UDP-glucose pyrophosphorylase: up-regulation in hypertrophic cartilage and role in hyaluronan synthesis

Cordula MAGEE, Maria NURMINSKAYA and Thomas F. LINSENMAYER[1]

Department of Anatomy and Cellular Biology, Tufts University Medical School, 136 Harrison Avenue, Boston, MA 02111, U.S.A.

Previously, we have performed subtractive hybridization to identify genes up-regulated in hypertrophic chondrocytes of the avian epiphyseal growth plate. In the present study, we report the identification of one of the clones as UDP-glucose pyrophosphorylase (UDPG-PPase) and propose a possible function for this enzyme in regulating hyaluronan (HA) synthesis in hypertrophic cartilage. We have cloned the 2.6 kb full-length cDNA for avian UDPG-PPase and confirmed its up-regulation in hypertrophic versus non-hypertrophic cartilage by Northern-blot analysis. The 6-fold increase in mRNA was paralleled by an equivalent increase in enzymic activity. The enzyme catalyses the conversion of glucose 1-phosphate into UDP-glucose, which is used to synthesize a number of cellular components, including HA. Overexpression of enzymically active UDPG-PPase in non-hypertrophic chondrocytes resulted in a 2–3-fold increase in total HA, as determined by a competitive binding assay and immunohistochemistry. In the developing growth plate, HA synthesis was elevated in the hypertrophic zone along with the up-regulation of the HA synthase (HAS)-2 gene. Our data suggest that an increase in both activities, UDPG-PPase and HAS-2, is required for non-hypertrophic chondrocytes to synthesize an amount of HA comparable with that in hypertrophic chondrocytes. Therefore we conclude that HA synthesis during chondrocyte differentiation is regulated at the level of the substrate-provider gene, UDPG-PPase, as well as the HAS genes.

Key words: chondrocyte, extracellular matrix, glycosaminoglycan, hyaluronan synthase.

INTRODUCTION

During endochondral bone formation, long bones are first formed as cartilage, which then becomes replaced by bone and/or a marrow cavity. The cartilage-to-bone transition involves several sequential steps based on the proliferative activity and morphological appearance of the chondrocytes, with young immature chondrocytes undergoing a phase of rapid proliferation, followed by maturation, hypertrophy and removal [1]. These morphological changes are accompanied by changes in their biosynthetic repertoire, resulting in alterations in the composition of the cartilage extracellular matrix. For example, non-hypertrophic chondrocytes synthesize and secrete large amounts of collagen type II, whereas hypertrophic chondrocytes have switched to producing collagen type X [2].

Previously, we [3] performed subtractive hybridization between non-hypertrophic and hypertrophic chondrocytes to identify genes that are up-regulated in the zone of hypertrophy. In the present study, we describe the isolation and further characterization of one of these genes, namely that encoding UDP-glucose pyrophosphorylase (UDPG-PPase), and examine its possible role in chondrocyte differentiation during embryonic development.

UDPG-PPase is the enzyme that catalyses the reversible conversion of glucose 1-phosphate into UDP-glucose. UDPG-PPase is a ubiquitously expressed protein and a necessary component of the cell's metabolic machinery, with a lack of the enzyme leading to cell death and lysis, as has been shown for the slime mould *Dictyostelium discoideum* [4]. The enzymic activity of UDPG-PPase can vary greatly between different tissues, with liver, for example, having a much higher enzymic activity than brain [5]. UDPG-PPase can also be differentially expressed during development. In *D. discoideum*, the enzyme becomes up-regulated 10-fold during fruiting body formation [6], with this up-regulation involving both RNA and protein synthesis.

The catalytic product of UDPG-PPase, UDP-glucose, can be used in the synthesis of a variety of molecules, such as glycogen, glycoproteins, glycolipids, proteoglycans and glycosaminoglycans, including hyaluronan (HA). The use of UDP-glucose is tissue dependent. In the lactating mammary gland, for example, UDP-glucose is converted into UDP-galactose for lactose synthesis, whereas in the liver, it is largely used for glycogen synthesis or is converted into UDP-glucuronic acid [7].

HA is present in most vertebrate tissues, particularly in the extracellular matrices or pericellular spaces in skin and the tissues of the musculoskeletal system. It is actively synthesized during embryonic development, in tissue repair and in regeneration processes, and is thought to have regulatory functions in cell growth, migration and differentiation (reviewed in [8,9]). In addition, HA serves as a space-filling molecule and lubricant in synovial joints. Of more relevance to the current study is its presence in cartilage as a linking component in the cartilage proteoglycan (aggrecan) complex [10,11], and as a component of the pericellular matrix surrounding the lacunae of hypertrophic chondrocytes [12,13].

HA synthesis requires two more enzymes in addition to UDPG-PPase. These are UDP-glucose dehydrogenase (UDPG-DH) and HA synthase (HAS). In bacteria, the genes encoding these enzymes are organized as the polycistronic HAS operon [14]; in eukaryotic organisms, it is unclear how expression of these genes is regulated and possibly co-ordinated.

