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Cloning of a Mouse Smoothened cDNA and Expression Patterns of Hedgehog Signalling Molecules during Chondrogenesis and Cartilage Differentiation in Clonal Mouse EC Cells, ATDC5

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Hedgehog (hh) family proteins appear to use the conserved targets in their signalling pathway including Patched (Ptc), Smoothened (Smo), and Gli. Although Indian hedgehog (Ihh) plays an important role in endochondral bone formation, the involvement of hh signalling molecules in skeletogenesis is unknown. We cloned a mouse (m) Smo cDNA and studied the expression patterns of Ihh, Ptc, Smo, and Gli mRNAs in mouse chondrogenic EC cells, ATDC5. The deduced amino acid sequence of mSmo consisted of 793 amino acids and was 98 and 93% homologous to the rat (r) Smo and human (h) Smo, respectively. In ATDC5 cells, the expression of *Ihh* mRNA paralleled that of type X collagen mRNA. *Smo, Ptc,* and *Gli* mRNAs were constitutively expressed throughout chondrogenesis and the subsequent cartilage differentiation processes except for the transient decrease in *Ptc* mRNA at the cellular condensation stage. Our data suggest that hh signalling molecules may be involved in chondrogenesis and cartilage differentiation in ATDC5 cells.

Secreted proteins of hh family play pivotal roles in limb bud development. Sonic hedgehog (Shh) is secreted from the zone of polarizing activity and establishes the anterior-posterior axis at the early stage of limb bud formation [1]. Ihh is expressed in the developing cartilage elements in mouse embryo [2]. Recently, Ihh is shown to be expressed in prehypertrophic chondrocytes in chick limb buds and regulates the rate of cartilage differentiation during skeletogenesis [3].

Signalling of hh family appear to use the conserved targets including Ptc, Smo, and Gli [3-8]. Mutations of *Ptc* and *Gli* genes each are responsible for the skeletal abnormalities in Gorlin [9] and Greig syndromes [10], indicating that these signalling proteins play important roles in the normal patterning in skeletogenesis. However, the expression patterns of these genes during chondrogenesis and cartilage differentiation remain unknown.

We previously reported that clonal mouse EC cells, ATDC5, keep track of the overt chondrogenesis in vitro[11,12]. In this paper we isolated a cDNA clone containing the mouse *Smo* coding sequence of 793 amino acids and examined the expression patterns of hh signalling molecules at the stages of chondrogenesis and cartilage differentiation in vitro in ATDC5 cells.

MATERIALS AND METHODS

Cells and culture conditions. ATDC5 cells were cultured as previously described[11,12]. Briefly, cells were maintained in a log growth phase in DMEM/Ham's F12 hybrid medium (Flow Laboratories, Irvine, U.K.) containing 5 %(v/v) FBS (GIBCO BRL, Gaithersburg, MD), 10 μg/ml human transferrin (Boehringer Mannheim GmbH, Mannheim, Germany), and 3×10^{-8} M sodium selenite (Sigma Chemical Co., St. Louis, MO) (the maintenance medium) at 37 °C in a humidified 5 % CO_2/95 % air atmosphere. Chondrogenesis and cartilage nodule formation could be induced only in a postconfluent phase when cells were cultured in the maintenance medium supplemented further with 10 μg/ml bovine insulin (Wako Pure Chemical, Osaka, Japan) (the differentiation medium). Cells were cultured in the differentiation medium for twenty one days until the growth of cartilage nodules ceased. Induction of calcification was achieved when medium was replaced with α-MEM (Flow Labora-

