MOLECULAR CLONING AND CHARACTERIZATION OF UDP-GLUCOSE DEHYDROGENASE FROM THE AMPHIBIAN XENOPUS LAEVIS AND ITS INVOLVEMENT IN HYALURONAN SYNTHESIS*

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Running title: Xenopus UDP-glucose dehydrogenase and HA synthesis

UDP-glucose dehydrogenase (UDG) catalyzes an NAD+-dependent, twofold oxidation of UDP-glucose (UDP-glc) to generate UDP-glcUA (1). In mammals, UDP-glcUA is used in the biosynthesis of hyaluronan (HA) and the glycosaminoglycans (GAGs) heparan sulfate and chondroitin sulfate (2). In addition, it is used in the liver where glucuronidation targets molecules for excretion (3). UDP-glcUA also serves as a precursor to UDP-xylene, which provides a major component of the cell wall polysaccharides in plants (4), and represents the initial sugar in GAG synthesis on proteoglycans. In many strains of pathogenic bacteria, such as group A streptococci and Streptococcus pneumoniae type 3, UDP-glcUA is used in the construction of the antiphagocytic capsular polysaccharide (5,6). UGDH is of biochemical mechanistic interest because it belongs to a family of sugar nucleotide-modifying enzymes that catalyze a net four-electron oxidation and serve as both alcohol dehydrogenases and aldehyde dehydrogenases (7).

The importance of UGDH is remarkable considering that its product, UDP-glcUA, is critical for GAG synthesis. GAG chains of proteoglycans and HA are ubiquitous components of extracellular matrices (ECM) and pericellular spaces, and an increasing body of information shows the role of GAGs in cell behavior, including signal transduction, cell proliferation, spreading, migration, and cancer growth and metastasis (8-10).

During development proteoglycans and HA have important roles (11). As UGDH synthesizes UDP-glcUA, one of the main UDP-sugar precursors, it is not surprising that alteration in UGDH...
expression causes evident phenotypes in developing embryos, as found by different authors in different model organisms. In particular in *Drosophila melanogaster*, UGDH is encoded by the *sugarless* gene and is required for heparan sulfate modification of proteins that control wing formation (12). In *C. elegans*, UGDH influences GAG synthesis, which is essential for vulval morphogenesis and embryonic development (13,14). In zebrafish, the enzyme is critical for normal cardiac development (15). In mouse, UGDH mutants arrest growth during gastrulation with defects in migration of mesoderm and endoderm (16). Moreover, a similar phenotype was found in mutants in the fibroblast growth factor pathway, highlighting that proteoglycans and GAGs facilitate signalling by mammalian growth factors.

Another well known model organism extensively used in developmental biology is the amphibian *Xenopus laevis*. Although several papers report the critical role of proteoglycans in *X. laevis* development (17-22), very limited information is available on GAG functions in amphibian embryogenesis (23-25). We have, therefore, characterized the *X. laevis* UGDH (xUGDH), the key enzyme in GAG biosynthesis in this model organism convenient for developmental studies. In this paper, we report the cDNA cloning of xUGDH, its biochemical kinetic parameters, its expression in developing embryos, and its genome organization. We have also extended our observations to *X. tropicalis*, an organism of the same genus as *X. laevis*, but with interesting potential for genetic studies (26).

**MATERIALS AND METHODS**

**xUGDH cloning -** A *X. laevis* EST data bank was searched for homologies with human UGDH cDNA (GenBank accession number NM_003359) using the World Wide Web based BLAST search engine (http://www.ncbi.nlm.nih.gov/blast/). IMAGE EST number 4202110 was found and was obtained from MRC geneservice (Cambridge, UK). DNA sequencing was done by an external service facility (BMR, Padua, Italy).

**Characterization of the xUGDH gene -** Introns of the *X. laevis* xUGDH gene were amplified with a pair of primers (Table 1) designed on the basis of mouse and human UGDH genomic sequences deposited in public data bases. PCR parameters were the following: denaturation at 94 °C for 30 s, annealing for 30 s at the temperature indicated in Table 1, elongation at 72 °C for 5 min for 35 cycles using 1.5 U of La-TAQ (a proofreading DNA polymerase from Takara) following the manufacturer’s conditions. Amplification of intron 8 was done using Phusion polymerase (Finnzymes) in 6% DMSO. PCRs were done on genomic DNA. Amplified intron lengths were determined by gel electrophoresis, and exon/intron boundaries were determined by sequencing the extracted bands from the gels.

**In silico** determinations of the intron/exon structure of the human, mouse and *X. tropicalis* UGDH genes were done by BLAST searches on the genomic databases. Sequence manipulations and contig constructions were done using Vector NTI Suite 6 software.

**Recombinant xUGDH expression and purification -** Cloning and transformation techniques were done essentially as described by Sambrook et al. (27). A histidine tagged xUGDH construct was generated by polymerase chain reaction (PCR). Briefly, the xUGDH ORF was amplified using Phusion DNA polymerase (Finnzymes) with the following primers: 

CCAAGGCTCGAGATGTTTCAGATTAAGAA

GATTT and

CCTTGGCTCGAGTTAAACTCTTTGTTTCTTATGAGGC (the sequences corresponding to Xho I recognition sites are underlined). The amplified product was digested with Xho I (Takara) and cloned into the Xho I linearized pET-19b (Novagen) plasmid. This construct (pHisxUGDH) was then sequenced; it encodes the full-length xUGDH protein with an additional six His-tag sequence at the N-terminus. For protein expression, the plasmid pHisxUGDH was transferred to the host BL21(DE3)pLysS *E. coli* strain (Promega). *E. coli* cells carrying the recombinant plasmid were cultivated at 37 °C in LB medium containing ampicillin and chloramphenicol (100 µg/ml and 34 µg/ml final concentrations, respectively). After an overnight growth (OD600nm>2.5), isopropyl-L-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM. The temperature was reduced to 30 °C and the cells collected after 24 h.

Cell extraction and His-tagged xUGDH purification were done using the B-TER...
purification kit (Pierce) following the manufacturer’s instructions. Some determinations were done on crude extracts that were prepared lysing the bacterial cells in B-Ter solution (Pierce) and recovering the supernatant after centrifugation.

xUGDH assay - The enzymatic activity of purified His-tagged xUGDH was determined by monitoring the change in absorbance at 340 nm that accompanies reduction of NAD⁺ to NADH. The assay conditions were the same as those previously described by Sommer et al. (28).