In the present study, we have cloned UDPG-PPase from chicken chondrocytes and found it to be up-regulated in hypertrophic versus non-hypertrophic cells. This up-regulation of UDPG-PPase occurs along with an increase in the expression

Abbreviations used: AP, alkaline phosphatase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, hyaluronan; HAS, HA synthase; b-HABP, biotinylated HA-binding protein; PFA, paraformaldehyde; poly(A)$^+$, polyadenylated; RACE, rapid amplification of cDNA ends; RT, reverse transcription; UDPG-DH, UDP-glucose dehydrogenase; UDPG-PPase, UDP-glucose pyrophosphorylase..

[1] To whom correspondence should be addressed (e-mail thomas.linsenmayer@tufts.edu).

of the HAS enzyme, and of HA synthesis itself. Additional evidence suggests that when the enzymic activity of either UDPG-PPase or HAS is increased in non-hypertrophic chondrocytes, this results in elevated HA synthesis. However, both enzymes must be up-regulated to produce a level of HA synthesis approaching that observed in the hypertrophic cells.

MATERIALS AND METHODS

Chondrocyte culture

Cartilage from 14- or 20-day chicken embryos (Spafas, Norwich, CT, U.S.A.) were dissected as described previously [15]. As a source of non-hypertrophic cartilage and chondrocytes, we used the caudal one-third of the sternum. This tissue remains cartilaginous throughout development and most of adulthood, and therefore is a good source of pure, non-hypertrophic chondrocytes. Zone 3 of the tibiotarsus, which is composed entirely of hypertrophic cells and can be cleanly dissected [1,16,17], was used as the source of hypertrophic cartilage and chondrocytes.

For cell culture, these tissues, from day-14 embryos, were dissociated in an enzyme mix containing 0.125 % trypsin (Gibco BRL, Gaithersburg, MD, U.S.A.), 0.08 % collagenase type I (Sigma, St Louis, MO, U.S.A.) and 0.06 % bovine testes hyaluronidase type I (Sigma) in a solution of Hanks buffered saline solution/Dulbecco's modified Eagle's medium (1:1, v/v) and 12.5 mM Hepes (pH 7.5). They were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10 % bovine calf serum (Hyclone Labs, Logan, UT, U.S.A.) and 50 units/ml penicillin and 50 units/ml streptomycin (Gibco BRL). The non-hypertrophic chondrocytes were not cultured for longer than 1 week, since, with extended time in culture and progressive passages, some of these cells can begin to undergo hypertrophy. The hypertrophic chondrocytes were passaged several times over a period of 2–4 weeks to ensure that they had reached the maximum stage of maturation. We have previously shown [18] that during this regimen, the relative production of the hypertrophic marker, collagen type X, synthesized by these chondrocytes progressively increases until it makes up > 90 % of the collagens being produced.

PCR

PCR was used to generate probes for Northern blotting, to analyse rapid amplification of cDNA ends (RACE) and to generate DNA inserts for the expression vectors used for transfection. The cycling programme consisted of one cycle of 94 °C for 1 min; 35 cycles of 94 °C for 15 s, 56 °C for 25 s, 72 °C for 1.5 min; and a final cycle of 72 °C for 10 min. The polymerase used was *Taq* DNA polymerase (Qiagen, Chatsworth, CA, U.S.A.). The sequences of the primers are given in the appropriate section below.

Northern blotting

For cell cultures from embryonic-day 14 chondrocytes, total RNA and polyadenylated [poly(A)$^+$] RNA was prepared using TRIzol (Gibco BRL) or the FastTrack kit (Invitrogen, Carlsbad, CA, U.S.A.). For tissues, total RNA was isolated from day-14 and day-20 embryo cartilages using the RNeasy Plant kit (Qiagen). A 2 μg portion of poly(A)$^+$ or 20 μg of total RNA was loaded per well, separated by gel electrophoresis, and transferred on to a positively charged nylon Hybond membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). Probes were generated by PCR, using the primers 5′-ATAACTTGGGTGCTACTG-TGGAC-3′ and 5′-GGTCCAAGATCCGAAGA-3′ to yield a 764 bp UDPG-PPase fragment (bp 866–1630), primers 5′-ATGTATTGTGAGAGGTTTATATG-3′ and 5′-CATACA-TCTAGCACCATGTC-3′ to yield a 1657 bp HAS-2 fragment (bp 563–2220), and primers 5′-GATCTGCACTACATGGT-3′ and 5′-GAGCCCATTGATCACA-3′ to yield a 101 bp glyceraldehyde-3-phosphate dehydrogenase (G3PDH) fragment (bp 165–266). PCR fragments were labelled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA, U.S.A.) using the HighPrime Labeling Kit (Roche, Indianapolis, IN, U.S.A.) and hybridized to the blots. Bound probes were detected by autoradiography. A digital image of the blots was recorded using the Eagle Eye II (Stratagene, LaJolla, CA, U.S.A.), and the intensity levels for the signals of UDPG-PPase, HAS-2 and G3PDH were determined using RFLPscan (Scanalytics, Billerica, MA, U.S.A.).

5′- and 3′-RACE

The partial cDNA fragment obtained previously by subtractive hybridization was extended to full length by 5′- and 3′-RACE, using a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, U.S.A.). RACE products were cloned into the pCR-TOPO II vector (Invitrogen). Positive clones were identified by PCR using gene-specific primers, analysed for size by PCR using vector primers and sequenced.

