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Rapid report

Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes¹

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Abstract

Growth plate chondrocytes progress through a proliferative phase before acquiring a terminally-differentiated phenotype. In this study we used Percoll density gradients to separate chick growth plate chondrocytes into populations of different maturational phenotype. By applying agarose gel differential display to these populations we cloned a cDNA encoding a novel 268 amino acid protein (3X11A). 3X11A contains two peptide motifs that are conserved in a recently identified superfamily of phosphotransferases. It is likely that 3X11A is a phosphatase, but its substrate specificity remains uncertain. 3X11A expression is upregulated 5-fold during chondrocyte terminal differentiation and its expression is approximately 100-fold higher in hypertrophic chondrocytes than in non-chondrogenic tissues. This suggests that 3X11A participates in a biochemical pathway that is particularly active in differentiating chondrocytes. © 1999 Elsevier Science B.V. All rights reserved.

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In higher vertebrates, longitudinal bone growth occurs at the epiphyseal growth plate. The process is termed endochondral ossification and is of fundamental importance in the growth and development of the appendicular skeleton. The sequence of cellular transitions that occurs within the growth plate have been characterised by a combination of morphological, ultrastructural and biochemical studies [1]. Briefly, chondrocytes emerge from a reserve zone at the epiphyseal edge of the growth plate and progress through a proliferative phase during which they have

a characteristic flattened morphology. After a number of cell divisions, proliferation ceases and the cells hypertrophy and gradually acquire a terminally differentiated phenotype. Differentiation is accompanied by increases in the expression of type X collagen, alkaline phosphatase (ALP) and other specific macromolecules [2–4]. Acquisition of the hypertrophic phenotype by growth plate chondrocytes appears to be essential for subsequent vascular invasion and replacement of the matrix by bone [5,6]. Consequently, the transition between proliferation and hypertrophy is a critical step during endochondral ossification. We wish to characterise the proliferative and hypertrophic phenotypes more fully and in particular to identify factors that may regulate chondrocyte differentiation and/or matrix resorption. To this

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end we have explored the use of Percoll density fractionation [7,8] as a method for separating chick growth plate chondrocytes into different phenotypic populations. By combining Percoll fractionation with agarose gel differential display we have identified a novel phosphatase that is highly expressed by differentiating chondrocytes.

Chondrocytes were isolated from growth plates of proximal tibiotarsi of 3-week-old chicks by collagenase digestion as described previously [9]. Discontinuous Percoll gradients were made up in Corning polystyrene culture tubes (16×125 mm) and consisted of 2 ml of each of 6 density solutions, 1.04–1.09 g/ml, prepared in 0.15 M NaCl. 1 ml aliquots containing 40–50×10⁶ chondrocytes were layered onto each gradient and centrifuged for 30 min at 400×*g* at 25°C. The Percoll fractionated chondrocytes (PFCs) were harvested by aspiration, washed in 20 ml 0.15 M NaCl, and resuspended in 1 ml of this solution. Samples were taken at this stage to assess cell number, size, viability and ALP activity. In some cases birds were injected with bromodeoxyuridine (BrdU) 1 h before death and cytospin preparations of cells obtained by Percoll fractionation were stained by immunofluorescence to detect proliferating cells [10]. The remaining cells were re-pelleted and stored at –70°C prior to RNA extraction. The mean cell volume of each Percoll fraction was measured on a Macintosh computer using the public domain software package NIH-Image 1.60. Alkaline phosphatase activity in cells from each fraction was measured as described previously [9].