Gene expression studies - Total RNA from 21 X. laevis embryos (7 from three different females) at different stages of development were extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol. One µg of total RNA from each extract was retrotranscribed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a dT(16) primer at a concentration of 500 µg/ml. The reaction was done in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 500 µM of a dNTP mix at 42 °C for 50 min. PCR amplifications of the cDNA samples were done using the first strand cDNA synthesis mix, 25 pmole of primer xUGDH up (CCCTTTGTGAGGCTACAGGA) and xUGDH low (CGGTGCAGATAACCATAGCA), 200 µM dNTPs and 1 U RedTaq polymerase (Sigma) in its own buffer. Reaction mixtures were subjected to 8 touchdown cycles with annealing temperatures from 58 to 54 °C and subsequently with cycles using the following parameters: denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, elongation at 72 °C for 40 s. As a control for genomic DNA contamination, all reactions were established with the control sample lacking reverse transcriptase. Normalization was done detecting cytoskeletal actin (upper primer: CTGAGTTCATGAAGGATACAGCA, lower primer: AAATTTACAGGTGTACCTGC) (29) with the above described conditions.

Quantitative Real-Time RT-PCR was used in aortic smooth muscle cells (AoSMC) transfection experiments (see below). Forty-eight hours after transfections, total RNA samples were extracted with Trizol (Invitrogen) and retrotranscribed using the High Capacity cDNA synthesis kit (Applied Biosystems) for 2 h at 37 °C. Quantitative RT-PCR was performed on an Abi Prism 7000 instrument (Applied Biosystems) using the Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. Probe and primers were developed from TaqMan gene expression assay reagents (Applied Biosystems). The following human TaqMan gene expression assays were used: HAS1 (Hs00155410_m1), HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), UGDH (Hs00163365_m1), and β actin (Hs99999993_m1). Fluorescent signals generated during PCR amplifications were monitored and analyzed with ABi Prism 7000 SDS software (Applied Biosystems). Comparisons of the amounts of each gene transcript among different samples were made using β actin as a reference. Standard curves were generated by serial dilution of cDNA, and, as calculations of PCR efficiency were very similar (about 90%) for each gene assayed, the relative quantitative evaluation of target gene levels was determined by comparing ΔCt (PE Applied Biosystems user bulletin number 2).

xUGDH expression in mammalian cells - The xUGDH ORF was amplified using Phusion DNA polymerase (Finnzymes) with the following primers:

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TACTCGAGACCATGTTCAGATTAAGAAGATT
TACTCGAGTTAAACTCTTTTGTTTCTTAAG
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The bold sequences correspond to Xho I recognition sites while the underlined adenosine represents the typical -3 purine of the Kozak consensus sequence. The amplified product was purified, A-tailed using RedTaq (Sigma) and cloned into a pTarget (Promega) vector. Expression plasmids were selected to have the insert in the sense orientation in order to synthesize the complete xUGDH protein (pTarget-xUGDH-sense) or to have the insert in the opposite direction used as the control vector (pTarget-xUGDH-antisense). DNA sequencing and in vitro transcription and translation (TNT in vitro transcription/translation system, Promega) were done to check the constructs. AoSMCs (Cambrex) were grown in SmGm2 complete (supplemented with 5% FBS) culture medium (Cambrex). The cultures were maintained in an atmosphere of humidified 95% air/ 5% CO₂ at 37 °C. 1 x 10⁶ cells between passages 2-5 were transiently transfected by means of a Nucleofector apparatus (Amaxa) and the Human Aortic Smooth Muscle Cells Nucleofector™ Kit using 5 µg of either the sense or antisense expression plasmid. After 48 h, transfected cells and conditioned cell culture media were collected. Cells were lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% triton X-100 supplemented with a protease inhibition
cocktail (Roche) using a cell scraper. Protein contents in the cell lysates were determined using the Bradford method, and UGDH activities were measured as described above.

Abrogation of human UGDH - Small interfering RNA (siRNA) was used to reduce human UGDH expression in AoSMCs. UGDH siRNA (5'-GGACUAAAAGAAGUGUAGGtt-3') and negative control siRNA #1 kit (scramble, code 4611) were purchased from Ambion. Transfections were done using a Nucleofector apparatus (Amaza) and the Human AoSMC Nucleofector™ Kit using a siRNA concentration of 50 µM of either UGDH siRNA or scramble siRNA. After 48 h of incubation, conditioned cell media were assayed for GAG contents by FACE analysis. The transfected cells were used for determination of UDP sugars contents and UGDH activities. Quantitative Real-Time RT-PCR was used to verify the reduction of UGDH mRNA expression.

GAG disaccharides and UDP-sugar precursors determination - HA and chondroitin sulfate disaccharides were determined by fluorophore assisted carbohydrate electrophoresis (FACE) analysis as previously described (30). UDP-glc and UDP-glcUA were quantified by capillary zone electrophoresis using the methodology outlined by Lehmann and colleagues (31). Briefly, 106 AoSMCs were lysed in 0.1 ml of PBS containing 0.5% Triton X-100. After centrifugation, the supernatants were deproteinized by acetonitrile treatment, lyophilized and resuspended in 90 mM borate buffer pH 9. Analyses were done using a 75 cm x 50 µm column, 25 kV voltage and detecting UDP sugar absorbances at 262 nm. The peak identity was assessed co-injecting the extracts with commercial UDP-glc and UDP-glcUA.

GAG determinations - To evaluate the amount of each GAG after induction or abrogation of UGDH, AoSMCs were transfected with pTarget-xUGDH constructs or siRNA, respectively, and labeled with [3H]glucosamine (25 µCi/ml) for 48 h in complete SmGm2E medium. Conditioned cell medium was recovered and the unincorporated [3H]glucosamine was removed by gel filtration using a PD10 column (Amersham). The cell layer was washed with PBS and scraped into 100 µl of PBS containing 1% Triton X-100, centrifuged at 10000g for 10 minutes at 4°C. The clear supernatant and the PD10 filtrated medium were digested with Proteinase-K (Finnzymes) at 50°C. After 8 h of incubation, Proteinase-K was inactivated at 90°C for 10 minutes. Each GAG solution was separated into four aliquots, and precipitated by adding 4 volumes of ethanol at -20°C for 18 h, and recovered by centrifugation. Two aliquots were resuspended in 0.1 M ammonium acetate pH 7 and digested with 100 mU/ml of hyaluronidase SD from Streptococcus dysgalactiae (Seikagaku) and 100 mU/ml of chondroitinase ABC (Seikagaku), respectively; one was resuspended in 10 mM Tris HCl pH 7, 4 mM CaCl2 and digested with 100 mU/ml of heparanase I, II, and III (Seikagaku). The last aliquot was used as an undigested control (reference). After 16 h of incubation at 37 °C, which allow complete degradation of 20 µg/ml of standard GAGs, 50 µg of chondroitin sulfate A (Seikagaku) was added to each digested sample as carrier and undigested GAGs were precipitated with ethanol. Specific GAGs were quantified by counting the radioactivity associated with digestion products (in the supernatant) and the undigested GAGs (pellet) with a liquid scintillation counter (Canberra Packard).

