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Human α-L-iduronidase: cDNA isolation and expression

(mucopolysaccharidosis type I/Hurler syndrome/lysosomal storage disorder/lysosomal hydrolase/alternative mRNA splicing)

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ABSTRACT **α-L-Iduronidase (IDUA; EC 3.2.1.76) is a lysosomal hydrolase in the metabolic pathway responsible for the degradation of the glycosaminoglycans heparan sulfate and dermatan sulfate. A deficiency of IDUA in humans leads to the accumulation of these glycosaminoglycans and results in the lysosomal storage disorder mucopolysaccharidosis type I. We have isolated and sequenced cDNA clones containing part of the human IDUA coding region and used PCR from reverse-transcribed RNA to obtain the full IDUA sequence. Analysis of the predicted 653-amino acid precursor protein shows that IDUA has a 26-amino acid signal peptide that is cleaved immediately prior to the amino terminus of the 74-kDa polypeptide present in human liver IDUA. The protein sequence contains six potential N-glycosylation sites. Northern blot analysis with IDUA cDNA detected only a single 2.3-kilobase mRNA species in human placental RNA; however, PCR analysis of fibroblast, liver, kidney, and placental RNA showed the existence of alternatively spliced mRNA from the *IDUA* gene. Southern blot analysis failed to detect major deletions or gene rearrangements in any of the 40 mucopolysaccharidosis type I patients studied. Expression of a full-length IDUA cDNA construct in Chinese hamster ovary cells produced human IDUA protein at a level 13-fold higher than, and with a specific activity comparable to, IDUA present in normal human fibroblasts.**

The lysosomal enzyme α-L-iduronidase (IDUA; glycosaminoglycan α-L-iduronohydrolase, EC 3.2.1.76) hydrolyzes the nonreducing terminal α-L-iduronide glycosidic bonds in the glycosaminoglycans heparan sulfate and dermatan sulfate (1, 2). IDUA has served as a model for processing and maturation events undergone by lysosomal enzymes (3–8). A deficiency of IDUA in humans results in the lysosomal storage disorder mucopolysaccharidosis type I (MPS-I; eponyms: Hurler, Hurler/Scheie, and Scheie syndromes), which is inherited as an autosomal recessive disease and shows wide variation of clinical presentation. Severely affected patients have mental retardation, somatic tissue complications, and a reduced life span, while mildly affected patients may have only mild somatic complications and a normal life span. Multiple different mutant alleles at the *IDUA* locus are thought to be responsible for the spectrum of clinical phenotypes (1, 9), but biochemical characterization of the residual IDUA activity has enabled discrimination only between the extremes of clinical phenotypes (10–12). The isolation of the *IDUA* gene was undertaken to provide a DNA probe for molecular analysis of mutations in MPS-I patients and for use in enzyme and gene therapy experiments in the canine model of MPS-I (13).

We have recently reported the immunopurification of the human IDUA enzyme as a mixture giving seven major bands, 74, 65, 60, 49, 44, 18, and 13 kDa, on an SDS/polyacrylamide gel, and we proposed that all were derived from a single precursor (7). The gene for human IDUA has been localized to chromosome 4p16.3 (14, 15). In this paper we report the isolation and sequencing of human IDUA cDNA clones and PCR products from reverse-transcribed RNA and the construction and expression of a clone that contains the entire coding region for human IDUA.† We also propose that six polypeptides of human liver IDUA are produced by proteolytic processing of the 74-kDa IDUA polypeptide. The isolation of a cDNA clone encoding canine IDUA was reported at a conference while this work was in progress (16).

MATERIALS AND METHODS

Polypeptide Isolation and Sequencing. All seven major polypeptides of IDUA (7) were directly sequenced from their amino termini as previously described (17). Tryptic peptide sequences from 150 μg of purified human liver IDUA were generated as previously described (18).