For total RNA extraction the cell pellets were homogenised in 1.5 ml Ultraspec (Biotecx) by repeated passage through a 25 gauge syringe needle. After extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack Resin (Biotecx), following the manufacturers protocol. The RNA was finally eluted in 100 µl RNase-free H₂O. To extract total RNA from tissue samples 0.5–1.0 g of tissue was homogenised in 10 volumes of Ultraspec using an Ultraturax homogeniser and total RNA purified as above. Agarose gel differential display was performed as described by Jefferies et al. [11] in 20 µl reactions containing the equivalent of 50 ng RNA. The cycling profile used was: 30 s at 94°C (1st cycle 2 min); 1 min at 40°C; 1 min at 72°C for 40 cycles followed

by 10 min at 72°C. The primers used to identify the gene we refer to as 3X11A were: DD3; GTGGAAGCGT and DD11; CACAGTGAGC. They were used at a concentration of 2 µM. Reaction products were resolved on 3% NuSieve 3:1 agarose gels (Flowgen) run in the presence of ethidium bromide (250 µg/l). Bands of interest were excised from the gels and cloned directly into the T-vector PCRII-Topo (InVitrogen). For sequence analysis plasmid minipreps were prepared (Qiagen) and sequenced on a LICOR 4200 automated sequencer (MWG-Biotech). Sequence database searches were conducted using FASTA and BLAST programs via the EBI World Wide Web server (URL: <http://www.ebi.ac.uk>).

The expression of specific genes was analysed by semi-quantitative RT-PCR. 500 ng RNA was reverse transcribed using the Superscript II preamplification system (BRL Life Technologies). All PCRs were performed in 20 µl reactions containing cDNA equivalent to 10 ng RNA, TLA buffer [12], 0.2 U Taqstart antibody (Clontech), 2 U Taq DNA polymerase and gene-specific oligonucleotide primers at 200 nM. All genes were assayed using the cycling profile: 1 min at 92°C (1st cycle 2 min); 1 min at 55°C; 1 min at 70°C. The number of cycles varied between genes and were chosen to ensure that band intensity was proportional to the amount of target. The primers and the number of cycles (in parentheses) used for each gene were β-actin, TGTGGTATCCATGAACTA and ATTCATCGTACTCCTGCCT (25); type II collagen, GCAGAGACCATCAACGGCGGT and CAGGAGCGAGGTCTTCTGCGA (17); type X collagen, GGAGAGTCTAGAGAACTATCAGG and ATTCTGAATTCGGCAGCTGGAGC (19); extracellular fatty acid binding protein (Ex-FABP), GACTTCTCCTGCGTGAGAAGG and ATCAACGCTGCATTCCTCCTGG (25); 3X11A, GCATTGAGTGCAAAGTGGAGG and GCTGCTTCTCTCCATCTCC (25 or 29). Oligonucleotide primers for 18S RNA were obtained from Ambion and required 14 cycles. 10 µl of each reaction were analysed on 1.5% agarose gels run in the presence of ethidium bromide (250 µg/l). The banding pattern on each gel was captured using a Bio-Rad Gel Doc image analysis system and PCR products were quantified by measuring signal intensity using NIH Image software.

