Identification of the rat xanthine dehydrogenase/oxidase promoter

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ABSTRACT

Inflammation and ischemia – reperfusion tissue injury are important pathophysiologic processes with a wide spectrum of clinical presentations; the enzyme xanthine dehydrogenase/oxidase (XDH/XO) is thought to play a key role in ischemia – reperfusion injury. Recent studies have shown the transcriptional regulation of XDH/XO by cytokines (Dupont et al., 1992, J. Clin. Invest. 89, 197 – 202). In the present study, the 5' structure of the XDH/XO gene and characterization of its promoter are undertaken providing an initial step to further elucidate the regulatory mechanism(s) of this enzyme. XDH/XO cDNA from rat bone marrow macrophage has been isolated and used to screen a rat genomic library in order to identify and characterize the promoter of the XDH/XO gene. By Southern analysis, XDH/XO was found to be a single copy gene in the rat genome. Primer extension, RNase protection, and anchor-PCR studies indicate the presence of multiple start sites within a 65 bp window located some 20 – 85 bp upstream of the translation initiator (ATG). Functional studies of the sequences up to 116 nt upstream of the translational start site, which encompasses the several transcriptional start sites, indicate that this region is sufficient to drive the expression of a luciferase reporter gene and is presumed to represent the promoter. Neither a TATA box nor a GC-rich region are present in close proximity to any of the transcriptional start sites; however, sequences with homology to known initiator elements are found within this 116 bp fragment. Several possible regulatory elements, including a NF-IL6 motif, are also located upstream of the transcriptional start site. This study represents the first description of the XDH/XO promoter from a vertebrate system.

INTRODUCTION

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) have important physiologic and pathophysiologic actions (1). XDH catalyzes the oxidation of hypoxanthine and xanthine to uric acid, accompanied by reduction of NAD to NADH. XO is thought to be derived from the XDH molecule, either by proteolytic cleavage or by oxidation of sulfhydryl groups (1). XO catalyzes the oxidation of xanthine and hypoxanthine by reduction of O₂ to superoxide radical (O₂⁻), which undergoes further dismutation to H₂O₂. The hydroxyl radical (·OH), which is highly reactive and can cause cell injury, can then be produced directly (2) or indirectly (3). The reactive oxygen metabolites generated from these reactions may contribute to ischemia – reperfusion tissue injury. Apparently, during ischemia, the rate of conversion of XDH to XO is accelerated while the availability of purine substrates is also increased due to ATP depletion. These events are thought to lead to increased generation of O₂⁻ by XO when molecular oxygen becomes available as an electron acceptor during reperfusion (4,5).

In addition to playing a key role in ischemia–reperfusion injury, XO may also be important in inflammation, and XDH/XO activity has been found in macrophages (6–10). A wide spectrum of models of inflammation are characterized by an increase in XO activity, including thermal burns, complement activation, the adult respiratory distress syndrome, and influenza pneumonia (11–14). While it had been proposed that higher activity of XO during inflammation resulted from a higher rate of conversion of XDH to XO, some studies have also suggested that the total amount of XDH is increased (16,17).

Since XO is derived by proteolysis or oxidation of XDH, the XDH gene is presumed to encode XO activity; however, little is known about the mechanism(s) of regulation of XDH/XO gene expression. The sequences of rat and mouse liver XDH/XO cDNA have been reported by Amaya et al. (15) and Terao et al. (16), respectively. Recently, Dupont et al. (17) reported that interferon gamma (IFN) is a potent inducer of XDH/XO in terms of activity, mRNA levels, and transcriptional rate in rat endothelial cells. Similar studies have also been performed in mouse liver (18). These studies suggested that the expression of XDH/XO gene could be up-regulated by IFN during inflammation.

Little is known about the genomic structure and regulatory elements that govern transcription of XDH/XO. While the sequences for XDH from Drosophila melanogaster (19) and Calliphora vicini (20) have been reported, their homology to the mammalian genes seemed to be limited to the coding region.
our knowledge, no mammalian XDH/XO genomic clone has been reported.

To explore the possibility of modulation of XDH/XO gene activity in inflammatory cells during inflammation or during ischemia—reperfusion injury, we have (1) cloned an XDH/XO cDNA from rat bone marrow derived macrophages, (2) cloned the 5' end of the XDH/XO gene from rat DNA and (3) identified the promoter region of this gene.