Oligonucleotides and Primers. All oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. ID47, 5′-AACTTCGAGACCTGGAACGAGCCCGACCAC-CACGACTTCGACAACGT-3′, designed from residue 2 to residue 17 of peptide 8 (see Fig. 2), was used for initial library screening. ID13, 5′-GCCCGGGCGGCA/GTCCACC/TTG-3′ (a mixture of four sequences; nucleotides separated by / are options at the same position), designed from residue 13 to residue 7 of the 74/13-kDa amino-terminal amino acid sequence (see Fig. 2), was used to screen Southern blots of the cosmid clone A157.1 (15). IDUA-specific primers used for PCR from cDNA were IDNT, ID39, ID56, ID57, ID58, ID60, and ID61 (see Fig. 2).

Library Screening. All libraries screened were of human origin and were purchased from Clontech. They were a leukocyte genomic DNA in EMBL3 (catalogue number HL1006) and the following cDNA libraries: colon (random primed, HL1034a), umbilical endothelial (HL1024b), umbilical endothelial 5′ stretch (HL1070b), and T-cell 5′ stretch (HL1068b). All libraries were plated at a density of between 40,000 and 55,000 plaques per 140-mm plate. The host cells used for each library were NM538 for the EMBL3 genomic library, C600 for the λgt10 cDNA libraries, and Y1088 for the λgt11 cDNA libraries. Probes were either labeled at the 5′ end (19) or labeled by primer extension of random oligonucleotide primers (20) and the Colony/Plaque*screen* filters (DuPont/NEN) were prehybridized, hybridized, and washed according to the manufacturer's instructions.

Sequencing. Specific oligonucleotides were made every 200–400 base pairs (bp) to sequence fragments in both directions fully (21). Compressed areas of G+C-rich se-

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Abbreviations: IDUA, α-L-iduronidase; MPS-I, mucopolysaccharidosis type I.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M74715).

quence were resolved by using 7-deazaguanosine (22). Direct PCR sequencing was by the linear PCR method (23).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from normal human placental, liver, and kidney tissue or cultured normal human fibroblasts as previously described (24). Poly(A)$^+$ RNA was obtained (25) from placental RNA and Northern blotting was carried out on 40 μg of total RNA and 10 and 40 μg of poly(A)$^+$ RNA as described (17).

cDNA Synthesis. Total RNA (3 μg) from normal fibroblasts was added to a reaction mix containing 1× Moloney murine leukemia virus (Mo-MLV) reverse transcriptase buffer (BRL), 40 units of RNAsin (Promega), 500 ng of random octamers, 0.5 mM deoxynucleotides (Boehringer Mannheim), and 200 units of Mo-MLV reverse transcriptase (BRL) to a final reaction volume of 50 μl. Incubation at 37°C for 1 hr was followed by hydrolysis of the RNA by the addition of 5 μl of 3 M NaOH and further incubation at 37°C for 30 min. The NaOH was neutralized by the addition of 1.25 μl of 10.3 M HCl, and the cDNA was precipitated and resuspended in 50 μl of water. Each PCR used 5 μl of cDNA.

PCR. PCR reagents were as described by Saiki *et al.* (26) except that the final concentrations of deoxynucleotides were 400 μM and 10% (vol/vol) dimethyl sulfoxide was present in the reaction mix. Forty cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and elongation at 72°C for 2 min were carried out. PCR products were analyzed on 4% (wt/vol) Nusieve GTG agarose (FMC) gels.

Construction of Full-Length IDUA cDNA. cDNA from a mixture of normal human fibroblast cell lines was used for PCR as described, using the primers ID60 and ID61. ID60 spans the initiating ATG codon and has a *Hin*dIII restriction site with a 4-bp GC clamp on the 5′ end. ID61 is ≈100 bp 3′ of a unique *Kpn* I restriction site (bases 818–823, see Fig. 2). Utilizing the *Hin*dIII and the *Kpn* I sites, we directionally cloned the PCR product in a pTZ19 vector that contained the rest of the IDUA coding sequence from the *Kpn* I site to the *Eco*RI cloning site of the clone λE8A. In all, 48 clones were analyzed and only one was found to be correct (full length). This insert was excised with *Hin*dIII and *Eco*RI and was directionally cloned in the expression vector pRSVN.07 (which drives expression of the insert from the Rous sarcoma virus long terminal repeat) to give pRSVNID21. This full-length IDUA cDNA insert was also subcloned in M13 and sequenced between the *Hin*dIII and *Kpn* I restriction sites, using IDUA-specific oligonucleotide primers to determine if any errors were present in the sequence.