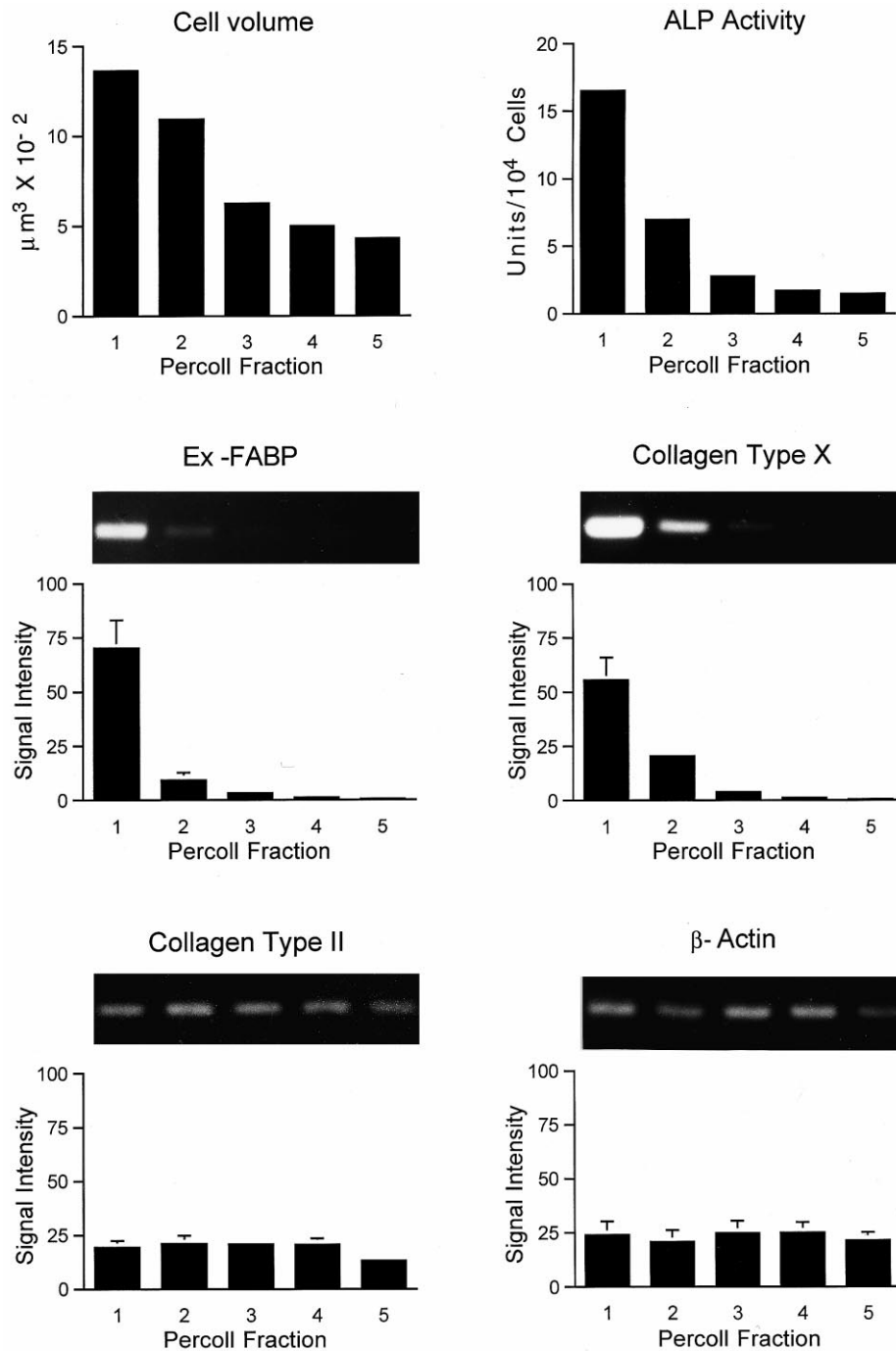


Fig. 1. Analysis of cell volume, ALP activity and marker gene expression in Percoll fractionated growth plate chondrocytes. In the gene expression studies each graph shows the mean \pm S.E.M. of the signal intensity measured in cells from three independent Percoll gradients, while the inset shows a typical banding pattern. Units for ALP activity are nmol pNPP hydrolysed per min. Signal intensities are in arbitrary units.

After Percoll fractionation (Fig. 1), fraction 1 contained the largest cells and fraction 5 the smallest in keeping with the results of other workers who have shown that the chondrocyte volume is inversely proportional to their buoyant density [7]. Alkaline phosphatase activity, which is a marker of chondrocyte hypertrophy [2], was highest in fraction 1 and decreased progressively in successive fractions. The expression of the hypertrophic markers Ex-FABP [13] and type X collagen was highest in the cells of fraction 1 and decreased dramatically in successive fractions, so that their expression was virtually undetectable in fractions 4 and 5. We estimate from the signal intensities that the expression of Ex-FABP and type X collagen decreased 92- and 130-fold between fractions 1 and 5. In contrast, the PFCs expressed type II collagen at a constant level, in keeping with its uniform distribution in the growth plate [2]. A proportion of the cells in fractions 3, 4 and 5 stained positively for BrdU with those of fraction 4 containing, by far, the highest percentage of labelled cells. No BrdU-labelled cells were found in fractions 1 and 2 (results not shown). These results indicate that fractions 1 and 2 contain predominantly terminally differentiated, hypertrophic cells and that proliferating chondrocytes are found in fractions 4 and 5. Fraction 3 is likely to contain a mixture of both cell types.