**MATERIALS AND METHODS**

**Materials**

Restriction enzymes and other DNA modification enzymes were purchased from Promega and/or New England Biolabs. The Sequenase 2.0 sequencing kit was obtained from United States Biochemical Corporation. Radioactive chemicals were purchased from NEN. Oligonucleotides were synthesized on a Milligen/Biosearch 7500 DNA synthesizer and the sequences are shown below.

**Isolation of rat bone marrow macrophage**

Rat bone marrow macrophages (RBMM) were isolated and cultured in alpha-minimal essential medium containing 10% fetal calf serum and 10% L-cell conditioned media (LCM) (21). Under the influence of LCM, bone marrow macrophage precursors differentiate. After 5—7 days, the cells exhibit macrophage morphology, plastic adherence, and expression of macrophage-specific mannose receptor activity (22,23).

**Isolation of cDNA clones of XDH/XO from RBMM**

A RBMM cDNA library was constructed using total RNA from bone marrow-derived macrophages after 7 days in culture. To screen the library, we relied upon known homologies between the rat liver and Drosophila XDH/XO (15). We synthesized primers from two regions of 100% base homology between the rat liver and Drosophila cDNAs. The 5' sense primer (oligonucleotide 885) and the 3' antisense primer (oligonucleotide 886) were used in a PCR reaction with the rat BMM cDNA library as template. A single PCR product of approximately 440 base pairs was generated. 1 x 10^6 plaques were screened by filter hybridization using C600 HFL as host, and using the 440 bp PCR product as probe. Nine positive plaques were identified.

**Isolation of a genomic clone of rat XDH/XO**

About 5–10 x 10^5 plaques of a SalI partial digest genomic library were screened by plaque hybridization. The 900 bp fragment from the XDH/XO cDNA (cDNA clone #20, see above) was labeled for the initial screening. The purified clone was subjected to restriction enzyme digestion and subcloned into Bluescript KS II plasmid for sequencing. The nucleotide sequence(s) reported in this paper has been submitted to the Genbank/EMBL Data Bank with accession numbers U08120, U08121, U08122, and U08123.

**Southern analysis**

Rat liver genomic DNA (20–50 µg) was digested with restriction enzymes and subjected to electrophoresis in a 1% agarose gel. The gel was transferred to Zeta-Probe (Bio-Rad), hybridized to cDNA probes from +5 to +863 (clone #20) and from +858 to +1518 (clone #10), and analyzed by autoradiography.

**Primer extension of XDH/XO mRNA**

Total RNA was extracted from rat bone marrow macrophages using Tri-reagent (Molecular Research Center Inc.). About 20 µg total RNA was used for primer extension together with 3 x 10^6 cpm of oligonucleotide primer (oligonucleotides 1292 and 694). Primer and RNA were annealed in buffer containing 20 mM Tris—HCl (pH 7.5), 250 mM NaCl, and 1 mM EDTA, heated at 85°C for 10 min, and transferred to a 50 or 60°C water bath for another 60 min. Reverse transcriptase buffer, 500 µM dNTPs, and 5 U of avian myeloblastosis virus reverse transcriptase were added for elongation. The extended products were separated by electrophoresis in a 5% polyacrylamide gel under denaturing condition and subsequently autoradiographed.

**Identification of the 5' untranslated region by anchor-PCR**

Anchor-PCR was performed as described by Troutt et al (24). In brief, primer extension was performed as mentioned above except the primer oligonucleotide (oligonucleotide 694) was not phosphorylated and the elongation step was performed at 50°C. Primer extended products (cDNA) were purified from excess primer oligonucleotides by using a Sephadex G-50 spin column. The anchor oligonucleotide (27) was blocked at the 3'—end with ddATP using terminal deoxynucleotidyltransferase and 5' end labeled with ATP using T4 polynucleotide kinase. Ligation of cDNA to the anchor oligonucleotide was carried out at room temperature for at least 24 h with 5 pmol of the 3' blocked and 5' phosphorylated anchor oligonucleotide, T4 RNA ligase reaction mix (50 mM Tris—HCl, pH 8.0, 10 mM MgCl2, 1 mM hexamine cobalt chloride, 20 µM ATP, 25% PEG 8000), and 5 U T4 RNA ligase (New England Biolab) in a total volume of 10 µl. Ligation reaction was terminated and amplified as described (24). The first PCR amplification was carried out as follows using 50 pmol of both internal oligonucleotide

