in liver tissues. Lanes 1, 2, and 3 show the XOR transcripts in mouse livers and lanes 4, 5, and 6 show the XOR transcripts in human livers.

RESULTS

Transcript Levels and Transcription Rates of the hXOR Gene

Are Repressed Compared with the mXOR Gene—XOR activity in human is substantially lower compared with other species including mouse (23–25). The low activity may be attributed, in part, to an inactive form of the enzyme (“demolybdo” and “desulfo”) (26, 27). Recent investigations suggest that unique gene expression mechanisms may also contribute to the low activity. To examine this possibility, we determined XOR transcripts in human and mouse livers, an organ rich in XOR activity, using β-actin expression to normalize for loading. As demonstrated in Fig. 1, the XOR transcript level is substantially lower in human than in mouse liver.

To compare nascent transcribed XOR transcripts from mouse and human liver nuclei, we determined the cycle number at the fluorescence threshold (Cn). The higher the Cn value, the lower the initial transcript copy number. Nascent transcribed β-actin transcripts were determined as internal standards. Nascent XOR transcript levels within a defined transcription time (30 min) were lower (6.97 ± 1.44 cycles, n = 3) in human than in mouse liver. This indicates that the transcription rate of XOR in human is substantially lower than that in mouse.

To determine whether repressed expression of hXOR (relative to mXOR) is restricted to the liver, we quantitatively compared the copy number of XOR transcripts in various tissues from human and mouse. As shown in Table I, compared with mouse, the Cn values of hXOR transcripts in the intestine, kidney, liver, and lung were higher by 7 cycles. This indicates at least 100-fold lower copy number of XOR transcripts in these human organs compared with those in the mouse.

Functional Analysis of 5’-Untranslated Regions of hXOR and mXOR Genes—To begin to investigate the mechanism for restricted expression of hXOR, we compared promoter activity of the 5’-untranslated region of hXOR with that of the mouse. The activities were examined in both HepG2 and HUVECs. In both cell types the 5’-untranslated region of hXOR had 2–3-fold lower activity compared with that of the corresponding region (−588 to −1) in mXOR (Fig. 2).

The 5’-Untranslated Region from −258 to −1 Contains Both Repressive and Stimulatory Regions Regulating Basal Promoter Activity of the hXOR Gene—To elucidate mechanisms for repressed expression of hXOR, we tested 5’-end step-deletional constructs in HepG2 and HUVECs for basal activity. Fig. 3 demonstrates that constructs F1, F2, F3, and F4 had minimal activity and the deletions from F4 to F6 caused markedly increased activity. Further 5’-step deletions from −258 to −78 (F6d1, d2, d3, d4, d5) determined that a 30-bp deletion from −258 to −228 (F6d1) caused a marked increase, and that the deletion from −228 to −198 (F6d2) caused a marked reduction of activity (Fig. 4). These results suggest this region (−258 and −228) represses basal promoter activity. In addition, the promoter fragment from −138 to −1 (F6d3) provided activity approximately two times background, indicating that this region contained core elements necessary and sufficient for minimal basal promoter activity of the gene.

Protein Interactions of DNA Fragments That Restrict Basal Promoter Activity—The deletional analysis indicated the region between −258 to −1 had multiple sites interacting with transcription factors including repressors and activators. To examine this possibility, EMSA were performed using nuclear protein extracts of HUVECs. As illustrated in Fig. 5A three approximately 110-bp oligomeric probes (probes 12, 34, and 56) were generated covering the regions between −258 and −146 (probe 56), −162 and −51 (probe 34), and −92 and −1 (probe 12). In these experiments, only the bands that were competed by unlabeled specific probes were defined as shifted bands. The results show two shifted bands for probe 56 (Fig. 5B), four shifted bands for probe 34 (Fig. 5C) and at least one shifted band for probe 12 (Fig. 5D). This confirms that multiple nuclear protein binding sites are present within the 5’-untranslated region between −258 and −1.