To identify genes that were differentially expressed within the growth plate we performed (in triplicate) a differential display analysis on RNA extracted from PFCs using 26 combinations of the 10-mer primers given in [14]. Using this approach we identified and cloned a 979 bp band which we named 3X11A (from the primer pair used in that experiment) that was derived from a transcript more abundantly expressed in fractions 1 and 2. Analysis of the DNA sequence of this clone indicated that it contained a potential open reading frame encoding a 268 amino acid protein with an M_r of 30442 and a pI of 6.81 (Fig. 2). Comparison of the 3X11A amino acid sequence with entries in protein sequence databases using the BLAST algorithm revealed similarity to phosphoserine phosphatase (PSP), the enzyme that catalyses the final step in the biosynthesis of serine from glycolytic precursors. An alignment of the amino acid sequence of the 3X11A protein with PSPs from 5 prokaryotic and 4 eukaryotic species (Fig. 3A) showed, however, that 3X11A had only 14–18% overall identity to PSPs suggesting that 3X11A is not the chick homolog of PSP. It was apparent from the alignment that the conserved residues were not distributed uni-

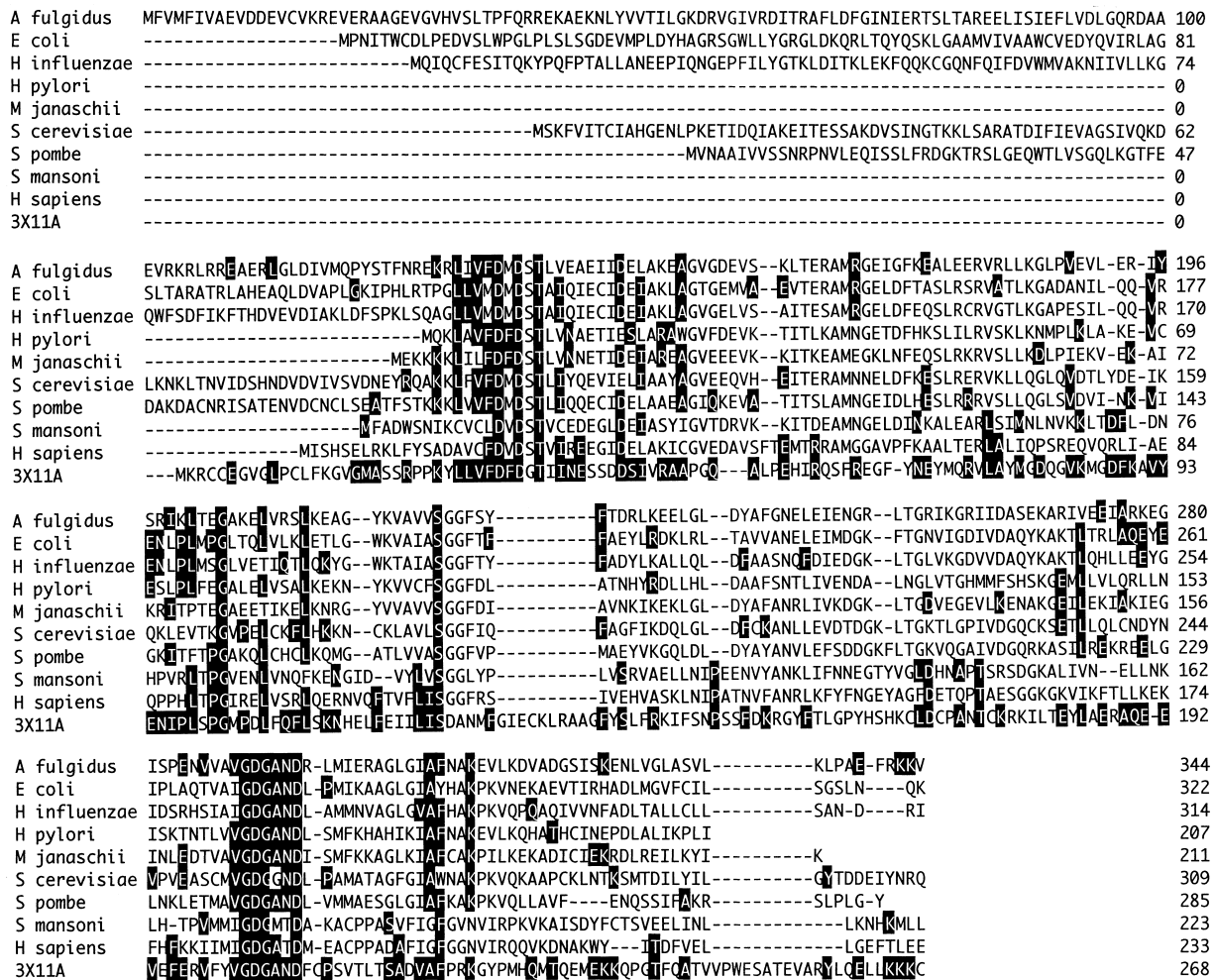
tion 3 is likely to contain a mixture of both cell types.