Primer extension

Primer extension was essentially carried out as described previously [19]. Briefly, a 23-mer antisense oligonucleotide (5′-AT-ACTCCAGCTCTTGCCGAATGA-3′) was end-labelled with [γ-^{32}P]ATP (ICN) using T4 polynucleotide kinase and incubated with 5 μg of poly(A)$^+$ RNA for 90 min at 65 °C in hybridization buffer [10 mM Tris/HCl (pH 8.3), 0.15 M KCl, and 1 mM EDTA]. Reverse transcription (RT) was performed for 1 h at 42 °C using Superscript II according to manufacturer's recommendations (Gibco BRL). Primer-extended products were RNase A treated, extracted with phenol/chloroform/3-methylbutan-1-ol (50:48:2, by vol.) and ethanol precipitated before being separated on a 5 % polyacrylamide gel.

Enzyme activity assay

UDPG-PPase activity was measured using an enzymic assay that results in the formation of NADPH as a product [20]. Briefly, cultured embryonic-day 14 chondrocytes were lysed in lysis buffer [0.08 M Tris/HCl (pH 7.5), 0.03 % Triton X-100, 100 μM PMSF and 10 μg/ml leupeptin (Sigma)], or day-14 and day-20 cartilage tissue was homogenized in lysis buffer. Samples were added to activity-assay buffer [50 mM Tris/HCl (pH 7.6), 0.67 mM UDP-glucose, 16 mM $MgCl_2$, 10 mM L-cysteine, 0.67 mM β-NADP, 0.01 mM glucose 1,6-bisphosphate, 0.25 unit/ml phosphoglucomutase, 0.25 unit/ml glucose-6-phosphate dehydrogenase and 1.67 mM PP_i], and the formation of NADPH was measured in a spectrophotometer at A_{340}. For the chondrocyte cultures, the UDPG-PPase activity was normalized to the amount of protein (units of enzyme/mg of protein) and to the numbers of cells (units of enzyme/cell). For the cartilage tissue, the UDPG-PPase activity was normalized to mg of total protein. One unit of enzyme results in the formation of 1 μmol of glucose 1-phosphate from UDP-glucose and PP_i per min at pH 7.6 at 25 °C.

Transfection

cDNA fragments encoding amino acid residues 12–508 of UDPG-PPase (primers 5′-ACGATGTCCCAGGCCGGCCCG-

T-3′ and 5′-GGTCCAAGATCCGAAGA-3′) and 1–553 of HAS-2 (5′-ATGTATTGTGAGAGGTTTATATG-3′ and 5′-CATAC-ATCTAGCACCATGTC-3′) were obtained by RT-PCR from total RNA of hypertrophic chondrocytes. The UDPG-PPase fragment was ligated into the TOPO-GFP expression vector (Invitrogen), and the HAS-2 fragment was ligated into the TOPO-myc vector (Invitrogen). Embryonic-day 14 chondrocyte cultures were transfected with UDPG-PPase, HAS-2, or both for 16–18 h using FuGene6 (Roche). For co-transfections, a 15-fold excess of HAS-2 expression plasmid was used. At 2 days post-transfection, cultures were either fixed and stained for HA, or the transfected cells were sorted by FACS and used for an HA competition binding assay. For FACS isolation of the cells transfected with the HAS-2 construct alone, cells were co-transfected with a vector encoding green fluorescent protein (GFP). Control, untransfected cells were also subjected to FACS.

Histological staining for HA

Free HA was visualized in cartilage tissue sections and cell culture using a biotinylated probe consisting of the HA-binding region of the cartilage proteoglycan aggrecan. The growth plate cartilage of 14- and 20-day chicken embryos was dissected, fixed for 20 min in 4% paraformaldehyde (PFA), and embedded in Tissue Tek O.C.T. compound (Electron Microscopy Sciences). Cryostat sections (6 μm) were permeabilized with 0.5% Triton X-100 for 10 min and blocked with 1% BSA. Some sections were incubated with chondroitinase ABC [50 m-units/ml in 0.1 M Tris/HCl (pH 8.0)] (Sigma) for 30 min at 37 °C prior to blocking to reduce the potential masking effect of proteoglycans. Sections were then incubated with 2 μg/ml biotinylated HA-binding protein (b-HABP; Seikagaku America, Falmouth, MA, U.S.A.) in 1% BSA for 1 h, followed by a 20 min incubation with alkaline phosphatase (AP)-conjugated streptavidin (Roche). Endogenous AP activity was blocked with 1 mg/ml Levamisole (Sigma) for 15 min, and bound b-HABP was visualized by the addition of Nitro Blue Tetrazolium ('NBT') and 5-bromo-4-chloroindol-3-yl phosphate ('BCIP') (Roche). All incubations were performed at 22 °C.

Transfected cell cultures were fixed in 4% PFA for 5 min and stained for free HA as described above, except that the bound b-HABP was visualized with rhodamine-conjugated streptavidin (Sigma).