xUGDH silencing during X. laevis development - Two antisense morpholino oligonucleotides (MOs; Gene Tools LLC) were generated on the basis of the xUGDH cDNA sequence: xUGDH MO CATGGTTTATCTTGCTGAGAACAGA, is complementary to the xUGDH translation start site and the adjacent coding sequence. A 5-mismatch MO, based on the xUGDH MO sequence, was used as a specificity control (CATcGTTTATgTTGgTGAcAAgAGA, where small caps indicate mismatched nucleotides). The optimal morpholino concentration to injected per embryo was established by independent pilot experiments (not shown) and was determined to be in the range of 10-20 ng/embryo. The MOs were injected bilaterally at the two-cell stage in order to down regulate the xUGDH activity in the whole embryo. All the experiments were performed by coinjecting the morpholino oligonucleotide and the GFP mRNA (300 pg/embryo) as a tracer, in order to be able to select, for the subsequent analysis, only the properly manipulated embryos. Embryos were injected in 0.1 x Modified Marc’s Ringer solution (MMR; 100 mM NaCl, 1.8 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM HEPES, pH 7.8) supplemented with 4% Ficoll and then cultured in 0.1 x MMR solution until the stage of analysis.
RESULTS AND DISCUSSION

xUGDH cDNA cloning - In this study we focused our attention on UGDH, a pivotal enzyme in the production of UDP-glCUA, an important precursor for GAG synthesis. It has been reported that UGDH is crucial in C. elegans and D. melanogaster morphogenesis (13,32), zebrafish cardiac valve formation (33) and also involved in mouse gastrulation (16). We previously reported the characterization of HA synthases (HAS1, HAS2, and HAS3) in X. laevis and showed their regulation during early stages of development (34, 35). It is also possible that UGDH, which provides one of the substrates for HAS, is regulated in the embryogenesis of this species. Therefore, we decided to clone X. laevis UGDH and characterize it.

To obtain the sequence of the X. laevis UGDH (xUGDH) mRNA, we initially performed a BLAST search using the human UGDH sequence against a X. laevis EST database. This revealed several ESTs with a high degree of identity with the human sequences. Then, we obtained the largest EST (dbEST id: 4202110) from MRC geneservice (Cambridge, UK) and derived the complete sequence deposited in the public database with GenBank accession number AY762616 (data not shown). This sequence has a 42 bp long 5’ UTR and a 1482 bp long ORF coding for a protein with 494 amino acids and a calculated molecular mass of 55,209.4 Da and a calculated isoelectric point of 6.49. A PROSITE analysis (http://www.expasy.org/prosite/) and a NetPhos analysis (http://www.cbs.dtu.dk/services/NetPhos/) of the xUGDH amino acid sequence revealed several putative phosphorylation sites. Although we did not further investigate the possibility of post translation modifications in this enzyme, previous studies have reported that UGDH may be phosphorylated in prokaryotic cells (35,36).

We searched for known signal peptides using the PSORT II program (http://www.psort.org/) without identifying any sorting sequence. xUGDH localization was predicted to be cytoplasmic with a 65.2% probability. Such a localization is consistent with a previous report that placed UGDH in the cytosol (13).

The deduced amino acid sequences of xUGDH and other known UGDH from several species are aligned (data not shown). The N-terminal region has the consensus sequence for NAD binding (i.e., GXGXXG) that is maintained with a high degree of identity among all the UGDH analyzed. The central region of the protein (i.e., GFGGSCFQKDVLN) has been proposed to be the catalytic domain (38), and all the aligned sequences show a high degree of identity with only a minor amino acid substitution in the UGDH belonging to C. elegans, D. melanogaster and soybean. Moreover the critical cysteine residue, number 276 of xUGDH, is conserved in all sequences, and it has been shown to be involved during the second half reaction of UGDH (i.e., conversion of UDP-aldehydoglucose to UDP-glCUA) (28). With respect to function/structure considerations, proline residues at positions 92 and 160 are believed to represent main bends in the protein structure (39-40), and they are conserved for UGDH from all species. Two lysine residues at positions 220 and 339 are also conserved, which correspond to lysine 219 and 338 of bovine UGDH. One of these lysine residues is probably catalytically involved in the first half reaction of the enzyme (i.e., conversion of UDP-glC to UDP-aldehydoglucose) (39,42). Moreover, using Conseq analysis (freely available at the Internet site http://conseq.bioinfo.tau.ac.il/) (43), regions spanning from amino acid 41 to 54, 128-135, 340-350 and 461-464 have been identified as important for structure or function of the protein (result not shown). Although this analysis is only a virtual prediction, it was done on all the known UGDH sequences deposited in the databases. Such regions could be useful to understand not only the catalytic mechanism of the reaction, but also to identify critical residues involved in an hypothetical regulatory mechanism that a pivotal enzyme such as UGDH could possess. Recently, for example, Conseq analysis has been successfully used to isolate important regions for protein-protein interactions (44).