**Sequences**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>737 (antisense)</td>
<td>−19 to −53</td>
<td>5'-TGAGCTCTTGAGAAGTAGTGATGATATCAGTCC-3'</td>
</tr>
<tr>
<td>885 (sense)</td>
<td>+16 to +39</td>
<td>5'-TTGGTCTTCTTGTGAAATGCAA-3'</td>
</tr>
<tr>
<td>825 (antisense)</td>
<td>+42 to +22</td>
<td>5'-CTTTTGTCCCTTCACAAAGA-3'</td>
</tr>
<tr>
<td>694 (antisense)</td>
<td>+84 to +58</td>
<td>5'-GACCGAAGATGTGGTTTCAGGTTCGCC-3'</td>
</tr>
<tr>
<td>1314 (antisense)</td>
<td>+107 to +87</td>
<td>5'-AGGCCCAACCTTCCCTCTTGAG-3'</td>
</tr>
<tr>
<td>1292 (antisense)</td>
<td>+141 to +121</td>
<td>5'-GCCCACCTTCTTCACACAGCAGA-3'</td>
</tr>
<tr>
<td>886 (antisense)</td>
<td>+450 to +421</td>
<td>5'-CTTACGCCCCAGAGTAGTTTCAGTCC-3'</td>
</tr>
<tr>
<td>296 (sense)</td>
<td>+436 to +456</td>
<td>5'-CTTTTGTCCCTTTCACAGCAGC-3'</td>
</tr>
<tr>
<td>Anchor oligonucleotide</td>
<td></td>
<td>5'-CTGGCTTGGCGGTGGCGAAATTGCAGCCAACAG-3'</td>
</tr>
</tbody>
</table>
(oligonucleotide 825) and complementary anchor oligonucleotide: 25 cycles of PCR with annealing temperature at 56°C for 1 min, elongation temperature at 72°C for 1 min, and denaturing temperature at 92°C for 1 min. The second PCR amplification was carried out with a more upstream internal oligonucleotide (oligonucleotide 737), in a similar fashion except no complementary anchor oligonucleotide was added, and the annealing step and elongation step was carried out at the same temperature (72°C) for 2 min. PCR amplified products were gel purified and subcloned into Bluescript KS II plasmid, which has been digested with EcoRV and terminally transferred with dTTP in the presence of Taq DNA polymerase. Positive clones were sequenced with Sequenase 2.0 from USB.

RNase protection

An EcoRI−FokI fragment (−282 to +26) and a reverse transcriptase−PCR product from oligonucleotide 486 and 1314 (−53 to +107) were each subcloned into Bluescript KS II. Uniformly labeled [α−32P]CTP antisense RNA (cRNA) was generated from HindIII-linearized plasmids with T7 RNA polymerase. A total of 4 × 10^6 cpm antisense RNA was co-precipitated with 30 μg RBMM total RNA, and resuspended in 20 μl hybridization buffer (40 mM piperazine-N,N'-bis[2-ethane sulphonic acid] (PIPES) (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% formamide). After hybridization at 54°C for 16 h, 300 μl of RNase digestion mix (300 mM NaCl, 10 mM Tris−HCl, pH 7.5, 5 mM EDTA, 100 U/ml RNase T1, and 5 μg/ml RNase A) was added and incubated at 37°C for 1 h. Proteinase K and SDS was added to a final concentration of 300 μg/ml and 1%, respectively, and further incubated at 37°C for 30 min after the digestion. Protected fragments were resolved in a 6% denaturing polyacrylamide gel electrophoresis and autoradiography.

Transcriptional activity of the putative XDH/XO promoter

Based on the restriction map in Fig. 3, upstream restriction sites were utilized for the construction of XDH/XO−luciferase reporter plasmids. For the XO-256 reporter plasmid, a SacI−Banl (−25 to −256) fragment was excised from a genomic subclone, and subcloned into a promoterless luciferase construct (−25 to −256) fragment was excised from a genomic Banl−SacI−EcoRI to yield two fragments of approximately 900 and 600 bp, consistent with known restriction sites present in the rat liver XDH/XO cDNA, which contains internal EcoRI sites at +884 and +1502, respectively. The 900 and 600 bp EcoRI fragments were further subcloned as described in Materials and Methods and are identified as cDNA subclone # 20 and # 10, respectively.