Expression of IDUA. CHO (Chinese hamster ovary) cells (strain DK1) were grown in Ham's F12 medium (GIBCO), 10% (vol/vol) fetal calf serum (GIBCO), penicillin at 100 μg/ml, streptomycin sulfate at 100 μg/ml, and kanamycin sulfate at 120 μg/ml at 37°C in a 5% CO_2 (vol/vol) atmosphere. CHO cells (1.2×10^7) were electroporated at 0°C by using a BRL Cell-Porator at a pulse of 330 μF and 275 V in the presence 15 μg of pRSVNID21. Cells were grown in nonselective medium for 48 hr and then 1:20 and 1:100 dilutions of the electroporated cells were selected in G418 sulfate (Geneticin; GIBCO) at 750 μg/ml. A bulk culture of resistant cells was extracted (14) and assayed for IDUA activity with the fluorogenic substrate 4-methylumbelliferyl α-L-iduronide (Calbiochem) (6). The Bio-Rad protein assay was used to quantitate the amount of protein in each sample according to the manufacturer's instructions. The monoclonal antibody Id1A was used for immunocapture (14) and immunoquantification in conjunction with a polyclonal antibody (12) to assay the specific activity of the expressed IDUA (7).

RESULTS AND DISCUSSION

All seven polypeptides of IDUA were subjected to direct amino-terminal sequencing, and three different amino-terminal sequences were found to be present. The 65-, 60-, and 18-kDa species have a common amino-terminal amino acid sequence, the 49- and 44-kDa another, and the 74- and 13-kDa species another. Assuming that all seven species represent part of a single IDUA polypeptide, we propose a model (Fig. 1), which differs from that previously proposed (7), showing three sites of proteolytic processing of the 74-kDa polypeptide to produce the seven major species of IDUA.

After tryptic digestion and separation by HPLC (18) of immunopurified IDUA, nine major peptides were sequenced. One tryptic peptide was the same as the 65/60/18-kDa amino-terminal sequence, and one of the two tryptic peptide species present in peak 3 was contained within the 49/44-kDa amino-terminal sequence. Incorporating choices based on human codon usage and assuming that the undetermined amino acid at position 16 of peptide 8 was a glycosylated asparagine residue (see Fig. 2), we used the sequence to design a 47-mer oligonucleotide (ID47) for library screening.

Using ID47 as a probe, we screened 500,000 clones of the EMBL3 human genomic library and obtained 8 clones. A genomic clone, λID-475, was purified and an ID47-positive 1.6-kilobase (kb) *Pst* I fragment was subcloned in pUC19 to produce pID89 (14). This 1.6-kb insert was then used to screen a number of cDNA libraries; this screening yielded only 1 clone, which contained an insert of 729 bp (λRPC1, bases 541–1269; see Fig. 2) from the λgt10 random-primed human colon cDNA library. The sequence of this clone was colinear with six peptide sequences, including the 49/44-kDa amino-terminal sequence, but the clone ended within peptide 9.

The λRPC1 insert was then used to screen a λgt11 human endothelial cDNA library. Twenty clones were isolated, and the insert of the longest clone, λE8A, was fully sequenced. The 1765-bp insert contained an open reading frame starting just before the position of the 65/60/18-kDa amino terminus (base 391 in Fig. 2) to a stop codon (base 2048). Six further tryptic peptides were matched to the translated DNA sequence but, significantly, the sequence of the 74/13-kDa amino terminus, a secondary tryptic peptide (peptide 2′), a signal peptide, and an initiating methionine were not present in this clone. Of the other clones, 7 ended at the same base at the 5′ end, while all the others were shorter. A 5′ probe