Genomic organization of xUGDH - By comparing the xUGDH transcript to the human and mouse genomic sequences available in public databases, we have inferred the positions of the introns of the xUGDH gene. For each hypothetical intron, we have designed, on the two flanking exons, a pair of specific primers (Table 1) to amplify by long amplification PCR a region expected to contain the intron. PCR products were separated by gel electrophoresis, and the exon-intron junctions were sequenced. As reported in Table 2A and B, the structure of the xUGDH gene is composed of 11 exons and 10 introns, and it matches that of mammalian UGDH genes (45). Notably, in the 5’ untranslated region of the human and mouse UGDH mRNA, we and others (45) found an additional intron of about 5700 bp. As this intron is located far upstream from the ATG start codon,
we did not investigate further its position in xUGDH gene. The major variability was detected in intron lengths, although all of them conserve their intron phase, indicating that the intron insertion points in the cDNA sequence have remained constant during xUGDH gene evolution. Interestingly, the consensus gt-ag (5'- 3') splice site sequences are not always conserved in the xUGDH gene as shown in the 5' splice acceptor sequence of intron 5. However, this non-canonical-splice site has been reported to be quite frequent and does not cause any deleterious mutations (46,47). Moreover, this non-canonical-splice site may be the main 5' intron acceptor site used during embryogenesis. In fact, no alternative splice isoforms of xUGDH transcripts have been found during X. laevis development by RT-PCR using two primers (i.e., xUGDH up and xUGDH low) flanking the intron 5 insertion point in the cDNA sequence. X. laevis is certainly a well recognized ‘model organism’ for cell and developmental biology, but it has the disadvantage of being tetraploid, which greatly complicates the creation of mutants and the analysis of gene regulation. In contrast, X. tropicalis, an amphibian of the same genus, is diploid and possesses a relatively small genome, whose sequencing is currently in progress. Therefore, X. tropicalis is destined to complement X. laevis as a model organism (26). Thanks to available preliminary data on the X. tropicalis genome (http://www.genome.jgipsf.org), we have determined the X. tropicalis UGDH genomic organization (Table 2B), and, as expected, the two genes are indeed very similar.

Recombinant xUGDH expression and characterization - To study the biochemical properties of xUGDH, we have generated the pHixxUGDH plasmid that codes for a six Histidine-tagged xUGDH. This plasmid was used to transform E. coli BL21(DE3)pLysS cells, and the recombinant protein expression was induced by IPTG treatment. Experiments were done to optimize the expression conditions, analyzing the effect of temperature, time of induction and collection, and IPTG concentration on xUGDH expression. The best conditions were obtained using E. coli cells grown overnight at 37 °C, induced with 1 mM IPTG, cultured at 30 °C and harvested 24 h after induction. The recombinant xUGDH overexpressed under these conditions was completely soluble and thus fully recovered in the crude extract. The specific UGDH activity in the crude extract of induced E. coli BL21(DE3)pLysS was about 50 fold higher than that of crude extracts of uninduced cells (data not shown).

The crude extract from IPTG-induced E. coli cells was affinity purified using a commercial spin column kit (Pierce). After the three elution steps suggested by the manufacturer, recombinant His-tagged xUGDH was eluted as a nearly single resolved band, and the final preparation was at least 90% homogeneous as judged by SDS-PAGE analysis (result not shown). The 54 kDa band corresponding to recombinant His-tagged xUGDH is in agreement with the molecular weight theoretically calculated from the translated xUGDH cDNA sequence. After the affinity chromatography step, the specific activity increased about twelve fold (data not shown).

As UGDH reduces two molecules of NAD to NADH during the conversion of a UDP-glc substrate to a UDP-glcUA, we followed the reaction by measuring NADH absorbance at 340 nm. To obtain kinetic constants ($K_m$ and $V_{max}$) for substrate and cofactor, we used the same experimental buffers and conditions previously described (28). Moreover, we did not remove the six His tagged sequence as it has been reported that it does not alter the property of the enzyme (28). The purified enzyme showed no reduction of NAD if incubated with either UDP-galactose, UDP-glcUA or UDP-N-acetylglucosamine indicating the purity and specificity of the enzyme (result not shown).

The steady-state kinetic parameters of His-tagged xUGDH have been calculated by incubating the enzyme with increasing concentrations of NAD in the presence of saturating UDP-glc substrate (Fig. 1A). Similarly, the dependence of reaction kinetics on the substrate was measured by increasing UDP-glc concentration in the presence of saturating NAD (Fig. 1C). For both substrates, saturation kinetics were observed. A double-reciprocal plot of the initial velocities revealed a $K_m$ of 0.9 mM for UDP-glc (Fig. 1B) and a $K_m$ of 0.3 mM for NAD (Fig. 1D). Both sets of conditions yielded a similar $V_{max}$ of about 10 nmol of NADH/min/mg of enzyme. These data are comparable to those of UGDH from other species (Tab 3).
number of embryos at different developmental stages. To visualize differences in the expression, different PCR cycles were done as outlined in Figure 2. Although the 531 bp long band corresponding to the amplicon of xUGDH was detectable in all the tested developmental stages, a more pronounced signal was visualized in the sample from 30 h post fertilization (hpf) that corresponds to an embryo at the tailbud stage. Such an expression pattern is related to the crucial function of UDP-glcUA. In fact, this molecule is a precursor for all GAGs (with the exception of keratan sulfate) including GAGs of proteoglycans and HA that are known to be essential for a proper development (48). Interestingly, the increase in xUGDH in the 30 hpf embryos corresponded with our previous finding that HA is dramatically elevated in embryos at this developmental stage (34). Moreover, the enzymes involved in HA synthesis (i.e., HAS2 and HAS3) are also up-regulated in this embryonic stage (34). Although the precise role of HA in X. laevis development is still to be defined, HA is critical during embryonic development of mammals. For example, mice unable to produce HA die in uterus from severe cardiac malformation (49,50).

**xUGDH expression and HA synthesis** - The data reported in this study strongly support the hypothesis that coordinated expression of UGDH and HAS may have a pivotal role in regulating HA levels. To elucidate the relationship between UGDH and HA synthesis and accumulation, we transfected human primary aortic smooth muscle cells (AoSMCs) with a xUGDH expression plasmid pTarget-xUGDH-sense. We also transfected the plasmid pTarget-xUGDH-antisense that did not code for any protein as a control. We hypothesized that an increase of UGDH activity that augments UDP-glcUA synthesis may increase the synthesis of HA. As shown in Figure 3A, UGDH activity increased in AoSMCs transfected with pTarget-xUGDH-sense while cells transfected with pTarget-xUGDH-antisense maintained a lower UGDH activity comparable with that of untransfected cells. The increment of UGDH activity of pTarget-xUGDH-sense transfected cells was in agreement with that reported for other mammalian cell lines transfected with mouse or bovine UGDH (42,51). To verify that an UGDH activity increase could actually increase UDP-glcUA, we quantified the UDP-glcUA:UDP-glucose ratios by capillary zone electrophoresis (Fig. 4). AoSMCs transfected with pTarget-xUGDH-sense, pTarget-xUGDH-antisense vectors, or not transfected showed UDP-glcUA:UDP–glucose ratios of 4, 0.8 and 0.7, respectively. This clearly indicates that the increase of UGDH activity augmented the cellular UDP-glcUA levels by about 4-fold with respect to control levels.