The sequence of the isolated RBMM cDNA compares closely with that of the rat liver cDNA isolated by Amaya et al. The sequence of the macrophage cDNA is 100% homologous to the liver cDNA until +1435. There are an additional 36 base pairs at this point in the rat macrophage clone. This sequence has a 10 base repeat (GAGGAGCTGC) at the beginning, +1435 to +1444, and the end, +1471 to +1480, of the 36 bp region. In addition, the second nucleotide following the second repeat (i.e. at position 1482) is changed, which results in an amino acid change from glutamine in the rat liver cDNA to histidine in the macrophage sequence. Comparison to other known XDH/XO genes (Fig. 1) indicates that the extra 12 amino acids in the rat macrophage clone is the norm. Although it is not possible to exclude expression of different but very similar genes, the simplest explanation for this observation resides either in differential splicing (25) in which the single base change has occurred in alternative exons, or in a cloning deletion in the rat liver cDNA which provides, thus far, the only example of an XDH/XO cDNA missing these 36 nucleotides (see discussion below). Certainly, the protein sequence deduced from the recently

**RESULTS**

Isolation of a cDNA clone for XDH/XO from rat bone marrow macrophages

Our overall goal was to study the regulation of the activity of XDH/XO in macrophages because of the important role that this enzyme may play in tissue injury during inflammation (1, 4−15). Thus, we aimed to investigate if transcription, processing, and regulation of the gene might be altered in any way in these cells. Therefore, we elected to isolate an XDH/XO cDNA from rat bone marrow macrophages (RBMM) to provide us with a reagent that could be used for screening a rat genomic library.

To screen a RBMM cDNA library, we relied upon known homologies between rat liver and *Drosophila* XDH/XO (15). We synthesized primers from two regions which show 100% nucleotide homology between the rat and *Drosophila* cDNAs. Since the transcriptional start site in the rat liver gene had not been identified, the adenine of the translational start site was assigned as +1. A single PCR product of approximately 440 base pairs was generated from oligonucleotide 885 and oligonucleotide 886 using the RBMM cDNA library as template. This fragment was used to screen the library and nine clones were obtained. One purified clone, GT10-XO-1, was digested with EcoRI to yield two fragments of approximately 900 and 600 bp, consistent with known restriction sites present in the rat liver XDH/XO cDNA, which contains internal EcoRI sites at +884 and +1502, respectively. The 900 and 600 bp EcoRI fragments were further subcloned as described in Materials and Methods and are identified as cDNA subclone # 20 and # 10, respectively.

Figure 1. Comparison of the 36 base pair insert in the rat bone marrow macrophage (RBMM) cDNA to other known XDH/XO cDNAs. Italics represent the extra 36 nucleotides found in the RBMM cDNA. Boldface letters represent the single base change-derived amino acid substitution. Underlined nucleotides represent the 10 base pair repeats in this region.
published mouse liver XDH/XO cDNA (16) differs by only one amino acid from that described by us for the RBMM gene (Fig. 1).

Complexity of the XDH/XO gene system in the genome
We have probed various restriction enzyme digests of rat genomic DNA with fragments from the XDH/XO cDNA. The results of such a Southern analysis are presented in Fig. 2A. Probing an EcoRI digest with the cDNA clone #10 (+858 to +1518) generated a single band of MW ca. 8 kb. A single band is also observed following a BamHI digest (data not shown). However, if the same blot is re-probed with cDNA clone #20 (+5 to +863), we detect a positive signal in three bands in an EcoRI digest, of MW ca. 4.7, 4.2 and 3.9 kb, as well as a like number of bands of MW ca. 8.0, 1.8 and 0.7 kb from a BamHI digest. There are several possible interpretations of these results. There might be a single gene for XDH/XO, which consists of coding sequences that are extensively interrupted toward the 5' end with fairly large introns which contain restriction sites leading to multiple fragments. On the other hand, there could be at least three different copies of XDH/XO-related sequences. Also, the existence of pseudogenes cannot be excluded at this point.