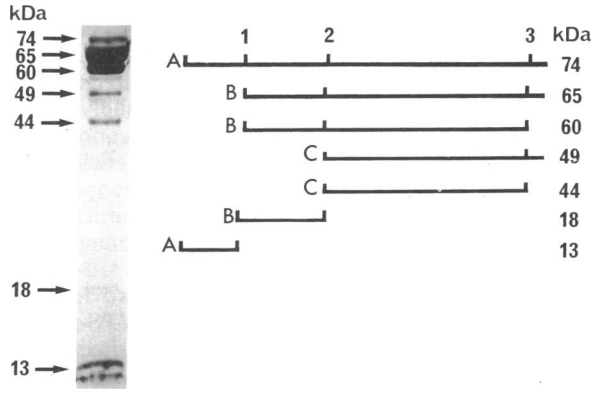

FIG. 1. A model to connect the seven major polypeptides in purified human liver IDUA present after SDS/PAGE as shown on the left, with the polypeptide sizes indicated in kDa (7). The three amino-terminal sequences present are represented by the letters A, B, or C next to the polypeptides. The proteolytic sites cleaved to produce the seven polypeptides from the 74-kDa polypeptide are numbered 1, 2, and 3.

derived from λE8A was used to screen another seven cDNA libraries. No clones were obtained from the screening of five of these cDNA libraries. Screening of two 5′ "stretch" cDNA libraries (umbilical endothelial and T-cell) resulted in a further 38 clones. PCR analysis of these clones showed that all ended at the same 5′ base as λE8A. Major secondary structures present in the IDUA mRNA may be responsible for the premature termination of these clones at their 5′ ends.

Using the polypeptide model for IDUA (Fig. 1), we hypothesized that the 74/13-kDa amino-terminal peptide sequence lay at the 5′ end of the IDUA mRNA. A mixed oligonucleotide, ID13, made to the 74/13-kDa amino-terminal sequence was used to probe Southern blots of the cosmid A157.1, which spans the area of the *IDUA* gene (15). A 2.8-kb *Bam*HI fragment was isolated and partially sequenced. The sequence contained an initiating methionine, a signal peptide, the 74/13-kDa amino terminus, and the start of the last unmatched tryptic peptide (peptide 2′ in Fig. 2). A number of oligonucleotides were made to this exon and used to PCR amplify normal fibroblast cDNA. A major PCR product was obtained between ID58 and ID61 and, using the oligonucleotides ID56 and ID57, was directly sequenced (23). The collated DNA sequence (Fig. 2) encodes a protein containing all amino-terminal and tryptic peptide sequences obtained from purified IDUA and is consistent with the model for IDUA (Fig. 1).

PCR of normal fibroblast cDNA at the 5′ end of the IDUA mRNA, using the oligonucleotides ID58 and ID61, produced a major product representing the sequence described (Fig. 2) and several minor products that also hybridized to an internal oligonucleotide, ID56 (data not shown). This indicates that the minor products were representative of alternative mRNA species from the *IDUA* gene, as has been reported for a number of other genes, including lysosomal hydrolases (27–29). We thought it important to identify the other IDUA mRNA species to determine which one encoded the active IDUA protein. The exon structure of *IDUA* has been determined (H.S.S., unpublished data). PCR of normal fibroblast cDNA using the oligonucleotide pairs ID56 to ID57 and IDNT to ID39 produced two products per reaction. The smaller products were isolated and directly sequenced; they showed alternative splicing of exons II and IV of *IDUA* (Fig. 3). The polypeptides from these alternatively spliced IDUA mRNA species would maintain the translation frame for the IDUA protein (see Fig. 3), leaving the primary sequence of the translated peptide identical to that of the deduced IDUA peptide except for the omission of 47 and 36 amino acids, respectively. Thus, the alternatively spliced mRNA species individually missing exons II and IV would produce peptide products of 606 and 617 amino acids, respectively.

Using the insert of λE8A as a probe against total placental RNA and poly(A)$^+$ RNA, we detected a single 2.3-kb band