The conditioned culture media of the AoSMC cultures were collected after 48 h of transfection, and their HA concentrations were measured by FACE analysis (Fig. 3B). AoSMCs produce high levels of HA (52,53). Interestingly, the HA disaccharide band derived from 10^6 pTarget-xUGDH-sense transfected AoSMCs was significantly stronger than that derived from the same number of control cells (i.e., transfected with pTarget-xUGDH-antisense or not transfected). The UGDH activity detected in control cells (Fig. 3A) could explain the basal HA signals in control cell culture medium (Fig. 3B). On the other hand, the increase of HA production in pTarget-xUGDH-sense transfected cells could be ascribed to the augmented UGDH activity in those cells. A similar result was obtained by Magee and collaborators (54) who discovered that the up-regulation of UDP glucose pyrophosphorylase stimulates HA synthesis in hypertrophic chondrocyte cultures. As UDP glucose pyrophosphorylase and UGDH belong to the same pathway that forms UDP-glcUA, it would be reasonable that the up-regulation of both of these enzymes can increase the concentration of HA synthesis precursors. Moreover, the \( K_m \) values of the three HA synthases are in the range of the cytoplasmatic concentration of UDP sugars (55) making the activity of HA synthetic enzymes responsive to the concentrations and the pool sizes of the cellular sugar nucleotides. Interestingly, densitometric quantification of the HA and the chondroitin 4 sulfate (CS-4S) disaccharide bands obtained from FACE analyses revealed that CS-4S synthesis did not change significantly after the xUGDH transfections (result not shown) indicating that the UGDH effect was selective for HA. Although UGDH is a central enzyme in the metabolism of GAGs, it may specifically regulate HA accumulation without altering other GAGs. In light of this specific HA increase, we performed quantitative RT-PCR analyses on transfected AoSMC cDNA and found that HAS2 and HAS3 mRNA levels were up-regulated about 2-fold only in pTarget-xUGDH-sense transfected cells while HAS1 mRNA levels were unchanged (Fig. 3C). This suggests that transfection of UGDH leads to an induction of HAS2 and HAS3 gene transcription or to a stabilization of HAS2 and HAS3 mRNAs. The specific increase of HAS2 and HAS3 fits well...
with the specific increase of HA in transfected AoSMCs (Fig. 3B).
The lack of increase in chondroitin sulfate may result from the fact that precursors for this GAG must enter the Golgi apparatus using several UDP-sugar transporters that have been shown to possess low $K_m$ values for their substrates (i.e., 1-10 µM) (56,57). While cytoplasmic UDP-sugar precursor concentrations could directly affect the activity of HA synthases located in the cell membrane, the UDP sugar transporters would be saturated at the cellular concentration of the UDP-sugar precursors thereby maintaining constant concentrations inside the Golgi apparatus and thus the constant activity of the Golgi GAG synthetic enzymes. The tight relationship between UGDH activity and GAG synthesis was recently outlined also in experiments in vivo with fibroblast-like synovial lining cells (58), and in vitro on immature and mature human articular cartilage explants (59), although the key importance of UGDH activity in GAG production was already suggested years ago (60,61).

Abrogation of human UGDH and HA synthesis - In order to demonstrate the putative link between UGDH activity and HA synthesis, we did siRNA experiments to reduce the endogenous UGDH in human AoSMCs. The greatest reduction of human UGDH mRNA was achieved by transfecting 50 µM of UGDH siRNA and incubating for 48 h. In these conditions quantitative RT-PCR showed a reduction of the human UGDH transcript of about 90% with respect to the controls (i.e., scramble siRNA and no DNA transfected cells) (Fig. 5A). Such a reduction of UGDH mRNA corresponded to a strong inhibition of UGDH activity. In fact, we were not able to detect any signals in the UGDH spectrophotometric assay used in this work indicating that the residual UGDH activity was below the detection limit of the assay (result not shown). To verify that the UGDH activity decrease could actually reduce UDP-glucUA contents, we quantified the UDP-glucUA:UDP-glucose ratios (data not shown). AoSMCs transfected with UGDH siRNA, scramble siRNA, or not transfected showed UDP-glucUA:UDP-glucose ratios of 0.25, 1.0 and 0.8, respectively clearly indicating that the inhibition of UGDH activity reduced the cellular UDP-glucose levels by 4-fold with respect to control levels. Further, the concentrations of HA in the conditioned culture media clearly showed a corresponding decrease (Fig. 5B) indicating that HA synthesis could be strictly regulated by the availability of the UDP sugars precursors at least in AoSMCs. This finding supports the proposed mechanism of the 4-methylumbelliferone- inhibition of HA synthesis; in fact the glucuronidation of 4-methylumbelliferone by endogenous UDP-glucuronyltransferase, induces a depletion of UDP-glucUA (62).

Moreover, chondroitin 4 sulfate was not influenced by changing availability of the precursors (see above). Interestingly, the inhibition of UGDH by siRNA did not change the levels of transcripts coding for HAS1, HAS2 and HAS3 quantified by quantitative RT-PCR (Fig. 5C). These data indicated that the expression of these enzymes cannot be reduced below a basal level even when one of their substrates is strongly decreased.

GAG determinations – To better elucidate the UGDH role in the control of GAG synthesis, we incubated AoSMCs with [3H]glucosamine in order to determine the GAG amount. We found that the total (i.e., medium and cell layer) incorporated radioactivity associated with GAGs was much higher in pTarget-xUGDH-sense transfected cells than in the controls (i.e., 0.16 cpm/cell versus 0.12 cpm/cell, respectively). On the other hand, the incorporated radioactivity associated with GAGs was lower in siRNA against UGDH treated cells than in the controls (0.06 cpm/cell versus 0.12 cpm/cell, respectively). The silencing of UGDH affected dramatically the GAG associated to the cell layer fractions in which the radioactivity incorporated in the GAG was undetectable. These data support the critical role of UGDH in promoting GAG synthesis.

Moreover, we quantified the percentage of the specific GAG family in conditioned medium (Tab. 4A) as well as the cell layer (Tab. 4B) samples. The determinations were performed counting the radioactivity in GAG resistant to hyaluronidase SD, chondroitinase ABC and Heparinase I, II, III digestions as described in Material and Methods section. For the percentage calculations, the total radioactivity which was digested was considered 100%.