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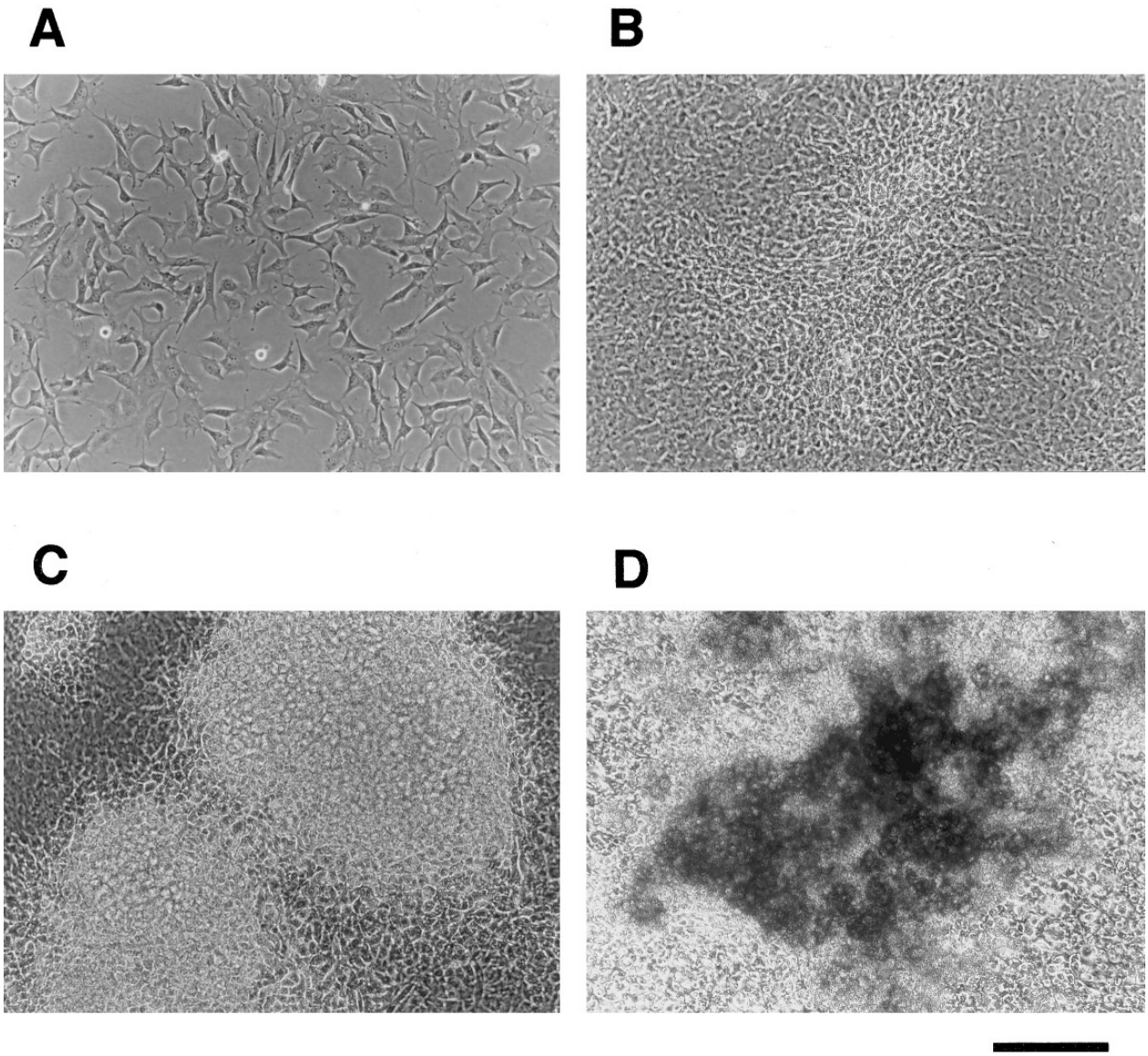

FIG. 1. Phase-contrast micrographs of ATDC5 cells at various stages. (A) Undifferentiated subconfluent stage (day 3); (B) the condensation stage (day 7); (C) cartilage nodules formation (day 21); and (D) calcification stage (day 45). Bar denotes 100 μm.

tories, Irvine, U.K.) containing 5 %(v/v) FBS, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite and 10 μg/ml bovine insulin (the calcification medium) and culture was continued at 37 °C in a humidified 3 % CO_2/97 % air atmosphere. In the present study we plated ATDC5 cells in a log growth phase in six-multiwell plastic plates at an initial cell density of 6×10^4 cells/well and cultured these cells for a total of forty two days with medium replacement every other day. Cells were cultured for the initial twenty one day-period in the differentiation medium in a 5 % CO_2/95 % air atmosphere and then for the subsequent twenty one day-period in the calcification medium in a 3 % CO_2/97 % air atmosphere. Under these conditions, undifferentiated ATDC5 cells reached confluence on day 5, cellular condensation occurred on day 7, cartilage nodule formation occurred on day 9, the growth of cartilage nodules ceased on day 21, and calcification began on day 35 (FIG. 1).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). ATDC5 cells were plated in six-multiwell plastic plates and cultured for three days in the differentiation medium. Cells remained subconfluent and undifferentiated during this culture period [11]. Total RNA was prepared by a single-step method according to Chomcznski and Sacchi [13], followed by the first-strand cDNA synthesis using SuperScript II RNase H^- reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The oligonucleotide primers and conditions for PCR amplification of *Gli* and *G3PDH* mRNAs were described previously [12,14]. A mouse homologue of Smo was identified by PCR with three different primers of degenerate oligonucleotides each corresponding to the conserved motifs in the human and rat Smo amino acid sequences: FD1, 5′-GCNATG-TTYGGNACNGG-3′ (forward direction); FD2, 5′-TGGGTNTGG-ACNAARGCNAC-3′ (forward direction); and RD, 5-GCNACNGGN-