Analysis of HA levels in cell cultures

Cellular HA was measured by a competitive binding assay using a modification of the protocol described by Kongtawelert and Ghosh [21]. First, 96-well microtitre plates (Maxisorp; Fisher Scientific, Pittsburgh, PA, U.S.A.) were coated with 0.1 mg/ml umbilical cord HA (Sigma) in 0.1 M $NaHCO_3$ (pH 9.6) overnight at 4 °C. Wells were then blocked with 1% BSA in PBS for 1 h at 22 °C. Whole-cell lysates of control and transfected chondrocyte cultures were incubated with 0.8 mg/ml b-HABP (Seikagaku America) in 50 mM Tris/HCl (pH 8.6) for 1 h at 22 °C, added to the HA-coated wells and incubated for an additional 1 h at 22 °C. For the standard curve, known amounts of purified HA were incubated with b-HABP before being added to the HA-coated wells. Wells were incubated with a peroxidase-conjugated mouse monoclonal anti-biotin antibody (1:2000 in PBS) for 1 h at 22 °C (Zymed, South San Francisco, CA, U.S.A.), followed by incubation with the substrate, *o*-phenylenediamine, in the dark. The colour reaction was stopped by the addition of 2 M H_2SO_4, and A_{490} was measured. The concentration of HA in the samples was then extrapolated from the standard curve.

RESULTS

Cloning of avian UDPG-PPase and its up-regulation in hypertrophic chondrocytes

To identify and isolate genes that are up-regulated in hypertrophic chondrocytes, in a previous study [3] we performed subtractive hybridization between cultured hypertrophic and non-hypertrophic cells. This resulted in a number of cDNA clones being identified that potentially represent genes undergoing up-regulation during hypertrophy. To identify the genes with the highest degree of up-regulation, a number of these clones were further screened by Northern-blot analysis.

In the present study, one of these genes we chose to investigate further has been identified as the avian homologue of the mammalian UDPG-PPase enzyme. By Northern-blot analysis, this gene was expressed in cultures of hypertrophic chondrocytes at a level approx. 6-fold higher than in cultures of non-hypertrophic cells (Figure 1A), with the size of the transcript being 2.6 kb. To obtain the full-length cDNA, we performed 3′- and 5′-RACE on the cDNA library, using the cDNA clone sequence to design the internal primers. The resulting RACE clone was 2642 bp. The 5′-end of the clone is shown in Figure 2(B), and primer extension revealed it to be just 6 bp short of the transcription start site (Figure 2A). The completeness of the 3′-end was shown by the presence of a poly(A) tail. The sequence has an open reading frame of 1.5 kb encoding a protein of 508 amino acids, with a predicted molecular mass of approx. 57 kDa. Sequence analysis at the amino acid level using a BLAST search revealed that the molecule had 91% similarity to the UDPG-PPase enzyme from human muscle, as encoded by the *UGP*2 gene [7].

To determine whether the up-regulation of the UDPG-PPase gene observed in chondrocyte cultures reflects the situation *in vivo*, we performed Northern-blot analysis with tissue RNA.

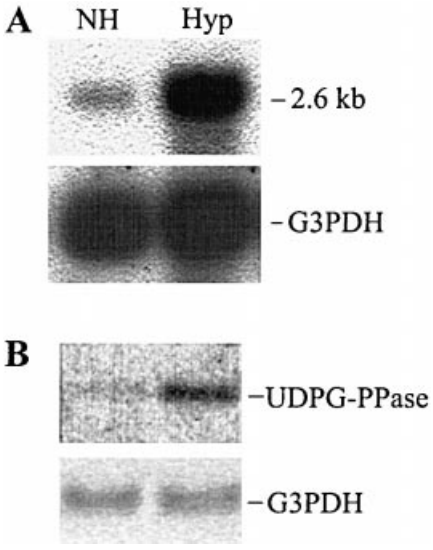

Figure 1 Up-regulation of UDPG-PPase mRNA levels in hypertrophic chondrocytes

Poly(A)$^+$ RNA (2 μg/lane) isolated from non-hypertrophic (NH) and hypertrophic (Hyp) embryonic-day 14 chondrocyte cultures (**A**), and total RNA (20 μg/lane) isolated from embryonic-day 20 cartilage tissue (**B**), was analysed by Northern blotting for the expression of UDPG-PPase and G3PDH. The band at 2.6 kb represents UDPG-PPase.

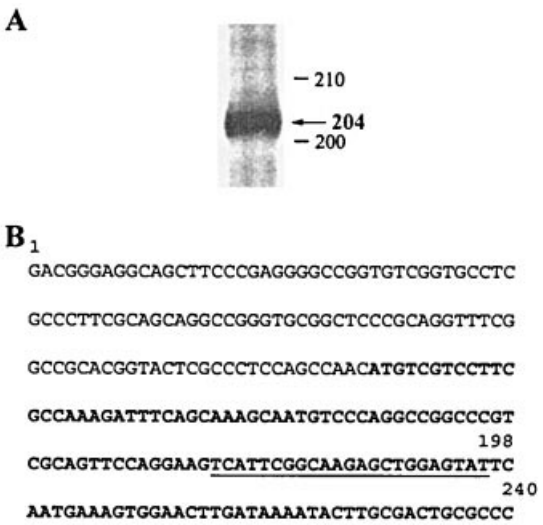

Figure 2 Primer extension-analysis of RNA isolated from cultured hypertrophic chondrocytes

(**A**) A 204 nucleotide major reverse-transcribed and extended product was obtained from poly(A)$^+$ RNA. (**B**) The nucleotide sequence of the longest UDPG-PPase 5′ RACE clone is shown. The position of the primer used for the primer extension is underlined. The beginning of the open reading frame is shown in bold.