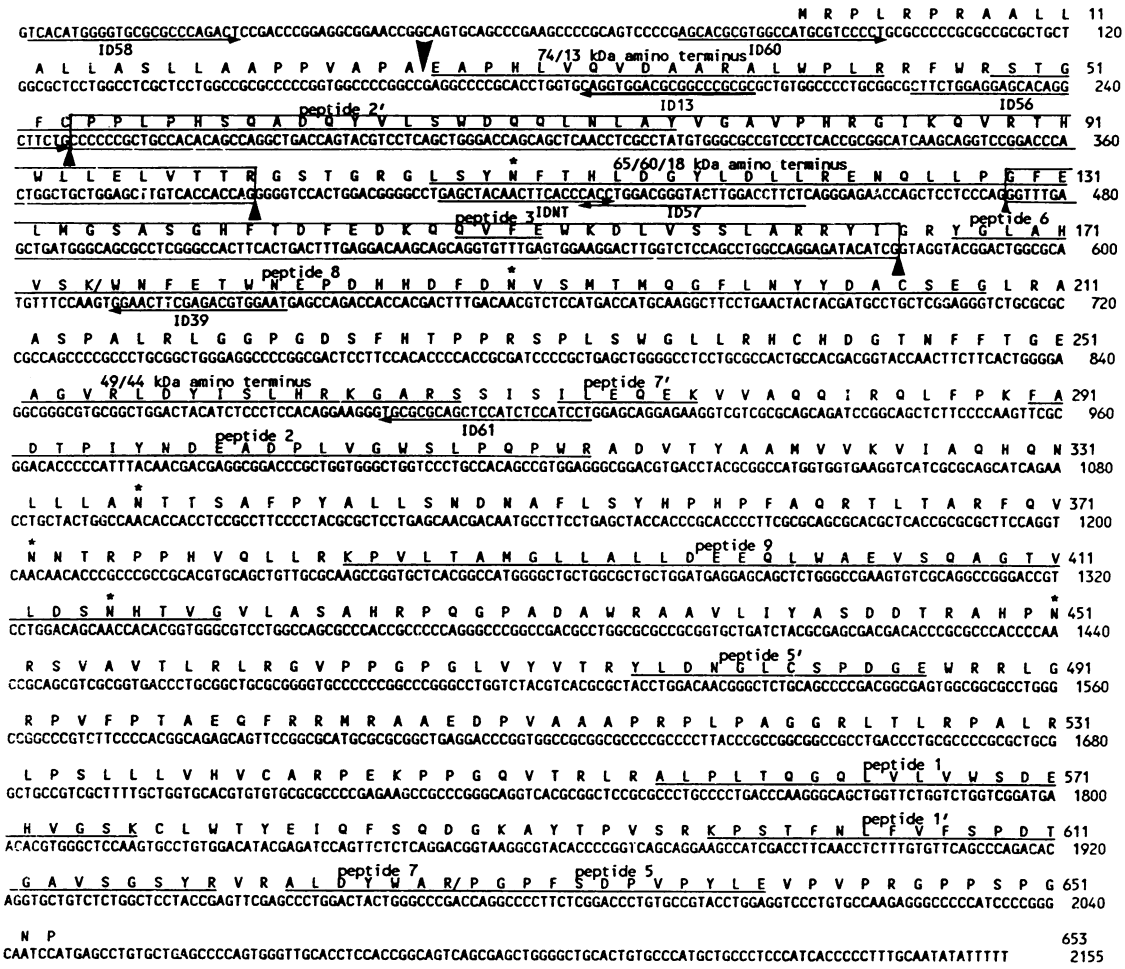

FIG. 2. Compiled nucleotide sequence for IDUA cDNA and the deduced amino acid sequence of the protein. The amino acid sequence is shown in single-letter code above the cDNA sequence. Nucleotide and amino acid numbers are in the right margin. The probable site of signal peptide peptidase cleavage is shown by a large arrow, and small arrows indicate exon junctions. Exons II and IV, which are alternatively spliced in some RNA transcripts, are boxed. Amino acids colinear with either amino-terminal peptide data or tryptic peptides are underlined and named above the sequence. Potential N-glycosylation sites are asterisked. Oligonucleotides used in this study are underlined below the nucleotide sequence with the arrows indicating either sense (→) or antisense (←). The cDNA clone λRPC1 extended from base 541 to base 1269 and λE8A extended from base 391 to the 3′ end of the sequence shown.