To distinguish between these possibilities, further Southern hybridization was done using short oligonucleotides which recognize localized sequences at different positions along the cDNA between +5 and +863 bp and on average are less likely to span multiple exons. The Southern analysis indicated that each oligonucleotide was able to recognize a specific, individual band which together make up the complement of bands recognized by the 5' portion of the cDNA (Fig. 2B). This indicates that the 5' heterogeneity can most simply be explained by intronic complexity in the 5' terminus of XDH/XO. Thus, we conclude that we are most likely dealing with a single copy gene which is extensively interrupted by introns, a conclusion which is directly supported by the cloning studies described below.

Isolation of rat XDH/XO genomic clones
We utilized the isolated cDNA clone #20 as a probe to screen a rat genomic DNA library. Two positive plaques were identified and subsequently purified to homogeneity. At the present time only one of these clones (XO genomic clone II) has been characterized. This clone contains approximately 23 kb of rat DNA. It hybridized with the 5' portion of the XDH/XO cDNA. In contrast, no hybridization was noted to the 3' portion of the cDNA (+858 to +1518). Accordingly, clone II was analyzed in detail as we were most interested in obtaining information about the promoter of this gene.

The restriction map of a 17 kb SalI–SalI fragment, obtained from a SalI digest of the 23 kb insert in clone II, is shown in Fig. 3. The positions of various oligonucleotides used in its analysis are also indicated in the figure. Comparison of the sequence information from the genomic subclones and the cDNA

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**Figure 2.** (A) Southern blot analysis of the XDH/XO gene. About 20 µg of rat liver genomic DNA were digested with restriction enzymes as indicated, subjected to 1% agarose gel electrophoresis and transferred to a nylon membrane. Two different cDNA clones (number as indicated) were randomly labeled with [α-32P]dATP and used for hybridization. Arrow markers indicate apparent molecular size in kilo-base pairs. (B) Southern blot of the XDH/XO gene. EcoRI (E) or PstI (P) digested rat liver genomic DNA were separated in a 1% agarose gel and transferred as described in Materials and Methods. Probes used for hybridization were generated from specific oligonucleotide primer (as shown) labeling on different genomic subclones. Arrow markers indicate apparent molecular size in base pairs.
clone revealed that the 5' portion of the XDH/XO gene has indeed been isolated, and that it is highly interrupted by introns. In fact the 17 kb SalI-SalI fragment contains only 191 bp of the rat liver cDNA sequences. The 191 bp exonic sequences are divided among three exons with sizes of 68 bp (-26 to +42, exon 1), 58 bp (+43 to +100, exon 2), and 65 bp (+101 to +165, exon 3), respectively. The estimated sizes for introns 1 and 2 are about 8 kb and 3 kb, respectively. The 3' limit of the genomic insert is in all probability at a SalI site, which is located just inside a third intron as indicated in Fig. 3. Although we know from Southern analysis that oligonucleotides for coding regions just downstream of the 191 bp exon position do not detect complementary sequences in the genomic clone II, we cannot exclude the possibility that the genomic insert in clone II contains an additional intron 3' to the SalI site prior to terminating at yet another, more downstream, SalI site. In fact, from the rat genomic DNA Southern analysis (Fig. 2B), we noted oligonucleotide 1292 and oligonucleotide 886 hybridized to different bands in the PstI digest. Since there is no PstI site located between oligonucleotide 1292 and oligonucleotide 886 in the cDNA sequence, these differences indicate the presence of an intron (intron 3) which lies between oligonucleotide 1292 and oligonucleotide 886, and evidently this intron contains a PstI site. Preliminary study indicates that the size of intron 3 is about 3 kb (unpublished data).

Sequence information on the genomic organization of the 5' end of the XDH/XO gene is presented in Fig. 4A. Most of the sequencing activity was concentrated on the exons and the regions immediately surrounding them. Analysis of the intron/exon borders is presented in Fig. 4B. Both acceptor sequences are fully canonical (AG) and two of the donor sequences are likewise (GT). In contrast, the sequence immediately following the third exon (CGTTG) is unusual and appears to diverge from the established rules. This raises the possibility that the gene we have cloned is in fact not spliced at this point.