This showed that UDPG-PPase was up-regulated approx. 4-fold in hypertrophic versus non-hypertrophic cartilage (Figure 1B).

Subsequently, to examine whether the increase in UDPG-PPase mRNA during hypertrophy was paralleled by an increase in enzymic activity, we measured the activity in non-hypertrophic and hypertrophic chondrocyte cultures. The UDPG-PPase activity was normalized both to cell number (units of enzyme/10^6 cells) and to the amount of protein (units of enzyme/mg of protein). Figure 3 shows that the up-regulation of enzymic activity in the hypertrophic cell cultures was 6-fold when normalized to cell number, which is consistent with the result observed with the mRNA described above. When normalized to the amount of protein (Figure 3), the increase in enzymic activity between non-hypertrophic and hypertrophic samples was 2-fold. A 2-fold up-regulation in enzymic activity was also observed in hypertrophic versus non-hypertrophic cartilage tissue when normalized to total protein (results not shown). The apparent difference in the up-regulation of enzymic activity in cultured cells illustrates the fact that hypertrophic chondrocytes are much larger than non-hypertrophic ones and therefore contain more protein.

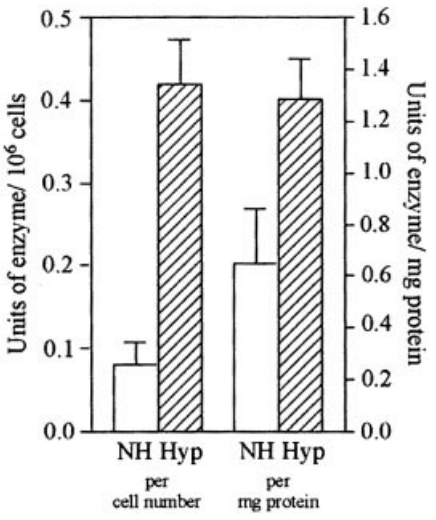

Figure 3 Enzymic activity of UDPG-PPase in hypertrophic versus non-hypertrophic chondrocytes

Cultured non-hypertrophic (NH; open bars) and hypertrophic (Hyp; hatched bars) chondrocytes were lysed and analysed for UDPG-PPase activity using an enzymic assay that links UDPG-PPase activity to the formation of NADPH. The activity was expressed in units of enzyme, and normalized to cell number or total protein. Results are expressed as the means ± S.D. of four separate experiments. $P < 0.05$ when the enzyme activity of Hyp was compared with NH.

Functional role of UDPG-PPase in HA synthesis

As mentioned in the Introduction section (and see the Discussion section), the product of the UDPG-PPase reaction can be used for the synthesis of a number of different molecules. In cartilage, three of these could be sulphated proteoglycans [16], glycogen [22,23] and HA [12,24]. We chose to focus on HA, since, as described in the Discussion section, work by others [16,22], together with our unpublished observations, made it unlikely that the large increase in UDPG-PPase detected in hypertrophic chondrocytes was reflected by a similar increase in either glycogen or proteoglycan.

Firstly, we examined histologically the distribution of free (non-proteoglycan-bound) HA in tissue sections of the tibiotarsal growth plate from 14-day embryos. For this, we employed an assay in which the tissue sections were incubated with a biotinylated probe consisting of the HA-binding region of the cartilage proteoglycan aggrecan. This probe specifically binds to free HA with high affinity [25].

Using this assay, unbound HA was detected primarily in the hypertrophic zone (Figure 4A); a finding similar to those previously observed by Pavasant et al. [12] in the juvenile rat growth plate. However, unlike in the rat, in which the HA reactivity is found predominantly in the pericellular region of the lacunae, in the avian hypertrophic cartilage we observed the unbound HA to be primarily intracellular, with a small amount throughout the matrix. This could reflect a species difference, a difference in the developmental stage examined (14-day chicken embryo versus 16-day neonatal rat), or both (see the Discussion section). To test whether the removal of potential masking proteoglycans within the matrix altered the distribution of staining, some tissue sections were digested with chondroitinase ABC prior to incubation with b-HABP. This slightly increased the reactivity within the matrix (results not shown), but it did not change the overall distribution of the staining, which still occurred preferentially within the hypertrophic zone.

To obtain information about the quantitative difference between HA synthesis in hypertrophic and non-hypertrophic cells, we assayed HA in extracts of cultures of these cell types using a modification of a competition binding assay. In this assay [21], the unbound HA in the samples competes with HA-coated wells for the binding to b-HABP. The up-regulation of cell-associated HA in hypertrophic versus non-hypertrophic chondrocytes ranged from 11-fold to more than 40-fold in different experiments. This was also true for the HA secreted into the medium, which showed a similar range (10–20-fold; results not shown).