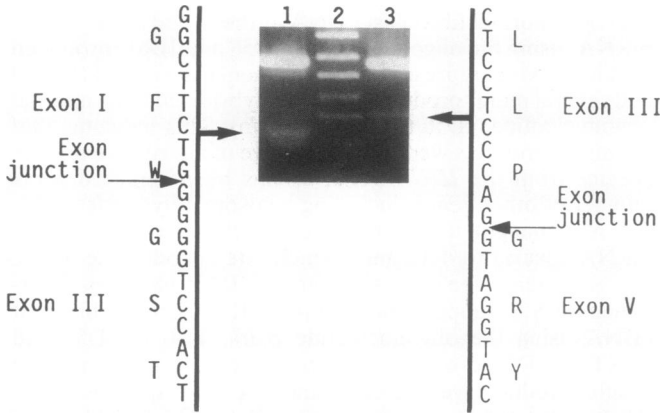

FIG. 3. PCR of reverse-transcribed normal fibroblast RNA, showing the alternative splicing of exons II and IV. Lane 1, PCR between ID56 and ID57, showing a major 225-bp product and a minor 84-bp product; lane 2, pUC19 *Hpa* II markers; lane 3, PCR between IDNT and ID39, showing a major 222-bp product and a minor 114-bp product. Partial sequences of the two minor products and their encoded amino acids are at the left and right of the figure. The position of the missing exon is indicated by the arrow labeled "Exon junction."

only when 40 μg of poly(A)$^+$ RNA was loaded in a single track. The strength of the signal also indicated that the mRNA for IDUA has a considerably lower abundance than the iduronate-2-sulfatase mRNA in placental RNA (16). Multiple PCR products of the same relative intensity were observed when reverse-transcribed liver, kidney, or placental RNA was used as template (data not shown), indicating that this splicing does not appear to be tissue specific and that these products may be minor mRNA species not detectable by Northern blot analysis. The alternative splicing of exon II introduces a tryptophan residue into the amino acid sequence at the splice junction, and the alternative splicing of exons II and IV both interrupt reported peptide sequences (peptide 2′ and the 65/60/18-kDa amino terminus of IDUA, respectively; see Fig. 2). Thus it was thought that the major PCR product was most likely to represent the full-length mRNA encoding IDUA. Expression of this putative full-length mRNA would establish that the nucleotide sequence presented here in Fig. 2 encodes enzymically active IDUA.

PCRs were performed with reverse-transcribed fibroblast RNA as template and the primers ID60 and ID61. The 840-bp PCR product was subcloned in the pTZ19 vector to produce a "full-length" IDUA cDNA clone. Sequence analysis of this full-length insert found four nucleotides that were different from the previously determined sequence. The differences, numbered as in Fig. 2, were A to C (base 276), G to A (base 402), T to C (base 440), and T to C (base 631). The first two differences alter the amino acid residues coded for by the cDNA from Gln to Pro (amino acid 63) and Arg to Gln (amino acid 105), respectively. The T to C (base 440) is a silent change that alters a Leu (amino acid 118) codon from TTG to CTG and introduces a second *Kpn* I site into the cDNA. Thus the cloned PCR product presumably resulted from partial digestion with *Kpn* I or the ligation of three fragments. The last change, T to C (base 631), is a silent change in the third base of an Asn (amino acid 181) codon. All of these differences may be polymorphic, but as two change amino acids, they may be transcription errors introduced by *Taq* DNA polymerase during PCR in the presence of high concentrations of dNTPs (400 μM) for 40 cycles (30). However, these conditions were essential to produce enough PCR product to conduct the experiment.

This full-length cDNA construct was subcloned in the expression vector pRSVN.07 to produce the construct pRSVNID21. CHO cells were electroporated in the presence of pRSVNID21, and G418-resistant colonies were selected and grown as a mass culture. Cellular extracts from control CHO cells, mixed normal human skin fibroblasts, and pRSVNID21-transfected cells were assayed for total IDUA activity by using the IDUA-specific fluorogenic substrate. CHO cell extract contained a low level of IDUA activity. Cellular extract from CHO cells transfected with pRSVNID21 gave a total activity 160-fold greater than the background (CHO cells) and 10-fold greater than the control normal human fibroblast activity (Table 1). To compare the specific activities of the recombinant and fibroblast IDUA, serial dilutions of the cellular extracts were assayed in parallel, using human IDUA-specific Id1A monoclonal antibody-based immunocapture (14) and ELISA assays (12). The CHO cell extract gave zero background in both assays. The ELISA result was normalized to the normal fibroblast extract and showed a 12.7-fold higher expression of human IDUA in the pRSVNID21 transfected CHO cells. The immunocapture assay showed that this results in an almost proportional increase in IDUA activity in the transfected CHO cells, demonstrating that the normal and recombinant enzymes have similar specific activities (Table 1). These results prove that the IDUA sequence used in this experiment codes for a protein that has a specific activity similar to the IDUA activity present in normal cultured human skin fibroblasts.

