Cloning and expression *in vitro* of human xanthine dehydrogenase/oxidase

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To study the expression of human xanthine dehydrogenase/oxidase (hXDH/XO), we cloned the cDNA covering its complete coding sequence and characterized it by translation *in vitro* in rabbit reticulocyte lysates and by transient expression in COS-1 cells. Two specific protein products with approximate molecular masses of 150 and 130 kDa were detected in both expression systems. These products are compatible with the molecular sizes of XDH/XO, and these peptides also showed immunoreactivity with polyclonal anti-hXDH antibodies. Significant XDH/XO enzyme activity (277 ± 54 pmol/min per mg of protein) was measured in lysates of transfected COS cells, whereas in control transfections the activities were below the detection limit of our assay (0.2 pmol/min per mg of protein). The COS cells expressed the enzyme predominantly ($89.8 \pm 0.3\,\%$) in the dehydrogenase form.

INTRODUCTION

Xanthine dehydrogenase/oxidase (XDH/XO; EC 1.1.1.204/1.1.3.22) catalyses the two last steps in purine catabolism in man, forming the end product uric acid from hypoxanthine and xanthine. The mammalian enzyme exists mainly as a dehydrogenase, which utilizes NAD^+ as the electron acceptor, but can be converted into an oxidase both *in vivo* and *in vitro*. The oxidase form utilizes molecular oxygen as the electron acceptor and releases substantial amounts of reactive oxygen metabolites under certain conditions, e.g. during tissue reoxygenation after hypoxia [1].

XDH is a homodimer with a subunit molecular mass of approx. 150 kDa. Conversion into XO occurs initially through thiol group oxidation and is reversible by treatment of the enzyme with thiol reagents [2]. Subsequently, irreversible conversion takes place through cleavage of an approx. 20 kDa fragment from each subunit [3,4], presumably catalysed by a calcium-dependent protease [5]. This conversion occurs during purification procedures unless the enzyme is protected by protease inhibitors [2]. Even if such precautions are taken, tissue preparations typically contain at least 10–15 % of their total XDH + XO activity in the oxidase form [6]. Thus it cannot be stated with certainty that all of the oxidase activity is derived by proteolytic maturation from the dehydrogenase form.

Recently we and others have mapped the gene encoding human XDH(hXDH)/XO to a single locus at 2p22 [7,8]. However, three discordant putative XDH/XO cDNA sequences for hXDH have been reported [9–11]. The sequence differences in these cDNAs, and in the corresponding nucleotide probes used, may also account for conflicting data on tissue-specific XDH/XO mRNA expression [10–12].

To clarify these issues we have cloned a cDNA containing the complete coding sequence of hXDH and demonstrated its identity by immunological and functional analyses of its protein product.

EXPERIMENTAL

Cloning of hXDH cDNA

The isolation of a 249 bp cDNA clone specific for hXDH (hXDH1) by PCR screening of a human mammary gland λgt11 cDNA library has been described [8]. This clone was used for producing cRNA probes for screening human liver and small intestine λgt11 cDNA libraries (Clontech) by standard methods [13]. Longer and overlapping clones were selected from among several obtained from different parts of the hXDH cDNA; clones 5a and 36 were derived from liver and intestinal libraries, respectively (Figure 1). They were subcloned into the Gemini7Z + vector (Promega). The 5′-end cDNA clone (hXDH27) was obtained by reverse transcription of RNA extracted from small intestine obtained at autopsy of a preterm infant. Random hexamer primers (Boehringer Mannheim) and Moloney murine leukaemia virus reverse transcriptase (Promega) were used, and the first-strand cDNA was amplified by using PCR primers specific for hXDH [9]. Unique linker sequences were included in the primers for cloning the 1.2 kb PCR product (bases −41 to 1128 according to the numbering of Ichida et al. [9]) into the pDIR vector (Clontech) in accordance with the manufacturer's protocol. After sequencing and cleavage by restriction endonucleases *Kpn*I and *Eco*RI, clone hXDH27 was ligated with clone hXDH5a at the unique *Eco*RI site (nt 862). This construct was ligated with clone hXDH36 at the *Sph*I site (nt 2481), and the 4.3 kb cDNA product containing the complete coding region of 3999 bp was cloned into the *Kpn*I site in the Gemini7Z + vector.