**Determination of the XDH/XO transcriptional start site**

The sequence upstream of the 5' cDNA terminus in the rat liver genomic clone is shown in Fig. 4A. Inspection of the sequence shows potential splice acceptor sites at -24 and -59 with respect to the adenine of the translation initiator codon. Since the organization of untranslated parts of the XDH/XO message is not known, the presence of potential splice acceptor sites raises the possibility that there might be another intron in the 5' untranslated region (UTR). The size of the XDH/XO mRNA observed in this study and two others (16,17) is approximately 5 kb, which is larger than the rat liver cDNA reported by Amaya et al. (15). Further, none of the rat cDNA sequences include...
any indication of the location of the polyadenylation site at the 3' end of the XDH/XO mRNA. Comparison of the recently published mouse liver XDH/XO cDNA to the rat liver XDH/XO cDNA indicates that the sizes of the coding regions of these two genes are very similar; 4005 bp in the mouse liver XDH/XO, and 3957 bp in the rat liver XDH/XO (not including the extra 36 bp found in this report and the extra 9 bp found in the 3' terminus of the mouse XDH/XO). In the mouse gene, however, the size of the 3' UTR has been identified (531 bp). Taking into account 250 bp for the average size of a poly A tail in mammalian mRNA, the total length from the translational start site to the end of the poly A tail in mouse XDH/XO is about 4800 bp. Thus, the estimated size for the 5' UTR in mouse liver XDH/XO, and by interpolation for the rat liver XDH/XO, is no more than 200 bp, and possibly somewhat less. Nonetheless, this is sufficient to extend beyond the splice acceptor sites discussed above, and certainly requires consideration of the hypothesis that there is an additional intron in the 5' UTR.

Accordingly, we have attempted to identify the transcriptional start site of the rat XDH/XO gene using several strategies. Initially, we employed primer extension analysis with oligonucleotide 1292. Instead of a defined, extended product as shown in the positive control from the phosphoenolpyruvate carboxykinase (PEPCK) gene, multiple extended products were observed (Fig. 5A). Two possibilities may account for this observation. First, since the 5' terminus of XDH/XO message contains somewhat repetitive sequences, these could lead to a strong secondary structure and interfere with primer extension. Second, there might be multiple transcriptional start sites in the XDH/XO gene.

To test the first possibility, another oligonucleotide primer (oligonucleotide 694), which is longer in length, was used to allow us to adopt a higher elongation temperature in primer extension. It was anticipated that the higher temperature would weaken any secondary structure in the 5' terminus, and allow more efficient processivity of the reverse transcriptase. As shown in Fig. 5A, multiple extended products were still observed. The most prominent extended products were about 20 and 40 bp beyond the ATG site. However, longer products, from 55–83 bp in length, could also be observed, though with a much weaker signal. This observation raises the distinct possibility that multiple start sites exist in the XDH/XO gene.

The second possibility was tested by RNase protection. Two cRNA probes were generated; one probe contained sequences from −53 to +107 and the other from −282 to +26 bp. The probes were annealed to mRNA isolated from RBMM. After the RNase digestion, several protected fragments of differing sizes were obtained (Fig. 5B). The −53 to +107 probe gave fragments of size 56, 88, 125, and 147 bp. The two longest fragments are consistent with transcripts containing sequence information from −19 and −41 bp upstream of the translation initiator. The two shorter fragments (i.e. mapping to sites downstream of the ATG)
may well reflect inability of the cRNA probe to hybridize extensively because of secondary structure in the XDH mRNA. The —282 to +26 probe yielded protected fragments of size 49, 52, 54, and 69 bp which correspond to RNA molecules extending from 23 to 43 bp upstream of the ATG codon.

The results from the primer extension and RNase protection are difficult to interpret. In all likelihood, strong secondary structure in the 5' portion of the XDH/XO mRNA is generating premature termination in primer extension as well as decreased hybridization in the RNase protection experiments, and we are unable to resolve whether we are detecting several different transcription initiation points or strong secondary structure. Even the observation that the start sites mapped by both techniques are the same is subject to the same concern.