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GCCCGTGAAGGAGCCCAACACAGAGGCCYTAACCGGGACCTTCAGCARRAGCGGTGCTCGAGAGTGATGGTCTTAAATGAAAAGGTGCTGTGAGGGGT 100
                                     M K R C C E G V
TGGGCTGCCATGCTGTTTAAAGGGTGTGGCATGGCCAGCTCCCGGGCTCCCAAGTACCCTCTGGTCTTCGATTTTGATGGGACCATCATCAACGAGAGC 200
  G L P C L F K G V G M A S S R P P K Y L L V F D F D G T I I N E S
AGTGATGACTCCATCGTGCGGGCAGCACAGGGCAGGCACTGCTGAGCAGCATCCGGCAGAGCTTCCGCGAGGGCTTCTACAATGAGTACATGCAGCGC 300
  S D D S I V R A A P G Q A L P E H I R Q S F R E G F Y N E Y M Q R
TCTGGCTCATGAGGGGACAGGGCGTCAAGATGGGGGACTTCAAGGCTGTCATGAGAACATCCCTGTCTCCGGGCATGCTGACCTCTCCAGTT 400
  Y L A Y M G D Q G V K M G D F K A V Y E N I P L S P G M P D L F Q F
CCTCTCCAGAACACAGGCTCTCGAGATCATCCTCATCTCCGACGCCAAGCATGTTTGGCATTGAGTGCAAACTGAGGGCGGGCGGTTTACTCCCTC 500
  L S K N H E L F E I I L I S D A N M F G I E C K L R A A G F Y S L
TTCCGCAAAATCTTCAGCACCCTCCAGCTTTGACAGAGGGGTTTACCTTCCACTTGGGGCCCTACCACAGCCACAGTGCCTTGACTGCCAGCCARCA 600
  F R K I F S N P S S F D K R G Y F T L G P Y H S H K C L D C P A N
CGTGCAAGCGCAAAATCCTAACGGAGTACCTGGCCGAGAGAGCGCAGGAGAGGTTGGAGTTCCGAGAGGGTGTTTTACGTGGGGATGGTGCCAA TGACTT 700
  T C K R K I L T E Y L A E R A Q E E V E F E R Y F Y V G D G A N D F
CTGCCCTTCGGTGACTTTGACTTCAGCTGACGTGCTTCCCTCGGAGGGCTACCCCATGCACAGATGACCCAGGAGATGGAGAGAGAGCAGCCTGGA 800
  C P S V T L T S A D V A F P R K G Y P M H Q M T Q E M E K K K C
ACCTTCCAGGCCACCGTTGTCCCTGGGAGTCGGCTACCGAGGTTGCCCGTATCTGCGAGAGCTCCTCAGAGAGAGTGTGAGGGATGGATGGCTCCAA 900
  T F Q A T V V P W E S A T E V A R Y L Q E L L K K K C
AGAGGCTTGTTCAGATAGCAGCTGGGAGGGAGAGGCTGGGAGATCACGTCTGCARCCCATGTTATTTGACT 979
    