To determine whether the up-regulation of UDPG-PPase was involved in this elevated HA synthesis, we overexpressed UDPG-PPase in non-hypertrophic chondrocytes by transfecting cultures

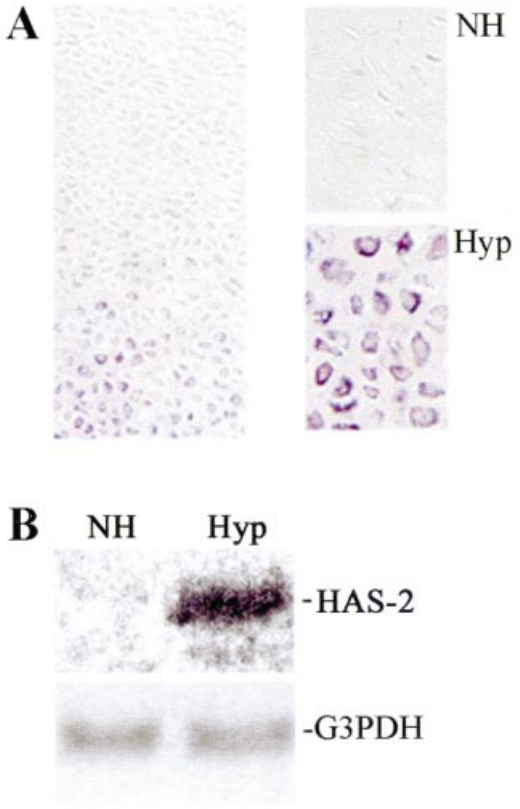

Figure 4 Up-regulation of HA and HAS in the hypertrophic zone of the epiphyseal growth plate

(**A**) Unbound HA in tissue sections of the epiphyseal growth plate of 14-day chicken embryos was detected with b-HABP and visualized with AP-conjugated streptavidin and Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate. Left panel: a low magnification (20 ×) picture of the growth plate at the transition from the non-hypertrophic to the hypertrophic zone. Right panels: high magnification (40 ×) images of the non-hypertrophic (NH) and hypertrophic (Hyp) zones. (**B**) Northern blots of HAS-2 and G3PDH in total RNA (20 μg/lane) isolated from 20-day embryonic non-hypertrophic (NH) and hypertrophic (Hyp) cartilage tissue.

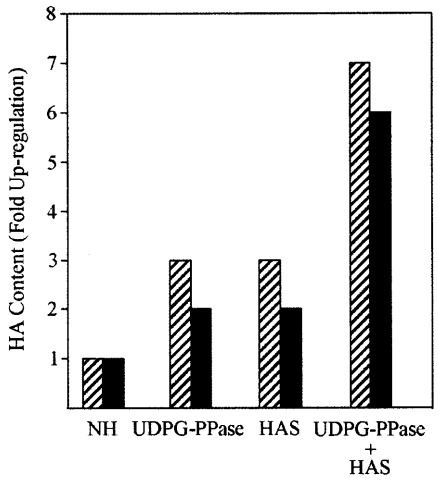

Figure 5 Effects of UDPG-PPase and HAS levels on HA synthesis by non-hypertrophic chondrocytes

Non-hypertrophic (NH) chondrocytes were transfected with expression vectors encoding either the UDPG-PPase–GFP fusion protein, HAS-2 (HAS), or both. At 2 days post-transfection, transfected cells were collected by FACS, lysed, and analysed for their HA content using a competition binding assay with b-HABP. Results are expressed as the ratio of transfected/untransfected NH cells. Two representative independent experiments are shown.

with a plasmid encoding the enzyme. The construct also contained the coding region of GFP, which allowed us to isolate the transfected cells within the population by FACS before subsequent analysis.

To verify that the fusion protein was enzymically active within the transfected non-hypertrophic cells, we assayed UDPG-PPase activity. The transfected cells showed an 8-fold higher activity than the control, non-hypertrophic cells (results not shown). We then examined the production of HA within the transfected cells using the competition binding assay described above. This showed the HA content of the transfected cells (Figure 5; UDPG-PPase) was 2–3-fold higher than that of the non-hypertrophic control cells (Figure 5; NH). Thus increasing the level of UDPG-PPase within chondrocytes leads to an increase in HA synthesis.

Although the transfection analyses with UDPG-PPase suggest that the UDPG-PPase is involved in the elevated synthesis of HA, the 2–3-fold increase in the transfected non-hypertrophic cells does not approach that in the cultured hypertrophic chondrocytes, which can range from 11-fold to more than 40-fold greater than in the non-hypertrophic cells (see above).

Up-regulation of HAS-2

We also examined the enzyme that catalyses the third and final step in HA synthesis, HAS. (The second enzyme in the pathway, UDPG-DH, has not been cloned in the chicken to date, and therefore we were unable to test whether transfection of this enzyme produced an up-regulation of HA synthesis.) Three vertebrate HAS genes have been identified [26–31], but work by others [32,33] has shown that HAS-2 is the most abundant form in cartilage. Therefore this was the form we examined.

Using RT-PCR, HAS-2 mRNA was detected in both hypertrophic and non-hypertrophic chondrocyte cultures (results not shown). However, by Northern-blot analysis, the mRNA for HAS-2 was present at appreciable levels only in the hypertrophic cartilage tissue (Figure 4B). Thus the mRNA for this enzyme appears to be highly up-regulated in hypertrophic chondrocytes.

To functionally examine the quantitative involvement of HAS-2 on HA production, we transfected non-hypertrophic cells with a construct encoding this enzyme. The transfectants produced 2–3-fold more HA than the untransfected cells (Figure 5; HAS); this was essentially the same increase as observed with the UDPG-PPase transfectants.