Our fundamental goal in attempting to define the transcriptional start site lay in our intent to identify the promoter for this gene. Since the region immediately upstream of the ATG contains two possible splice acceptor sites (at —24 and —59), if we could obtain evidence that the primer extended products extended into known 5' sequences beyond —59, then we could conclude definitively that transcription initiation must have occurred at a nearby site. Hence, the possibility that the promoter and an unidentified first exon were located in an undefined upstream position could be eliminated. Accordingly, we have utilized anchor-PCR to isolate those primer extended products which are greater than 59 nts. These PCR products were sequenced and the identified transcriptional start sites are summarized in Fig. 3B, along with the other transcriptional start sites identified by primer extension and RNase protection. The results indicate that only immediately 5' sequences are found in the mRNA and there is no evidence of additional splicing. Thus, we conclude that, at least in RBMM, the XDH/XO promoter is located 20–85 bp upstream of the ATG. Also, we would argue that there is tentative evidence for the existence of a number of separate start sites in this region as summarized in Fig. 4A.

### Identification of the XDH/XO promoter

Inspection of the sequences upstream of the 65 bp domain of transcription initiation indicates the presence of a considerable concentration of cis-acting binding sites typically found in eucaryotic promoters (Fig. 4A). These include the sites for the ubiquitous factors AP-2, CCAAT factor, and NF-IL6. In addition to the ubiquitous sites, there is a 10/11 match to the a-interferon stimulatory response element (ISRE, 31) as well as a perfect match to an Ets-1 binding site (26). There is, however, no canonical TATA binding site or GC-rich sequence (Sp1 transcription factor binding site) in close proximity to the transcription initiation regions.

To test whether the region upstream of the translational start site has functional promoter activity, a fragment of DNA upstream of the ATG (from —25 to —256 bp) was subcloned in front of a luciferase reporter gene (XO-256), and assayed by transient transfection. Plasmid pRSV/βGal was co-transfected with the luciferase reporter constructs as an internal control. As shown in Fig. 6A, the XO-256 luciferase construct provided an approximate 50-fold increase in activity compared to the promoterless construct (the parent plasmid used for initial subcloning). Further, insertion of additional XDH/XO upstream fragments (XO-500, XO-3000, and XO-6000) 5' of the luciferase reporter gene, in general, leads to a decrease in promoter activity (Fig. 6A). This indicates the presence of possible negative regulator(s) upstream of the 250 fragment.

**Figure 6.** (A) Deletion analysis of the rat XDH/XO promoter. Constructs containing various XDH/XO upstream fragments were subcloned in front of luciferase reporter gene, and transiently transfected into HeLa cells. β-Galactosidase reporter gene was co-transfected as an internal control. Luciferase activity was normalized to β-galactosidase and used to represent the transcriptional activity of XDH/XO promoter. (B) Deletion analysis within the 256 bp rat XDH/XO promoter. Various restriction enzymes were used to generate a series of XDH/XO reporter constructs and then transiently transfected into HeLa cells as described. Promoterless construct represents the parent plasmid (pSVOARPL-ΔA5). At least three independent experiments were done and all observations were performed in triplicate. The results are all normalized to a value of 100 for the XO-256 construct.

**Figure 7.** Comparison of upstream nucleotide sequences of XDH/XO to known initiator sequences. Arrow indicates the transcription start site of known initiators. Cross mark, asterisk, and arrow head represent start sites found in rat XDH/XO by primer extension, RNase protection and anchor-PCR, respectively. PBGD, porphobilinogen deaminase; DHFR, dihydrofolate reductase; rpS16, small ribosomal protein 16.
Further deletion analysis within the 256 bp fragment was subsequently performed (Fig. 6B). A smaller fragment, -116 bp upstream of the ATG, was identified as containing basal XDH/XO promoter function. This -116 bp fragment leads to a 30-fold increase in promoter activity compared to the promoterless construct. Further deletion to position -73 abolished the promoter activity. The -25 to -116 fragment was sub-divided into two fragments, from -73 to -116 and from -73 to -25. Neither subfragment was capable of directing transcription. This indicates that either, (1) the two subfragments contain binding activities which need to communicate in some way or (2) a critical binding domain around -73 was destroyed when the basal promoter was dissected into two halves.