The quantifications were done on untransfected AoSMCs (Tab 4A and B, row control) or transfected with pTarget-xUGDH-sense (Tab. 4A and 4B, row sense) or with siRNA against human UGDH (Tab. 4A and B, row RNA UGDH). We found that HA is the most produced GAG in the medium as well as in the cell layer. Interestingly, when we overexpressed the xUGDH we found a significant increase in both medium and cell associated HA content while the synthesis of other GAGs were only slightly modified (Tab. 4A and
4B). On the other hand, when we abolished the expression of UGDH in AoSMC by siRNA we found a decrease of medium HA without altering the synthesis of other released GAGs whereas in the cell layer associated GAGs the incorporated radioactivity was under the detection limit (Tab. 4A and 4B) indicating that cell surface associated GAGs could have a more rapid turnover in comparison to GAGs secreted into the medium. Interestingly, as the sum of percentages indicated in the table rows represents the GAG associated radioactivity, it is noteworthy that in the medium fractions almost 100% of the radioactivity was GAG associated while in the cell fractions the radioactivity accounted for about 80%. This value reached about 100% when UGDH was induced and this increase (~20%) was specifically caused by HA synthesis.

As observed from our previous results (Fig. 3B and 5B), we showed that HA is the GAG that is more sensitive to UDP-glcUA concentration inside the cytoplasm and, therefore, the cell could regulate specifically the HA synthesis controlling the UDP-glcUA availability.

In vivo xUGDH silencing - X. laevis embryos were injected bilaterally with xUGDH MO complementary to the 5'UTR of xUGDH mRNA in order to prevent its translation. Control and xUGDH MO treated embryos were coinjected with mRNA coding for the green fluorescent protein (GFP) allowing the selection of only those embryos injected in both sides, before the subsequent analysis. Embryos treated with xUGDH MO had severe malformations at the end of gastrulation that lead to a failing in the blastopore closure. In the subsequent developmental phases xUGDH MO injected embryos are not able to complete the neurulation step correctly as the neural tube remained posteriorly opened (90% of injected embryos n=120) (Fig 6A). In contrast control embryos (injected with the same dose of the 5-mismatch MO) showed a normal development (100% of injected embryos n=130) (Fig 6B). The embryos progressively died and did not reach the tailbud stage. It has been shown that during gastrulation, both in X. laevis and mouse embryos, mesodermal cells enter and move within a HA-rich environment (63,64). Moreover, the HAS2 is expressed in the involuting mesoderm in X. laevis embryos (35) and appears to be critical in the zebrafish gastrulation process (65). Therefore our xUGDH knock down functional data are consistent with a role of HA in the gastrulation movements and suggest an important role of the xUGDH activity during early X. laevis development.

In conclusion, we have reported the cloning of the UGDH cDNA sequence from the amphibian X. laevis and found that the gene structure and biochemical properties confirmed data obtained in other species. We described an up-regulation of xUGDH mRNA during the tailbud stage of X. laevis development that was correlated with the elevated HA production typical of the developing embryo at this stage. We also demonstrate a critical role of xUGDH activity in Xenopus early development. Functional down regulation of xUGDH, in fact, results in early embryonic lethality. Moreover, we reported that an increase in UGDH may be responsible for HA accumulation at least in an in vitro cellular model. These results support the hypothesis that the synthesis of HA requires the activation of a complex mixture of enzymes, not only the synthetic proteins, but also the enzymes involved in metabolism of the UDP-sugar precursors.

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FOOTNOTES

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Xenopus laevis UDP-glucose dehydrogenase cDNA complete sequence has been deposited in GenBank database under the accession number AY762616.

The abbreviations used are: UGDH, UDP-glucose dehydrogenase; HA, hyaluronan; ECM, extracellular matrix; GAG, glycosaminoglycan; UDP-glc, UDP-glucose; UDP-glcUA, UDP-glucuronic acid; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase(s); HAS, HA synthase(s); EST, expressed sequence tag; UTR, untranslated region; MO, morpholino.

FIGURE LEGENDS

Table 1
Sequence of the primers used in this work to amplify the indicated intron.

Table 2
Structure of the xUGDH gene.
A. Schematic representation of the xUGDH gene. Lines indicate introns; filled boxes exons in the open reading frame; open boxes exons in the 5' and 3' UTRs. Dotted lines and box represent the exon and the intron of the human UGDH gene located in the 5' UTR (not characterized in the *X. laevis* gene).

B. Intron sizes were determined by gel electrophoresis, and position numbering is based on exon coding sequences where A of the ATG start codon is +1. Uppercase letters are exonic sequences, and lowercase letters are intronic sequences. n.d., not determined. *Hs*, *Homo sapiens*; *Mm*, *Mus Musculus*; *Xl*, *Xenopus laevis*; *Xt*, *Xenopus tropicalis*. Analyses were done on the basis of sequences AC021148 and AC108506 for human and mouse, respectively. The *X. tropicalis* UGDH genomic sequence was derived from sequence scaffold 10698, 7440 and AASO427206.b1.

**Table 3.**
Comparison of $K_m$ values (expressed in mM) of UGDH from different species.

**Table 4A**
Conditioned medium GAGs content in AoSMCs.
AoSMCs (control) or AoSMCs transfected with either pTarget-xUGDH-sense (sense), or pTarget-xUGDH-antisense (antisense), scramble siRNA (scramble), or siRNA against human UGDH (siRNA UGDH) were treated as outlined in the Material and Methods section. GAG contents are reported in percent of the total radioactivity digested in the sample. The total GAG column represents the percentage of the total radioactivity sensitive to enzymatic digestions. The reported data are the mean of two independent experiments.

**Table 4B**
Cell associated GAGs content in AoSMCs.
AoSMCs (control) or AoSMCs transfected with either pTarget-xUGDH-sense (sense), or pTarget-xUGDH-antisense (antisense), scramble siRNA (scramble), or siRNA against human UGDH (siRNA UGDH) were treated as outlined in the Material and Methods section. GAG contents is reported in percent of the total radioactivity in the sample. nd not detected. The total GAG column represents the percentage of the total radioactivity sensitive to enzymatic digestions. The reported data are the mean of two independent experiments.

**Fig. 1.** Kinetics of xUGDH using variable amount of UGDH with a saturation quantity of NAD (10 mM) (A), or variable amounts of NAD with a saturation quantity of UGDH (5 mM) (C). $K_m$ values for UGDH and NAD were calculated by the method of Lineweaver and Burke for data show in B and D, respectively. Thirty micrograms of purified xUGDH from fraction E3 (Fig. 3) were used for each determination. The plots represent the mean of three independent experiments.