Since the transfections with neither the UDPG-PPase nor the HAS-2 resulted in the large range in HA production observed for the hypertrophic cells themselves, we performed double transfections of non-hypertrophic cells with constructs of the two enzymes. The double transfections produced a 6–7-fold increase in HA synthesis (Figure 5; UDPG-PPase + HAS), which was approx. 70 % of the lowest value observed for the authentic hypertrophic cells (which was an 11-fold increase). This suggests that, whereas the increased synthesis of HA observed for hypertrophic chondrocytes involves the co-operative effect of up-regulation of both UDPG-PPase and HAS-2, other as yet unknown factors are also likely to be involved (see the Discussion section).

Finally, we examined whether this increased synthesis of HA by the transfected cells produced morphologically detectable

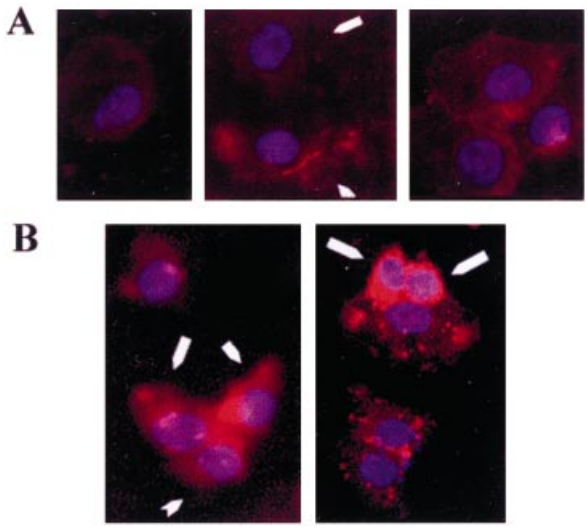

Figure 6 Increased levels of UDPG-PPase result in higher levels of HA

HAS-2 and GFP (**A**) or UDPG-PPase–GFP and HAS-2 (**B**) were overexpressed in non-hypertrophic chondrocytes. Cultures were fixed with 4% PFA 2 days post-transfection, and the HA was detected using b-HABP followed by visualization with rhodamine-conjugated streptavidin. Arrows designate the transfected cells as identified by GFP. Nuclei are stained with Hoechst. Magnification, 40 ×.

changes in their cell-associated HA. The transfected cells were identified by the presence of GFP, and the cell-associated HA was detected by the binding of b-HABP. Since, in this assay, the cells were not permeabilized, the HA being visualized was extracellular and in close association with the cells. The cells transfected with HAS-2 alone, as determined by the presence of GFP (Figure 6A; arrows), showed an HA signal that was slightly stronger than that observed for the untransfected cells (Figure 6A; unmarked cells). This was consistent with the moderate, 2–3-fold increase in HA production measured previously using the competition binding assay. Cells co-transfected with both UDPG-PPase and HAS-2 (Figure 6B; arrows), however, exhibited a greatly elevated HA signal, confirming the need for the up-regulation of both enzymes.

DISCUSSION

The results of the present study demonstrate that UDPG-PPase is up-regulated in the hypertrophic zone of the avian growth plate during embryonic development, with a 6-fold increase in enzymic activity (per cell) during the progression from non-hypertrophic to hypertrophic chondrocytes in culture. This is accompanied by a similar increase in the corresponding mRNA, suggesting that the regulation is achieved at the transcriptional level, rather than through activation or derepression of the enzyme itself. The avian transcript is 2.6 kb and contains an open reading frame encoding a 508-amino-acid protein. The UDPG-PPase molecule is highly conserved across species, with the avian homologue showing 91% identity at the amino acid level with the enzyme from human muscle.

During chondrocyte hypertrophy and cartilage growth, the increased levels of UDP-glucose, resulting from the 6-fold increase we observed in UDPG-PPase activity, could potentially result in, or be involved in, a large increase in the production of three different types of molecules, namely glycogen [22], proteoglycan [16] and HA [12].

We think it unlikely that either glycogen or proteoglycan are directly affected by the up-regulation in UDPG-PPase, as neither of these appear to be appreciably increased in hypertrophic chondrocytes. By electron microscopy, we could not detect an accumulation of glycogen granules in the chondrocytes within the growth plate (results not shown), whereas others [22] have observed an enrichment of glycogen within a small subpopulation of cells termed 'dark chondrocytes' by the same method. These cells, however, which have certain characteristics of apoptotic and necrotic cells, are distributed throughout all of the regions of the growth plate and therefore would not result in an appreciable increase in glycogen in the hypertrophic region.

Likewise, for sulphated proteoglycans, there does not seem to be a large increase in their synthesis by hypertrophic chondrocytes. Kim and Conrad [16], in an extensive study using chondrocyte cultures similar to those employed here, quantitatively measured the incorporation of sulphate into proteoglycans derived from the non-hypertrophic and hypertrophic zones. They also examined an intermediate zone, the zone of maturation (which they termed zone 2a). The relative synthesis between the zones varied depending on whether the cells were in primary culture or were passaged. However, the sulphate incorporation into the cells from the hypertrophic zone was never more than 30% greater than that in the non-hypertrophic zone, which is unlikely to account for the large increase we observed in UDPG-PPase.