The cDNA clones were sequenced in both strands by using Sequenase Version 2.0 (United States Biochemical Corp.) in

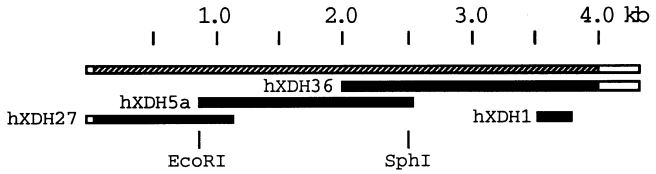

Figure 1 Subclone composition of hXDH cDNA

The open boxes depict the flanking non-coding regions. Our first hXDH clone (hXDH1) is also shown.

Abbreviations used: hXDH, human xanthine dehydrogenase; XO, xanthine oxidase.

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The nucleotide sequence reported in this paper will appear in the EMBL and GenBank Nucleotide Sequence Databases under the accession number U39487.

accordance with the manufacturer's protocol. The final cDNA sequence was confirmed with the AutoRead Sequencing kit and analysed on an ALF DNA Sequencer (Pharmacia) at the Institute of Biotechnology, University of Helsinki. The sequences were assembled by using the Staden Package program [14] on a Sun workstation.

Translation *in vitro*

After sequencing, the hXDH cDNA was translated *in vitro* by using TNT T7-coupled rabbit reticulocyte lysate (Promega) and ^{35}S-labelled methionine (1000 Ci/mmol; Amersham) in a volume of 50 μl in accordance with the manufacturer's protocol. The translation products were revealed by electrophoresis on a 7.5 % (w/v) polyacrylamide/0.1 % SDS denaturing gel, followed by drying of the gel and a 1 h exposure to Kodak BioMax autoradiography film at room temperature. Prestained high range protein standards (BioRad) were used.

For protein expression in COS-1 cells (American Type Culture Collection no. CRL 1650), hXDH cDNA was cloned into the *Kpn*I site of the simian virus 40 early promoter-driven SVpoly expression vector [15]. The cells were transfected by using DEAE-dextran and chloroquine with the use of standard procedures [16]. After 72 h, cells were washed with PBS, collected by incubating at 37 °C for 15 min in PBS containing 0.5 mM EDTA, and transferred to 10 ml centrifuge tubes. After centrifugation (5 min at 190 ***g***) the cell pellet was resuspended and sonicated on ice in 500 μl of 50 mM potassium phosphate buffer, pH 7.8, containing 0.5 mM dithiothreitol, 1 mM EDTA, 5 μg/ml leupeptin, 0.1 μM pepstatin, and 1 mM PMSF. The cell extracts were then used for XDH/XO enzyme assay and Western blotting.

XDH/XO assay

For the assay of total XDH + XO activity, cell extracts were incubated with 45 μM [^{14}C]xanthine (Amersham, specific radioactivity 50–60 mCi/mmol) and 150 μM NAD^+ in a total volume of 100 μl at 37 °C for 30 min. XO activity was measured by omitting NAD^+ from the mixture. The reactions were stopped by adding 10 μl of 4.2 M perchloric acid and transferring the tubes to ice. The acid was removed by using Alamine–freon extraction [17], and the product uric acid was separated from the substrate by HPLC (Shimadzu) with a reverse-phase column (HPLC Technologies, Ltd.) and isocratic elution with 50 mM potassium phosphate, pH 4.5, at a flow rate of 1 ml/min. The fraction of the eluate corresponding to the uric acid peak was collected and counted by liquid scintillation counting (Rackbeta, LKB-Wallac). The enzyme activity is expressed as pmol/min per mg of total protein in each sample.

Western blotting

From the cell lysates, a 5 μl sample was size-fractionated by gel electrophoresis on a 7.5 % (w/v) polyacrylamide/0.1 % SDS gel after denaturation by heating in a 2-mercaptoethanol-containing loading buffer. The proteins were electrotransferred to Immobilon-P polyvinylidene difluoride filters (Millipore) and incubated for 2 h with polyclonal rabbit anti-hXDH antibodies [18]. After washing of the filters, immunocomplexes were detected by alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) and a standard Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colour reaction. High range protein size standards (BioRad) were visualized by staining with 0.25 % Coomassie Blue.

RESULTS

cDNA sequence

Several independent cDNA clones were isolated from human liver, small intestine and mammary gland libraries, and these clones were used to construct a full-length XDH/XO cDNA clone as described in the Experimental section. All clones from these libraries were identical in their overlapping regions, indicating that they were products of a single gene and providing no evidence of alternative splicing of XDH/XO mRNA.