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Fig. 2. Nucleotide sequence of 3X11A and its deduced amino acid sequence. An N-terminal pentapeptide corresponding to a motif that is conserved in the active site of a superfamily of phosphotransferases is underlined. A C-terminal, hexapeptide corresponding to a second active site motif also conserved in these enzymes is double underlined.

a.



b.



Fig. 3. Comparison of 3X11A with related proteins. Sequences were aligned using the DNASTAR program MEGALIGN. (A) Alignment of the 3X11A sequence with those of phosphoserine phosphatases from the species indicated. Shading indicates where a residue is conserved between 3X11A and at least one phosphoserine phosphatase. (B) Alignment of the sequence around the conserved motifs in 3X11A with the corresponding regions in other members of the phosphotransferase superfamily. Shading indicates where a residue is conserved between 3X11A and at least one other enzyme. Motif 2 is not present in β-phosphoglucomutase, deoxyglucose 6-P phosphatase or glycerol 3-P phosphatase.

formly throughout the sequence but were clustered around two amino acid motifs, DFDGT, residues 32–36 (motif 1) and GDGAND, residues 202–207 (motif 2) (Fig. 3A). By performing a second database search using only these motifs we identified a number of other proteins that are members of a recently identified superfamily of enzymes catalysing phosphotransferase reactions [15,16]. This superfamily includes the P-type ATPases, a large class of enzymes that use the free energy of the hydrolysis of ATP to transport cations across cell membranes; a number of phosphatases that catalyse the generation of inorganic phosphate from a variety of phosphomonoester substrates and two phosphomutases that catalyse the transfer of a phosphoryl group between adjacent carbon atoms of hexose monophosphates [15,16]. The reactions catalysed by the members of this superfamily appear to proceed through a similar mechanism. Consequently, the active sites of these enzymes share structural features and this is reflected in the conservation of certain active site residues corresponding to the two motifs identified above. Fig. 3B shows an alignment of the sequence around the conserved motifs in 3X11A, with the equivalent regions from representative members of each class of enzyme in the superfamily. With the exception of

phosphoserine phosphatase, 3X11A has no similarity outwith these motifs. The first aspartate in the DFDGT motif is totally conserved in all members of this superfamily and is transiently phosphorylated during the reaction. The DFDGT motif of 3X11A matches the consensus motif DFDXT that is conserved in the phosphatases and phosphomutases in this superfamily (Fig. 3, [16]) and is distinct from the totally conserved DKTGT motif of the P-type ATPases. Together with the absence of transmembrane domains in its sequence, this indicates that 3X11A is not a P-type ATPase. The second motif is less well conserved in this superfamily (Fig. 3A). However, we consider that 3X11A is likely to be a phosphatase rather than a phosphomutase since the structure of its GDGAND motif more closely matches that of the phosphatases and P-type ATPases (GDX[NT]D) rather than that of the phosphomutases. The reaction catalysed by 3X11A remains unknown.