Site-directed Mutations of the Region between −258 and −228 Relieve the Repressed Promoter Activity—To identify the binding sites for nuclear proteins that repress promoter activity we mutated the regions between −258 and −228 (Fig. 6 represents one of three independent experiments). Loss of repressor activity by the mutations in constructs F6 m5, F6 m6 and F6 m7 suggests that the sequence “acacaggtgg” (−242 to −230) represents the core sequence necessary for repression. Of note, this sequence contains a consensus E-box (ACAG-GTGT) that is known to bind various repressors including the zinc finger-homeodomain transcription factor AREB6 (38, 39).

In comparison with F6 m5, F6 m6 and F6 m7 that show a marked relief, the constructs F6 m1, F6 m2, and F6 m8, that contain mutations flanking the E-box, provided only modest relief from the repression.

Site-directed Mutations of the Mutative TATA-like Element Also Relieve the Repressed Promoter Activity—Sequence analysis indicated that the 5’-untranslated region of hXOR, but not mXOR contains a putative TATA-like element. To characterize the contribution of this element to the repressive activity, we mutated sites within the element and examined for activity. As shown in Fig. 7, the mutation caused a marked increase in promoter activity. Thus converting the TATA site into a non-TATA site relieved repressor activity.

Analysis of Interactions between Nuclear Proteins and Sequences Containing the Putative TATA-like Element—To begin to investigate which factors were responsible for the repressive effects of the TATA-like element, interactions between nuclear proteins and probe 34 containing the element (as illustrated in Fig. 5A) were analyzed using competition studies. In these experiments a subset of oligomeric probes derived from the region of probe 34 was generated and used as competitors (Fig. 8A). Fig. 8B demonstrates that unlabeled probe 34 competes

![Figure 1](https://www.jbc.org/)
completely with bands I, II, and IV (lane 10). Probe 34-d also competes with bands I, II, and IV (lane 6), while probe 34-e competes with bands I and II (lane 7), and probe 34-f competes with bands II and IV (lane 8). The shifted band III was partially competed by the unlabeled probe 34 but not by any of the subset of probes, suggesting that the shifted band III is non-specific. The results indicate that the region from −138 to −89 is required and sufficient for the shifted band I, the sequence
from $-125$ to $-51$ is required for the shifted band IV, and $-125$ to $-89$ is required for shifted band II. Neither probe 34-b nor the TATA flanked by non-$hXOR$-related sequences competed the shifted bands. This suggests that $hXOR$-specific sequences flanking the core region (from $-125$ to $-89$) are required for nuclear protein binding.

**TFIID Binds the Putative TATA-like Element and Protects It from DNase I Digestion**—TATA-binding proteins forming a TFIID complex initiate RNA polymerase II-driven transcription by forming a complex with other transcriptional factors (40, 41), but may also be involved in repressing promoter activity (42). To test whether TFIID interacts with the TATA-like element of $hXOR$, the binding of TFIID to the TATA-like element was examined using EMSA with antibodies to TFIID and footprinting analysis. Fig. 9 shows that antibodies to TFIID, but not irrelevant antibodies, blocked a specific band shift. This indicates that TFIID is involved in the nuclear protein binding to the probe 34 containing the putative TATA-like element.

To further characterize the binding of TFIID to the TATA-like element, DNase I footprinting analysis was performed using of hrTFIID. The labeled probes covered the region from $-162$ to $-51$ that contains the TATA-like element and its flanking sequences. Fig. 10 demonstrates that a 26-bp region (from $-117$ to $-92$) containing the TATA-like element was protected from DNase I digestion by hrTFIID. The footprints were observed on both strands in repeated experiments.