This cDNA sequence (Figure 2) was over 99 % identical with that reported by Ichida et al. [9] with a total of five nucleotide differences ($A^{572} \rightarrow T$, $A^{691} \rightarrow G$, $T^{1122} \rightarrow A$, $G^{3449} \rightarrow C$ and $C^{3888} \rightarrow G$], resulting in three amino acid changes in the polypeptide. However, there are major differences between our cDNA sequence and that reported by Wright et al. [10] (overall similarity 60 %). The similarity of our sequence with the third hXDH cDNA sequence submitted by Xu et al. [11] is 94 % in the coding region.

Translation *in vitro* and immunoblotting

A single major polypeptide band of approx. 150 kDa was detected by autoradiography after translation of the XDH/XO cDNA in the reticulocyte lysate in the presence of [^{35}S]methionine (Figure 3). This corresponds to the subunit molecular mass of hXDH. A fainter band corresponding to approx. 130 kDa was also seen. This is in accordance with the molecular mass of the oxidase form after the proposed proteolytic conversion. However, on electrophoresis on a more concentrated gel (15 % polyacrylamide) followed by autoradiography, no bands migrating in the 20 kDa size range were seen (results not shown).

To evaluate whether the primary translation product is the 150 kDa moiety, the translation reaction was performed in the presence of protease inhibitors (1 μM PMSF, 5 μg/ml leupeptin and 0.1 μM pepstatin): the 130 kDa polypeptide band became fainter but did not disappear (Figure 3). In the presence of 1 mM EDTA to inhibit Ca^{2+}-dependent proteases, no translation product was obtained (results not shown). Thus we cannot prove definitively that the 130 kDa protein is a proteolytically modified form of the primary translation product.

The translation products both in the reticulocyte lysates and in extracts from transfected COS cells were immunoreactive in Western hybridization and the pattern of polypeptide bands from the COS cell lysates was similar to that seen in SDS/PAGE and autoradiography analyses of the programmed reticulocyte lysates. Furthermore an identical pattern of immunoreactive polypeptides was also detected in a homogenate prepared from normal human liver (Figure 4).

Enzyme activities

Neither XDH nor XO activity was measurable in the reticulocyte lysates expressing the XDH/XO cDNA, suggesting deficient post-translational processing in this system.

In contrast, in lysates of COS-1 cells 72 h after transfection, significant XDH + XO activity was detected (specific activity 277 ± 54 pmol/min per mg of protein). The relative proportion of XO activity of total XDH + XO activity was 10.2 ± 0.3 % (the results are expressed as means $\pm$ S.D. from three separate transfection experiments, each measured in duplicate). In cells transfected with the SVpoly vector alone, or in control transfections without DNA, XDH + XO activities were below the detection limit of the assay (0.2 pmol/min per mg of lysate protein).

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                                                                                GGTACCTGGAGTTCGGGGACCCCAACCTGTGACA

ATG ACA GCA GAC AAA TTG GTT TTC TTT GTG AAT GGC AGA AAG GTG GTG GAG AAA AAT GCA 60
Met Thr Ala Asp Lys Leu Val Phe Phe Val Asn Gly Arg Lys Val Val Glu Lys Asn Ala 20

GAT CCA GAG ACA ACC CTT TTG GCC TAC CTG AGA AGA AAG TTG GGG CTG AGT GGA ACC AAG 120
Asp Pro Glu Thr Thr Leu Leu Ala Tyr Leu Arg Arg Lys Leu Gly Leu Ser Gly Thr Lys 40

CTC GGC TGT GGA GAG GGG GGC TGC GGG GCT TGC ACA GTG ATG CTC TCC AAG TAT GAT CGT 180
Leu Gly Cys Gly Glu Gly Gly Cys Gly Ala Cys Thr Val Met Leu Ser Lys Tyr Asp Arg 60

CTG CAG AAC AAG ATC GTC CAC TTT TCT GCC AAT GCC TGC CTG GCC CCC ATC TGC TCC TTG 240
Leu Gln Asn Lys Ile Val His Phe Ser Ala Asn Ala Cys Leu Ala Pro Ile Cys Ser Leu 80