To confirm that 3X11A is indeed differentially expressed in the growth plate we designed specific oligonucleotide primers and used these in semi-quantitative RT-PCR assays for 3X11A expression in PFCs (Fig. 4A). A single band of the predicted size (336 bp) was detected. The signal intensity was constant in fractions 1–3 and decreased progressively in frac-

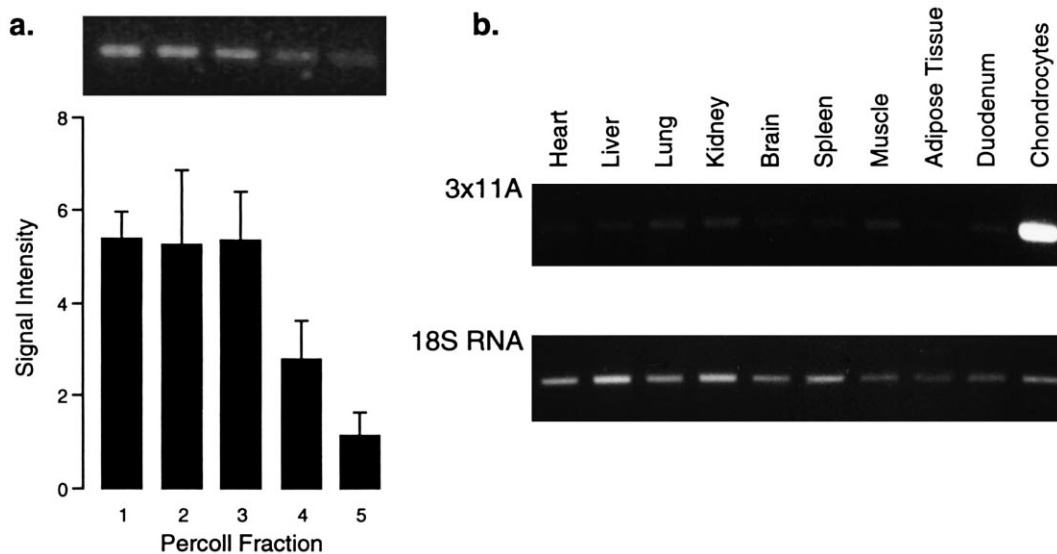


Fig. 4. Pattern of expression of 3X11A. (A) 3X11A expression in growth plate chondrocytes. The graph shows the mean signal intensity (\pm S.E.M.) measured in RNA purified from cells obtained from 3 independent density gradients. The signal intensity is in arbitrary units. The inset shows the banding pattern from a typical gel. (B) Distribution and abundance of 3X11A in chick tissues. 3X11A expression was measured by RT-PCR using specifically designed primers in RNA purified from the tissues indicated.

tions 4 and 5. These results therefore confirm those of the differential display analysis, and based on the difference in intensity of the bands in fractions 1 and 5 we estimate that the expression of 3X11A is up-regulated 5-fold during chondrocyte differentiation. To determine the tissue distribution of 3X11A transcripts, similar assays were performed on RNA extracted from various tissues. By increasing the cycle number to 29, 3X11A expression was detected at a uniform level in cardiac muscle, liver, lung, kidney, brain, spleen, skeletal muscle and adipose tissue (Fig. 4B). However, 3X11A expression was markedly elevated in terminally differentiated growth plate chondrocytes compared to all other tissues assayed. From the band intensities we estimate that 3X11A expression in these cells was some 100-fold higher than in the non-chondrogenic tissues. This pattern of expression would seem to indicate that 3X11A participates in a biochemical pathway that is particularly active in differentiating chondrocytes. Since 3X11A appears to be a phosphatase, we speculate that it may be involved in the generation of inorganic phosphate during matrix mineralisation. This role is generally attributed to ALP but this enzyme can be removed from some preparations of matrix vesicles without impairing their ability to mineralise [17]. Additionally, a number of studies have shown that in addition to ALP, growth plate chondrocytes express several distinct phosphatases and ATPases that are involved in mineralisation [18,19], although the specific endogenous substrates have not yet been identified. Further progress in understanding the role of 3X11A in chondrocyte biochemistry must await the identification of the reaction catalysed by this enzyme.

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