**DISCUSSION**

XOR is a key enzyme in the catabolism of purines. Over the past century the enzymology of XOR has been intensively investigated. Despite a welter of information about its mechanisms of action, very little is known about factors that control its expression. Of note, relative to most other species, low XOR activity has been shown in a variety of human tissues. Post-translational mechanisms only partially explain the low activity (26, 27). We hypothesized that repression of XOR expression is another mechanism contributing to the low activity in humans.

**The Expression of hXOR Gene Is Transcriptionally Repressed**—In support of the hypothesis, in the present investigation we demonstrated that XOR transcript levels and transcription rates were substantially lower in human than mouse tissues. Moreover, the lower activity of the human promoter compared with that of the mouse in hepatocytes and vascular endothelial cells support the contention that a generalized repression of $hXOR$ gene expression may contribute to the low activity in humans.

**An E-box Regulates the Promoter Activity of the hXOR Gene**—Studies to characterize the basis for repression of $hXOR$ transcription demonstrated that the core region from $-138$ to $-1$ is required for basal promoter activity and the sequence from $-258$ to $-228$ is required for repressive regulation of the basal activity. The requirement for both the core region and repressive sequence is not specific for a given cell type.

We determined that the sequence acacaggtgtgg ($-242$ to $-230$) is critical for restricting promoter activity of the $hXOR$ gene. This “core” sequence contains a consensus E box element (ACAGGTGT) located at $-240$. Site-directed mutations of the E-box element eliminated the repressive activity. This observation is consistent with previous studies demonstrating that the E-box may play a critical role as a regulatory site (43). Several transcription factors containing helix-loop-helix motifs have been identified that bind to the E-box, and augment or repress gene transcription. These include AREB6, USF, and TFII-I. AREB6, a zinc finger homeodomain protein, inhibits transcription by interacting with the repressor NC2. AREB6 is expressed in HepG2 and HUVECs (data not shown). In neuro-
blastoma cells, the repressive effect of AREB6 on the human 70-kDa heat shock gene promoter requires the sequence GTTTC in conjunction with the ACAGGTGT (38). AREB6 is the only known factor whose regulation of promoter activity requires the presence of this conjuncted sequence (38, 39). This sequence is present 21 bp upstream (−226) of the E-box in hXOR gene and preliminary studies have demonstrated binding of nuclear proteins to the E-box site and a requirement for the GTTTC for the binding (data not shown). Further studies will be necessary to determine whether AREB6 is indeed involved in repressing promoter activity of hXOR.

The TATA-like Element Also Regulates the Repressed Promoter Activity of the hXOR Gene—Recently, a novel transcriptional activity was reported (42) that represses transcription from promoters containing the TATA-like element, but activates that lacking the TATA-like element. Compared with the TATA-less rat or mouse XOR, the region between −138 and −1 of hXOR contains a TATA-like element (30, 44–46). Site-directed mutagenesis demonstrated that conversion of the TATA

FIG. 6. Site-directed mutational analysis of the repressive region of the hXOR promoter. The left panel illustrates the constructs between the region from −258 to −226 of the hXOR promoter-luciferase reporter with wild type of promoter sequences (F6) or the site-directed mutations (F6 m1 - F6 m8). The right panel demonstrates the promoter activity of hXOR promoter-luciferase reporter constructs with wild type or various mutated sequences. The pGL3-basic vector without the inserts was used as a background control (Basic).

hXOR promoter region between −258 and −1. Probe 34 contained the putative TATA-like element. B, two shifted bands (I and II) were observed using probe 56 as indicated by arrows and were competed with specific (lane 3, unlabeled probe 56) but not nonspecific competitors (lane 4). C, four shifted bands (I, II, III, and IV) were observed using probe 34 as indicated by arrows. Three bands (I, II, and IV) were competed with specific (lane 3, unlabeled probe 34) but not nonspecific competitors (lane 4). D, a shifted band was observed using probe 12 as indicated by an arrow and was competed with specific (lane 3, unlabeled probe 12) but not nonspecific competitors (lane 4).
to a non-TATA site relieved repression. These results indicate a functional requirement of the TATA-like element in repressive regulation of hXOR.