CAC CAT GTT GCA GTG ACA ACT GTG GAA GGA ATA GGA AGC ACC AAG ACG AGG CTG CAT CCT 300
His His Val Ala Val Thr Thr Val Glu Gly Ile Gly Ser Thr Lys Thr Arg Leu His Pro 100

GTG CAG GAG AGA ATT GCC AAA AGC CAC GGC TCC CAG TGC GGG TTC TGC ACC CCT GGC ATC 360
Val Gln Glu Arg Ile Ala Lys Ser His Gly Ser Gln Cys Gly Phe Cys Thr Pro Gly Ile 120

GTC ATG AGT ATG TAC ACA CTG CTC CGG AAT CAG CCC GAG CCC ACC ATG GAG GAG ATT GAG 420
Val Met Ser Met Tyr Thr Leu Leu Arg Asn Gln Pro Glu Pro Thr Met Glu Glu Ile Glu 140

AAT GCC TTC CAA GGA AAT CTG TGC CGC TGC ACA GGC TAC AGA CCC ATC CTC CAG GGC TTC 480
Asn Ala Phe Gln Gly Asn Leu Cys Arg Cys Thr Gly Tyr Arg Pro Ile Leu Gln Gly Phe 160

CGG ACC TTT GCC AGG GAT GGT GGA TGC TGT GGA GGA GAT GGG AAT AAT CCA AAT TGC TGC 540
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TTC ACG CCC CTG GAT CCA ACC CAG GAG CCC ATT TTT CCC CCA GAG TTG CTG AGG CTG AAA 660
Phe Thr Pro Leu Asp Pro Thr Gln Glu Pro Ile Phe Pro Pro Glu Leu Leu Arg Leu Lys 220

GAC ACT CCT CGG AAG CAG CTG CGA TTT GAA GGG GAG CGT GTG ACG TGG ATA CAG GCC TCA 720
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ACC CTC AAG GAG CTG CTG GAC CTC AAG GCT CAG CAC CCT GAC GCC AAG CTG GTC GTG GGG 780
Thr Leu Lys Glu Leu Leu Asp Leu Lys Ala Gln His Pro Asp Ala Lys Leu Val Gly 260

AAC ACG GAG ATT GGC ATT GAG ATG AAG TTC AAG AAT ATG CTG TTT CCT ATG ATT GTC TGC 840
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CCA GCC TGG ATC CCT GAG CTG AAT TCG GTA GAA CAT GGA CCC GAC GGT ATC TCC TTT GGA 900
Pro Ala Trp Ile Pro Glu Leu Asn Ser Val Glu His Gly Pro Asp Gly Ile Ser Phe Gly 300

GCT GCT TGC CCC CTG AGC ATT GTG GAA AAA ACC CTG GTG GAT GCT GTT GCT AAG CTT CCT 960
Ala Ala Cys Pro Leu Ser Ile Val Glu Lys Thr Leu Val Asp Ala Val Ala Lys Leu Pro 320

GCC CAA AAG ACA GAG GTG TTC AGA GGG GTC CTG GAG CAG CTG CGC TGG TTT GCT GGG AAG 1020
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CTC AAC CCC GTG TTC ATG GCC AGT GGG GCC AAG CTG ACA CTT GTG TCC AGA GGC ACC AGG 1140
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TTC AAG CAG GCC TCC CGG AGA GAA GAT GAC ATT GCC AAG GTA ACC AGT GGC ATG AGA GTT 1320
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Glu Leu Leu Gln Asp Val Cys Ala Gly Leu Ala Glu Glu Leu His Leu Pro Pro Asp Ala 500

CCT GGT GGC ATG GTG GAC TTC CGG TGC ACC CTC ACC CTC AGC TTC TTC TTC AAG TTC TAC 1560
Pro Gly Gly Met Val Asp Phe Arg Cys Thr Leu Thr Leu Ser Phe Phe Phe Lys Phe Tyr 520

CTG ACA GTC CTT CAG AAG CTG GGC CAA GAG AAC CTG GAA GAC AAG TGT GGT AAA CTG GAC 1620
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CCC ACT TTC GCC AGT GCA ACT TTA CTG TTT CAG AAA GAC CCC CCA GCC GAT GTC CAG CTC 1680
Pro Thr Phe Ala Ser Ala Thr Leu Leu Phe Gln Lys Asp Pro Pro Ala Asp Val Gln Leu 560