**Fig. 2.** Expression of the mRNAs coding for xUGDH during embryogenesis at the indicated hours post fertilization (hpf) or days post fertilization (dpf). Different RT-PCR cycles were used to visualize an increase in xUGDH expression at 30 hpf. Cytoskeletal actin was used as a reference. Bottom panel, quantitative densitometric analysis (mean ± S.D.) of gels from two independent experiments. xUGDH:β-actin ratio at 0 hpf was fixed as 1.

**Fig. 3.** A. UGDH activities in AoSMC extracts transfected with pTarget-xUGDH-sense (sense), pTarget-xUGDH-antisense (antisense) or without DNA (control). xUGDH activities are expressed in mmol NADH min$^{-1}$ mg protein$^{-1}$. Transfections were done in triplicate, and data are expressed as mean +/- standard error (SE). B. FACE analyses on conditioned culture media of AoSMCs transfected as described above. ∆HA, HA disaccharide; ∆CS-OS, Chondroitin disaccharide; ∆CS-4S, Chondroitin 4 sulfated disaccharide; ∆CS-6S, Chondroitin 6 sulfated disaccharide. The analyses were done in triplicate, and one representative result is shown. C. Quantification of mRNA coding for HAS1, HAS2 and HAS3 by RT-PCR in AoSMCs transfected with pTarget-xUGDH-sense (sense), pTarget-xUGDH-antisense (antisense) or without DNA (control) after 48 h of incubation. Transfections for gene expression analyses and the quantifications were repeated three times with identical results both using β-actin (shown) or GAPDH (not shown) as reference genes. Unpaired Student’s t-test was performed for statistical analyses using Origin 7.5 software (Microcal Software). Gene expression is expressed in arbitrary units.
Capillary zone electropherogram showing the UDP-Glc and UDP-GlcUA separations. (A) Separation of standard (70 µM) of UDP-Glu and UDP-GluA in 90 mM borate buffer pH 9. (B) Separation of extracts from AoSMC transfected with pTarget-xUGDH-sense vector. (C) Separation of extracts from AoSMC transfected with pTarget-xUGDH-antisense vector. AoSMC extracts were prepared after 48 h from transfection. In the cellular extracts, arrows precisely indicate the UDP-glc and UDP-glcUA peaks.

Fig. 5. A. UGDH mRNA quantification by RT-PCR in AoSMCs transfected with no siRNA (control), a scramble siRNA (scramble) or siRNA against human UGDH (siUGDH). Transfections were done in triplicate, and data are expressed as mean +/- standard error (SE). B. FACE analyses of conditioned culture media of siRNA AoSMCs transfected as described above (see Fig. 3 legend for disaccharides designations). The analyses were done in triplicate, and one representative result is shown. C. Quantification of mRNA coding for HAS1, HAS2 and HAS3 by quantitative RT-PCR in AoSMCs transfected with no siRNA (control), a scramble siRNA (scramble) or siRNA against human UGDH (siUGDH). Transfections for gene expression analyses and the quantification were repeated three times with identical results using both β-actin (shown) or GAPDH (not shown) as reference genes. Gene expression is expressed in arbitrary units.