It was proposed that the mechanism of this repressor activity of the TATA-like element involved the interaction between TFIID and repressors (42). The requirement of a TATA-like element in repressive regulation of hXOR promoter may involve a similar mechanism of interaction of TFIID and repressors. Analysis of DNA-nuclear protein interactions showed three specific band shifts within the region from −138 to −51, indicating multiple transcription factors binding or interacting within the region. Further studies with a subset of specific competitors demonstrated that the shifts required a common or core region (from −125 to −89) that contained a centered TATA-like element. To examine TFIID binding to the region, we studied interactions between TFIID and the core region from −138 to −51. The results demonstrated that antibodies to TFIID blocked a specific band shift and TFIID protected the TATA-like element from DNase I digestion by binding to the region. In addition, competition studies demonstrated that the sequences flanking the TATA-like element of hXOR are required for the binding of nuclear proteins to the TATA-centered region. Taken together, the results suggest that there is a DNA-nuclear protein complex formed via interactions between several transcription factors and regulatory sites within the core region (−125 to −89) that produce a repressive activity of the TATA-like element. In the complex, TFIID may play a central role via binding to the element.

Models for composite core promoters containing both TATA elements and initiators, such as the adenovirus major late

**Fig. 7.** Functional analysis using site-directed mutagenesis of the putative TATA-like element. The left panel illustrates the site-directed mutation of the TATA-like element. The hXOR promoter-luciferase reporter with the wild type TATA-like element was constructed as a control. The right panel demonstrates the activity of hXOR promoter-luciferase reporter gene constructs with (TAmut) or without (F6) mutation of the TATA-like element.

**Fig. 8.** Competition analysis of EMSA using nuclear protein extracts of HUVECs. A, the subset of DNA probes used for the analysis. Six probes (34-a to 34-f) were generated by PCR from the promoter region between −162 and −51 (probe 34). B, effect of competitors (34-a, -b, -c, -d, -e, -f, and TATA) on nuclear protein binding to probe 34. The competitor TATA probe contained a TATA element flanked by non-hXOR-related sequences.
promoter and human β-globin promoter (47–50), have been shown to provide alternative preinitiation complex assembly pathways. In the model, the stabilization of TFIID binding to the TATA element either by TFII-I binding to the initiator or by interacting with TFII-A provides differential mechanisms in gene regulation by specific activators or repressors (51, 52). In the TATA-less rat XOR, a series of four initiators have been reported that induce promoter activity by interacting with the initiator-binding proteins TFII-I or YY-1 (45, 46, 53, 54). In the TATA-containing hXOR promoter, the fourth initiator of the rat XOR promoter is conserved and overlaps partially with the TATA element. Therefore, hXOR is an example of a composite core promoter. TFIID binding to the TATA-like element of the hXOR may be stabilized by TFII-I or YY-1 binding to the initiator, thereby providing the opportunity for differential regulation of hXOR. It has been reported that TFII-I is able to bind to initiators and the consensus E-box and YY-1 have repressive effects on gene transcription (47, 52). In future studies of mechanisms of repressive regulation of the hXOR promoter, it will be important to determine the role of the initiator and its binding proteins TFII-I and YY-1, including their possible interactions with the E-box.

To summarize potential mechanisms for the repressed expression of hXOR, a model is proposed and illustrated in Fig. 11. In the model, the region from −138 to −1 provides a core for formation of a complex containing TFIID that is required for basal promoter activity. In a speculated bent DNA structure, the repression of hXOR by interacting with TFIID or by binding to AREB6 may inhibit the gene transcription via indirect (through a protein-protein interaction with repressor NC2) or direct interactions with the TFIID-containing complex. NC2 has been shown to negatively regulate gene transcription by interacting with TFIID and AREB6. Based on EMSA, other unknown transcription factors that may be involved in the regulation of hXOR are labeled with capital A–E.

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