Instead, our present results suggest that the elevated UDP-glucose in hypertrophic chondrocytes contributes to an increase in the synthesis of HA. The morphological observations, visualized by the binding of the b-HABP to tissue sections of the tibiotarsal growth region of 14-day embryos, clearly show an up-regulation of free (non-proteoglycan-bound) HA in the growth region. This is consistent with a previous report [12] using a similar assay to examine the distribution of HA in the tibia of the neonatal rat. In the hypertrophic region of the rat epiphyseal growth plate, the strongest HA signal was in the pericellular region of the lacunae, whereas in the embryonic chick, we observed it within the hypertrophic chondrocytes themselves. This may reflect differences between the two species (chicken versus rat), differences in the stage of development (embryonic versus neonatal), or both. Studies on cartilaginous growth regions have reported species temporal-developmental differences for other developmental parameters within the growth plate [34]. For example, in rodents the hypertrophic cartilage of developing embryos becomes heavily calcified soon after it forms even in the earliest embryos. In the developing chicken, however, such calcified cartilage cannot be detected until several weeks post-hatching. The reason for this difference is unknown. However, it has been suggested that this may reflect a more rapid rate of growth and development in the chicken embryo, such that the hypertrophic cartilage is removed before it has had time to undergo appreciable calcification [34]. The difference between the presence of the pericellular ring of HA seen within the lacunae of the hypertrophic cartilage of the neonatal rat, and its absence in the chicken embryo, may possibly reflect a similar difference in developmental rates. In the chicken embryo, the rapid formation and removal of the hypertrophic chondrocytes and their surrounding matrix may preclude the deposition of a pericellular layer of HA. However, it seems likely that chicken chondrocytes synthesizing high levels of HA do have the potential for producing a heavy layer of cell-associated material. As observed in the present study (Figure 6), when cultured chondrocytes were co-transfected with the UDPG-PPase and HAS, they showed an appreciable accumulation of cell-associated, extracellular HA.

Quantitative analyses of HA in the cultures of non-hypertrophic cells versus hypertrophic ones also showed a large increase in HA production by the hypertrophic cells. There was, however, a wide range with the values ranging from 11-fold to more than 40-fold. This could reflect heterogeneity in the population of hypertrophic cells isolated from the hypertrophic region and used to initiate the cultures, since the cells within the hypertrophic region are not a homogeneous population, but a continuum of cells undergoing hypertrophy.

HA production involves three major enzymes: UDPG-PPase, UDPG-DH and HAS. In bacterial cells, such as *Streptococcus*, the three enzymes form a single operon, which ensures their co-ordinate regulation. In higher organisms, the potential co-ordinated regulation of these enzymes has not been extensively investigated. We have shown in the present study that the up-regulated expression of UDPG-PPase in hypertrophic versus non-hypertrophic cartilage is paralleled by a similar increase in mRNA for HAS-2, which, among the three genes for mammalian HAS, has been reported as being the major form in cartilage [32,33]. As UDPG-DH has not been cloned in chicken to date, we could not investigate the expression of this enzyme. Its mRNA level, however, is also likely to be elevated in hypertrophic chondrocytes, as Spicer et al. [35] have reported that the increased HA production by human fibroblasts in response to interleukin-1β was accompanied by a rise in UDPG-DH mRNA.

In the present study, neither UDPG-PPase nor HAS over-expressed by itself was sufficient for non-hypertrophic cells to produce amounts of HA similar to that of hypertrophic chondrocytes. Only the cells overexpressing both enzymes were capable of synthesizing HA at levels approaching those of the hypertrophic zone of the growth plate. These findings are consistent with the possibility that UDPG-PPase may contribute to the regulation of HA synthesis by controlling the availability of UDP-glucose. Therefore we propose that co-ordinated up-regulation of at least two enzymes of the HA synthesis pathway is required for the synthesis of physiological levels of HA by hypertrophic chondrocytes. Possibly, up-regulation of the third enzyme in the pathway, UDPG-DH, may also be involved in the production of HA by hypertrophic chondrocytes.

Nevertheless, our present results have shown that elevated levels of UDPG-PPase by itself are capable of increasing HA production by non-hypertrophic chondrocytes, presumably by providing more precursor for the subsequent enzymic reactions in the HA synthesis pathway. In support of this, it was recently reported [32] that, in response to growth factors, increased HA production need not be accompanied by an increase in HAS expression. The authors concluded that HA synthesis appears to be regulated at multiple levels, and that modulation of HAS expression represents only one parameter. As suggested by the present results, control of UDPG-PPase expression, and thus precursor levels, provides another level of regulation.

HA has been suggested to play a role in lacunae enlargement and therefore in long bone growth [12,36]. According to this hypothesis, the secretion of the glycosaminoglycan, HA, by hypertrophic chondrocytes into the pericellular space produces a swelling pressure resulting in increased lacuna size. Our observation suggests that the increased intracellular HA levels may also participate in the enlargement of the hypertrophic chondrocytes themselves, but our attempts to test this hypothesis have produced equivocal results. Nevertheless, if either of these hypotheses is correct, the production of HA must be under tight control to ensure that the expansion of the growth plate is appropriate for the different regions of the body. Our observations strongly suggest that UDPG-PPase production is involved in this control.

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