TTC CAA GAG GTG CCC AAG GGT CAG TCT GAG GAG GAC ATG GTG GGC CGG CCC CTG CCC CAC 1740
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CTG GCA GCG GAC ATG CAG GCC TCT GGT GAG GCC GTG TAC TGT GAC GAC ATT CCT CGC TAC 1800
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ATA GAT ACA TCA GAA GCT AAG AAG GTT CCA GGG TTT GTT TGT TTC ATT TCC GCT GAT GAT 1920
Ile Asp Thr Ser Glu Ala Lys Lys Val Pro Gly Phe Val Cys Phe Ile Ser Ala Asp Asp 640

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Lys Lys Gly Phe Ser Glu Ala Asp Asn Val Val Ser Gly Glu Ile Tyr Ile Gly Gly Gln 740

GAG CAC TTC TAC CTG GAG ACT CAC TGC ACC ATT GCT GTT CCA AAA GGC GAG GCA GGG GAG 2280
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GGC AAG GAG ACC CGG AGC ACT GTG GTG TCC ACG GCA GTG GCC CTG GCT GCA TAT AAG ACC 2460
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CTG TGC AAA ACC AAC CTT CCC TCC AAC ACG GCC TTC CGG GGC TTT GGG GGG CCC CAG GGG 2760
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ATA ATT CCC ACC AAG TTT GGA ATA AGC TTC ACA GTT CCT TTT CTG AAT CAG GCA GGA GCC 3060
Ile Ile Pro Thr Lys Phe Gly Ile Ser Phe Thr Val Pro Phe Leu Asn Gln Ala Gly Ala 1020

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AGG GTG TCC CTG CTC CGC GAC TGC CCC AAC AAG AAG GCC ATC TAT GCA TCG AAG GCT GTT 3780
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Gly Val Pro Glu Asn Cys Lys Pro Trp Ser Val Arg Val *

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Figure 2 hXDH cDNA sequence and deduced amino acid sequence

The nucleotide differences and resulting amino acid changes with respect to the sequence reported by Ichida et al. [9] are shown in bold and underlined.

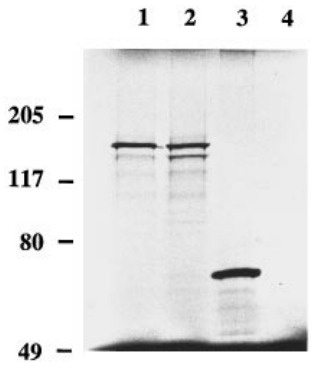

Figure 3 Autoradiograph of [^{35}S]methionine-labelled hXDH translation product in rabbit reticulocyte lysate

Lane 1, translation with protease inhibitors (see text) in the reaction; lane 2, translation without protease inhibitors; lane 3, control translation of luciferase cDNA (product approx. 61 kDa); lane 4, control translation without DNA. The positions of BioRad prestained high range standard sizes are indicated on the left in kDa.

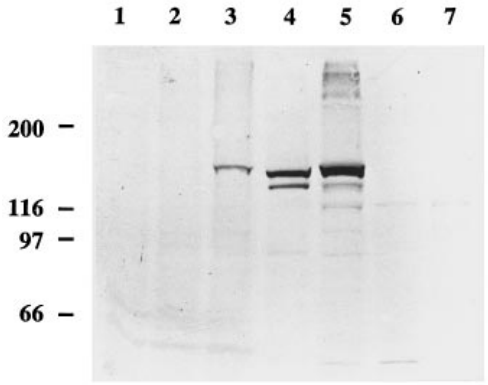

Figure 4 Western blot of hXDH/XO expressed *in vitro* by using polyclonal rabbit anti-hXDH antibodies

The first three lanes are from the rabbit reticulocyte lysate: lane 1, control reaction with no DNA added; lane 2, control reaction with luciferase cDNA; lane 3, hXDH cDNA; lane 4, human liver homogenate as positive control (150 μg of total protein). The next three lanes are lysates of transfected COS-1 cells: lane 5, SVpoly vector + hXDH cDNA; lane 6, SVpoly vector alone; lane 7, control transfection without plasmid. The positions of BioRad prestained high range standard sizes are indicated on the left in kDa.