We therefore conclude that the total coding sequence for IDUA has an open reading frame of 1959 bp encoding a peptide of 653 amino acids. A signal peptide of 26 amino acids with a consensus cleavage site (31) was present immediately adjacent to the mature amino terminus of the protein (74/13-kDa amino terminus). Thus the mature human IDUA protein of 627 amino acids has a molecular mass of 70,029 Da, which is consistent with the previous estimates of IDUA size after allowing for post-translational modifications (5–8). All major peptide species sequenced are present in the translation of the open reading frame, totalling 234 amino acids (42%) of the 627 amino acids of the mature IDUA. This includes several peptides that were present as minor sequences in peptide peaks (secondary peptides—e.g., peptide 7′). The presence of all three amino-terminal sequences from purified human liver IDUA in the peptide sequence presented in Fig. 2 supports the hypothesized model of proteolytic processing of the 74-kDa IDUA polypeptide (Fig. 1). Of six potential glycosylation sites present (Fig. 2), the Asn of the potential sites in the 65/60/18-kDa amino-terminal sequence and peptide 8 was not detected in sequencing and may therefore be glycosylated. The potential glycosylation site at the very end of peptide 9 also was not defected, but this may be due to a weak signal towards the end of the sequence rather than a glycosylated residue. No significant homology was found between the human IDUA amino acid sequence and proteins in the GenBank, National Biomedical Research

Table 1. Expression of IDUA

Cell type	IDUA activity*		Relative IDUA protein†	Relative IDUA specific activity‡
	Total	Captured§		
CHO	1	ND	ND	—
CHO with pRSVNID21	160	152	12.7	12.0
Normal human fibroblasts	16	12.6	1	12.6

ND, none detected.
*Activity is in pmol × 10^{-2} per min per mg of cell protein.
†The amount of human IDUA protein captured in the ELISA assay per mg of cell protein normalized against human fibroblasts.
‡Expressed as IDUA activity relative to IDUA protein.
§IDUA activity captured in the immunocapture assay.

Foundation, or Swiss-Prot data bases (all releases of May 1991).

A cDNA for canine IDUA has been isolated; it codes for a protein of 617 amino acids with a signal peptide of 25 amino acids (16). Purified IDUA from canine testis contains two polypeptides, 68 and 63 kDa, and the canine 68-kDa and human 74-kDa amino termini both begin with Glu. The amino terminus of the canine 63-kDa species is Leu-106 corresponding to the human liver 65/60/18-kDa amino terminus of Leu-107. Thus, the mature canine IDUA is 35 amino acids shorter than the mature human IDUA in a region encoded 3′ to exon III of the human *IDUA* (Fig. 2). This may represent a difference between species, or the reported canine cDNA may be an alternatively spliced message that lacks the sequence of exon IV, or it may result from a yet-to-be-described alternative splicing event.

Alternatively spliced IDUA mRNA species may be splicing errors or they may produce IDUA "deletion" products with functions different than those of the full-length IDUA. It is possible that these IDUA "deletion" products have different substrate preferences compared with the full-length IDUA. Forms of IDUA with different substrate preferences have been reported (32, 33). It will be interesting to determine whether the translated peptides that result from the alternatively spliced IDUA mRNA have a function, and more importantly, whether they play any part in the pathology and various clinical phenotypes of MPS-I. Southern blot analysis of *Pst* I-digested DNA from 40 MPS-I patients with the full-length cDNA for IDUA did not detect any mutations (data not shown). The availability of the full-length cDNA sequence for human IDUA should make it possible to characterize MPS-I mutations and to determine how much of the clinical variability reflects different mutations and how much reflects other genetic or environmental influences. Furthermore, large-scale expression of IDUA should provide enzyme for evaluation of enzyme therapy in the dog model for MPS-I, and the cDNA in the appropriate vectors may be used for experimental gene therapy in the same model.

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