DISCUSSION

Xanthine oxidase is a molecule of considerable clinical interest because of its potential role in generating reactive oxygen species in ischemia—reperfusion tissue injury and in inflammation (1). XO is thought to be derived from a proteolysis/oxidation conversion of the protein product of XDH. Recent studies have suggested that in addition to cytokine-mediated XDH/XO conversion, inflammatory cytokines such as interferon might induce transcription of the XDH/XO gene (17,18). However, no XDH/XO promoter has been identified and it has not been possible to study the regulation of the gene in detail.

In this report, we describe the isolation of a XDH/XO cDNA from a rat bone marrow macrophage library. Using this cDNA clone as a probe, we have isolated the 5'-most three exons of the XDH/XO gene from a rat liver genomic library. Further, we have identified the transcriptional start sites and the promoter within this genomic clone. In addition, several observations in this report provide insight into the basal regulation of the XDH/XO gene.

We have presented evidence based on Southern analysis that there appears to be only one gene for XDH/XO. However, the cDNA isolated from RBMM while very similar to that isolated by Amaya et al. (15) from rat liver, is nonetheless clearly different. The macrophage cDNA contains an additional 36 nucleotides (which are also seen in cDNAs from other species). In addition, there is a single nucleotide substitution downstream of the 36 nucleotide insertion. Given the observation that there is no indication of multiple genes, as well as the finding that the XDH/XO gene is extensively interrupted with introns, we tested for the existence of differentially spliced messages. Using a reverse transcriptase—PCR approach, we have found that there is no evidence of differential splicing. In tissues that express high XDH/XO activity (small intestine, liver, and RBMM), we observed no differences in terms of their sizes of PCR products, or the restriction digests of the amplified products (unpublished data). However, the expression of a very minor form of XDH/XO that is missing the 12 amino acids could not be excluded. Other possible explanations for the missing 12 amino acids are either allelic variation between different rat species or a cloning variation due to the presence of 10 bp repeats flanking the 36 bp insert that led to a cloning artifact.

Primer extension of the XDH/XO mRNA indicates multiple transcriptional start sites which lie within a 65 nucleotide window upstream of the translational start site. However, in primer extension experiments of this type, if the message has a region of strong secondary structure, premature termination of reverse transcriptase may occur (28). Based on an energy minimization algorithm (29), there is an extensive secondary structure at the 5' end of the XDH/XO message. If this is the case, then one would expect that the shortest extended cDNA will have the highest yield and vice versa. Indeed this was observed as reported in Fig. 5A. In addition, sequence analysis of primer extended products amplified by PCR indicates that at least two of the start sites contain uninterrupted sequences from a position immediately upstream of the splice acceptor sites in the 5' UTR providing an indication that splicing to these sites is not occurring. Observations from the RNase protection assay were consistent with the primer extension and anchor-PCR. Similar indications of strong secondary structure at the 5' terminus are seen in the RNase protection assay.

There is no canonical TATA box or GC-rich region within normally expected distances from any of these transcriptional start sites in the XDH/XO gene. Comparison of sequences around the transcription initiation sites with other genes lacking TATA boxes reveals a considerable degree of homology (Fig. 7). These initiator sequences have been known to direct transcription start in a TATA-less gene. Also, cooperation between such an initiator region and an upstream regulator greatly enhances the promoter activity (30). This notion was substantiated in the XDH/XO gene when the putative non-TATA initiation sequence (−73 to −25) which most resembles the canonical initiator was coupled to a luciferase reporter gene, and shown to direct a significant level (30-fold induction) of transcriptional activity in the presence of upstream regulators (−116 to −73). In fact, a putative CCAAT transcriptional factor binding site has been identified within this −116 bp fragment. Deletion of the putative CCAAT binding site abolished the promoter activity of the XDH/XO gene; however the CCAAT sequence by itself could not initiate transcription.

We conclude that the bulk of the initiation of the XDH/XO mRNA occurs over a definable 65 bp region of the gene. The sequences immediately upstream of what we are calling the basal promoter were then examined for ability to modulate the basal transcriptional activity. We find that sequences out to around −250 bp elevate transcriptional activity to 50-fold above the basal promoter. Extension of this analysis to around −6000 bp revealed a modest diminution of transcriptional ability, suggesting the presence of additional modulating elements. Recent observations (unpublished data) have provided evidence that substantial up-regulation in response to PMA occurs in this latter domain.

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REFERENCES