DISCUSSION

The present data show that our hXDH cDNA clone codes for a protein with XDH/XO activity as well as immunoreactivity with anti-XDH antibodies. These data also show that monkey-derived COS-1 cells, but apparently not rabbit reticulocyte lysates, possess the mechanisms necessary to incorporate the requisite cofactors (Fe–S centres, flavin moiety and molybdopterin) in the human XDH/XO polypeptide chain as well as those needed to fold the protein into its proper functional form.

The nucleotide differences between our clone and that reported by Ichida et al. [9] are minimal; these cDNAs most probably represent products of the same gene. The differences at positions 572 and 691 could represent genetic polymorphism or could be due to artifacts arising from the reverse-transcription or PCR amplification used in both studies to obtain the 5′-end cDNA clones. In addition to these two sites, only one nucleotide change (at position 3449) would result in a change in the amino acid sequence. None of the predicted amino acid differences are in the cofactor or substrate binding regions of the molecule.

Although both Xu et al. [11] and our group have mapped the gene for XDH to the same locus, there were significant differences in these two cDNA sequences. Our approach to gene mapping was fluorescent hybridization *in situ*, whereas Xu et al. [11] have used a PCR-based approach for gene localization. At the region of their PCR primers (1816–1839 and 1862–1886) our sequences are identical. However, the nucleotide variation in other regions of the cDNA reported by Xu et al. [11] is rather extensive and therefore probably cannot be explained by polymorphism of a single gene. Two other potential mechanisms that could account for these dissimilarities are alternative exon usage and the presence of duplicate genes within the locus. However, because the sequences of all the cDNAs that we cloned from several different tissues were identical, these explanations seem unlikely. In any case, it will be interesting to see whether the expression of the cDNA cloned by Xu et al. [11] also produces a protein with properties of XDH/XO, and how the functional properties of this protein will compare with the enzyme that we have cloned. It is worth noting that at the region of the clone used by these authors for producing cRNA probes for Northern hybridization (bases 2605–3301), similarity to our sequence is remarkably low (approx. 85%). It is therefore possible that this could account for their unexpected finding of abundant mRNA expression in most of the tissues studied, whereas in other reports mRNA expression [12] as well as enzyme activity [19] have been observed predominantly only in the intestine and the liver.

The major differences between our XDH clone and that reported by Wright et al. [10] suggest two different genes, and the protein corresponding to their cDNA may be a related enzyme, e.g. another member of the family of molybdopterin-containing flavoproteins, as previously speculated [20,21]. Characterization of the protein coded by this cDNA should clarify this issue.

The proportion of XO activity in the total XDH + XO activity in transfected COS cells is in line with previous findings, in which tissue preparation was optimized to prevent conversion of XDH to XO [6] as an artifact. This 'constitutive' XO activity has earlier been shown to consist mainly of the irreversibly (proteolytically) converted enzyme rather than the conformationally altered dehydrogenase [6,22, 23]. Because we did not separately assay the reversible and irreversible XO forms, the ratio of XO activity to XDH activity cannot be compared with the relative abundance of the 150 and 130 kDa peptide bands. We were unable to block completely the production of the 130 kDa polypeptide by protease inhibitors, or to detect a putative approx. 20 kDa cleavage product. This suggests that other mechanisms, such as alternative translation initiation, might also be responsible for giving rise to the 130 kDa peptide. As the sequence flanking hXDH AUG^{+1-3} (UGACAAUGA) diverges substantially from the consensus sequence for optimal eukaryotic translation initiation [CC(A/G)CCAUGG] [24], 'leaky ribosomal scanning' of the mRNA could be envisaged. Other approaches will be necessary to explore whether, and at which site, XDH is proteolytically cleaved, and whether XO is a primary translation product of the XDH/XO gene.

Note added in proof (received 18 January 1996)

In a recently published erratum [25], Xu et al. report corrections to their cDNA sequence. The similarity between their corrected sequence (GenBank accession no. U06117) and the sequence reported in this paper is 99.6%.

We thank Dr. Lars Paulin at the Institute of Biotechnology, Helsinki, for valuable assistance in sequencing the cDNA clones, Dr. Anu Jalanko for helpful discussions and for providing the SVpoly vector, and Dr. Kalle Saksela for valuable comments on the manuscript. We also thank Ms. Ritva Löfman and Ms. Sari Linden for skilful technical assistance. This study was supported by the Foundation for Pediatric Research (M.S.), and by the Sigrid Juselius Foundation and the Academy of Finland (K.O.R.).

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Received 14 September 1995/16 November 1995; accepted 29 November 1995