xUGDH down regulation during early X laevis development. A. Dorsal view of representative X. laevis embryos at late neurula stage (20 hpf) injected with xUGDH Mo. The neural tubes remain posteriorly opened as indicated by the arrows. Control embryos at the same developmental stage, injected with the control morpholino (xUGDH mismatched oligo) have normally completed the neurulation step and show in B. The distribution of the injected morpholino oligos is visualized by GFP fluorescence as shown in A’ and B’.
| Intron | Primer      | Sequence 5'-3'                                      | Annealing temp. °C |
|--------|-------------|----------------------------------------------------|-------------------|
| Intron 1 | XEso1_up    | CGTGATGCCCACAATGTGTC CAGCTTCTGTATAGCTCCGTCT        | 53                |
|        | XEso2_low   |                                                    |                   |
| Intron 2 | XEso2_up    | GCCGAGGCAAGAATCTGTCTT ATCAAATATCCGCGAATGC          | 52                |
|        | XEso3_low   |                                                    |                   |
| Intron 3 | XEso3_up    | TGCCAGAAGGATTGCTCCAAT TCACATAGTCACGCACAGC          | 53                |
|        | XEso4_low   |                                                    |                   |
| Intron 4 | XEso4_up    | ACCGGGTGTTATATCCTGGGTGTT ATTCATCTCGCCTCTG          | 53                |
|        | XEso5_low   |                                                    |                   |
| Intron 5 | XEso5_up    | ATGCTTTCTGTGCGAGAGA TTGCGACTTTCTGACGTTTC          | 53                |
|        | XEso6_low   |                                                    |                   |
| Intron 6 | XEso6_up    | GCTGTCTCTGCAAAGGATGTCTTG TGCAAAAAACAGGAGACCA       | 53                |
|        | XEso7_low   |                                                    |                   |
| Intron 7 | XEso7_up    | CAAGGATTATCGATTGCTGTTT CCTTGGAAACTTTGGGATCA        | 53                |
|        | XEso8_low   |                                                    |                   |
| Intron 8 | XEso8_up    | CCTCATGGATGAAAGTGGCA CCATGGTGTCAGATTACA           | 54                |
|        | XEso9_low   |                                                    |                   |
| Intron 9 | XEso9_up    | GCTGGTTCTACATTCTACCGATT TATTTTGACGCTCCGAAAGTA     | 53                |
|        | XEso10_low  |                                                    |                   |
| Intron 10| XEso10_up   | AGAATGTATGGTGAGCCAGCA GCAGGTCTGGCAAACCGAAT         | 53                |
|        | XEso11_low  |                                                    |                   |
| INTRON | SPECIE | EXON INTRON BOUNDARY SEQUENCE | LENGTH | PHASE | POSITION |
|--------|--------|-------------------------------|--------|-------|----------|
| 1      | Hs     | ATTTATGAGghtaacata tctttttagCCAGGACTA | 7166   | 0     | 162      |
|        | Mm     | ATTTATGAGghtaaactat cttcctctagCCTGGGATTA | 1327   | 0     | 162      |
|        | Xt     | ATTTATGAGghtaatgcc tttttacagCCTGGGTTG | nd     | 0     | 162      |
|        | Xi     | ATTTATGAGghtaatgacc tcttcacagCCTGGGTTG | 1700   | 0     | 162      |
| 2      | Hs     | TTTATTTTCgtgtaagtatt ctacatcagGTGAATACT | 3221   | 0     | 264      |
|        | Mm     | TTTATTTTCgtgtaagtatt tctcactagGTGAACACA | 391    | 0     | 264      |
|        | Xt     | TTTATTTTCgtgtaagtatt tattgacagGTCAACACT | 87     | 0     | 264      |
|        | Xi     | TTTATTTTCgtgtaagntc tctttcagGTCAACACT | 96     | 0     | 264      |
| 3      | Hs     | AATTTACAGgtataaaaa ggattctagGTGCTGTCC | 110    | 0     | 465      |
|        | Mm     | AATTTACAGgtatgaaga ctgttctagGTGCTGTCC | 91     | 0     | 465      |
|        | Xt     | AATTTACAGgtatagaact atctcactagGTGCTGTCT | 297    | 0     | 465      |
|        | Xi     | AATTTACAGgtatagaact tcttcacagGTGCTGTCT | 1000   | 0     | 465      |
| 4      | Hs     | TCCAAACTGgttagtata ttttttagGCAGCAAAT | 445    | 0     | 663      |
|        | Mm     | TCCAAACTGgttagtata tcttttaagGCAGCAAAT | 378    | 0     | 663      |
|        | Xt     | TCCAAACTGgttagtata ttttttagGTGCTGTCC | 86     | 0     | 663      |
|        | Xi     | TCCAAACTGgttagtata ttttttagGCAGCAAAT | 400    | 0     | 663      |
| 5      | Hs     | CCAGTGTTGghtaatccta tttctgcaagGTGTGGTG | 1099   | 1     | 811      |
|        | Mm     | CCAGTGTTGghtaatccta tttcttctagGTGTGGTG | 2291   | 1     | 811      |
|        | Xt     | CCAGTGTTGghtaatccta ttttttagGTGCTGTCC | 531    | 1     | 811      |
|        | Xi     | CCAGTGTTGghtaatccta atatagctagGTGCTGTCC | 1300   | 1     | 811      |
| 6      | Hs     | TGCCAGCAAggtattaatc tttctactagGGAATCTTC | 2816   | 2     | 1037     |
|        | Mm     | TGCCAGCAAggtattaatc tatttttagGTGCTGTCC | 1640   | 2     | 1037     |
|        | Xt     | TGCCAGCAAggtaticaat ttttttagGTGCTGTCC | 448    | 2     | 1037     |
|        | Xi     | TGCCAGCAAggtaticaat ttttttagGTGCTGTCC | 750    | 2     | 1037     |
| 7      | Hs     | TGATACAAGgtacagtgggct tttctatatagGGAATCTTC | 247    | 2     | 1037     |
|        | Mm     | TGATACAAGgtacagtgggct ttttctctagGGAATCTTC | 833    | 2     | 1037     |
|        | Xt     | TGATACAAGgtacagtgggct ttttttagGTGCTGTCC | 426    | 2     | 1037     |
|        | Xi     | TGATACAAGgtacagtgggct ttttttagGTGCTGTCC | 750    | 2     | 1037     |
| 8      | Hs     | ATGACCAAGgtaaggcttg gtttttagatGTAATGGGC | 728    | 1     | 1171     |
|        | Mm     | ATGACCAAGgtaaggcttg ttttttagGTGCCAGAC | 398    | 1     | 1171     |
|        | Xt     | ATGACCAAGgtaaggcttg ttttttagGTGCCAGAC | 905    | 1     | 1171     |
|        | Xi     | ATGACCAAGgtaaggcttg ttttttagGTGCCAGAC | 2300   | 1     | 1171     |
| 9      | Hs     | ATGGTTAAAGgtaaggcttg ttttttagatGAAATGGGA | 431    | 0     | 1263     |
|        | Mm     | ATGGTTAAAGgtaaggcttg ttttctagatGAAATGGGA | 85     | 0     | 1263     |
|        | Xt     | ATGGTTAAAGgtaaggcttg ttttttagatGAAATGGGA | 967    | 0     | 1263     |
|        | Xi     | ATGGTTAAAGgtaaggcttg ttttttagatGAAATGGGA | 1400   | 0     | 1263     |
| 10     | Hs     | GGCTTCCAGgtaatcagtttttagatGTAACACA | 3621   | 0     | 1374     |
|        | Mm     | GGCTTCCAGgtaatcagtttttagatGTAACACA | 2761   | 0     | 1374     |
|        | Xt     | GGCTTCCAGgtaatcagtttttagatGTAACACA | nd     | 0     | 1374     |
|        | Xi     | GGCTTCCAGgtaatcagtttttagatGTAACACA | 2000   | 0     | 1374     |
TAB. 3

| Species   | $K_m$ NAD | $K_m$ UDP-glc | Ref.   |
|-----------|-----------|---------------|--------|
| *X. laevis* | 0.3       | 0.9           | this work |
| *H. sapiens* | 0.35      | 0.1           | 28     |
| *G. gallus*  | 0.9       | 0.5           | 66     |
| *C. elegans* | 0.2       | 0.2           | 13     |
| *E. coli*    | 1         | 0.05          | 67     |
| *S. pyogenes* | 0.06      | 0.02          | 68     |
|                  | ΔHA Medium | ΔCS Medium | ΔHS Medium | Total GAG % |
|------------------|-----------|-----------|-----------|-------------|
| Control sense    | 77        | 13        | 9         | 99          |
| antisense        | 83        | 9         | 7         | 99          |
| siRNA scramble   | 78        | 13        | 9         | 100         |
| siRNA UGDH       | 79        | 12        | 8         | 99          |
|                  | 68        | 14        | 10        | 92          |
|                  | ΔHA Cell | ΔCS Cell | ΔHS Cell | Total GAG % |
|------------------|----------|----------|----------|-------------|
| Control sense    | 40       | 6        | 30       | 76          |
| antisense        | 62       | 8        | 27       | 97          |
| siRNA scramble   | 44       | 5        | 30       | 79          |
| siRNA UGDH       | nd       | nd       | nd       | 0           |
FIG. 1
FIG. 2
FIG. 3
FIG. 4
FIG. 5
FIG. 6
Molecular cloning and characterization of UDP-glucose dehydrogenase from the amphibian Xenopus laevis and its involvement in hyaluronan synthesis
Davide Vigetti, Michela Ori, Manuela Viola, Anna Genasetti, Eugenia Karousou, Manuela Rizzi, Francesco Pallotti, Irma Nardi, Vincent C. Hascall, Giancarlo De Luca and Alberto Passi

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