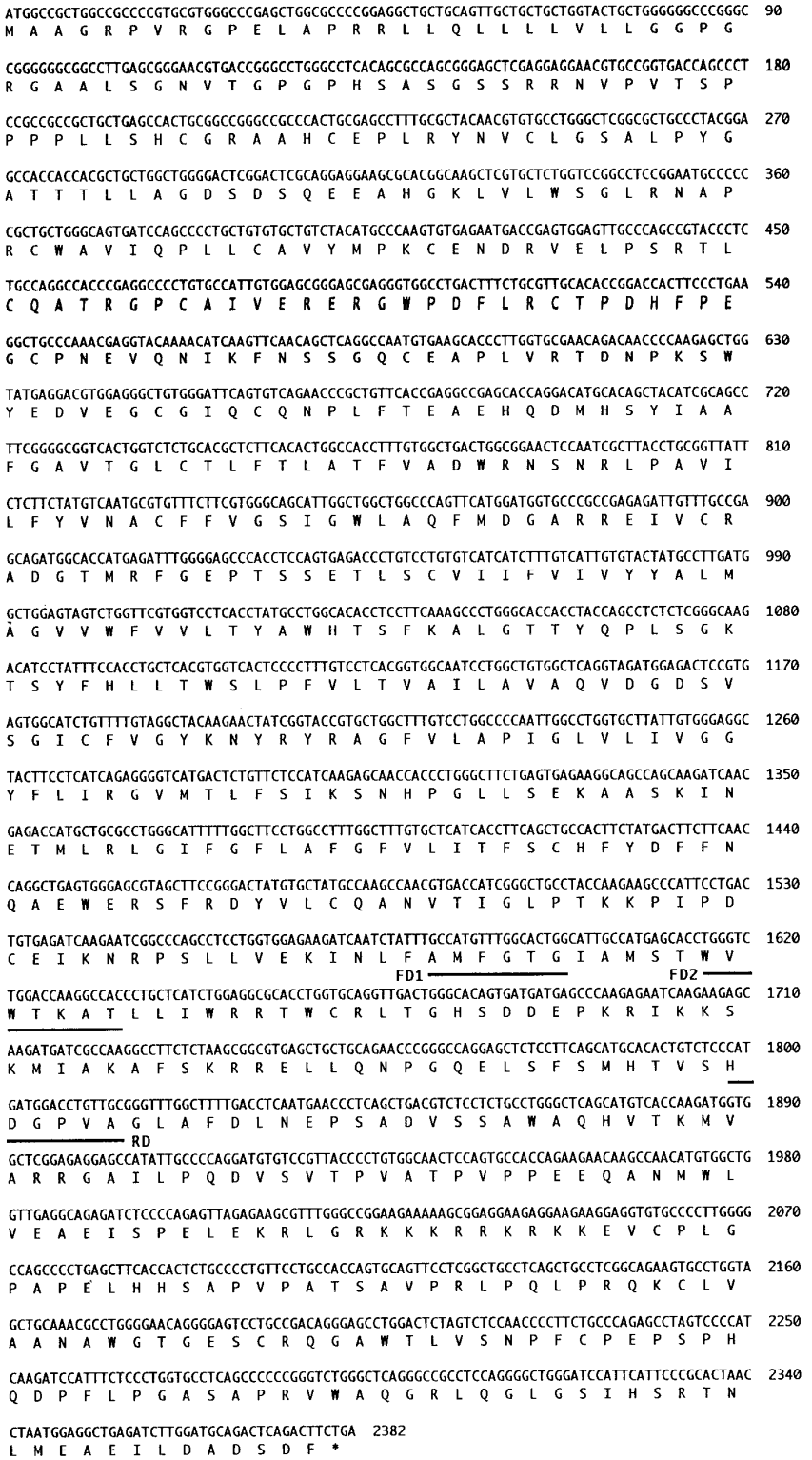

FIG. 2. Nucleotide sequence of *mSmo* and the deduced amino acid sequence. The underlines indicate the amino acid sequences used to design degenerate oligonucleotide primers for the nested-PCR.

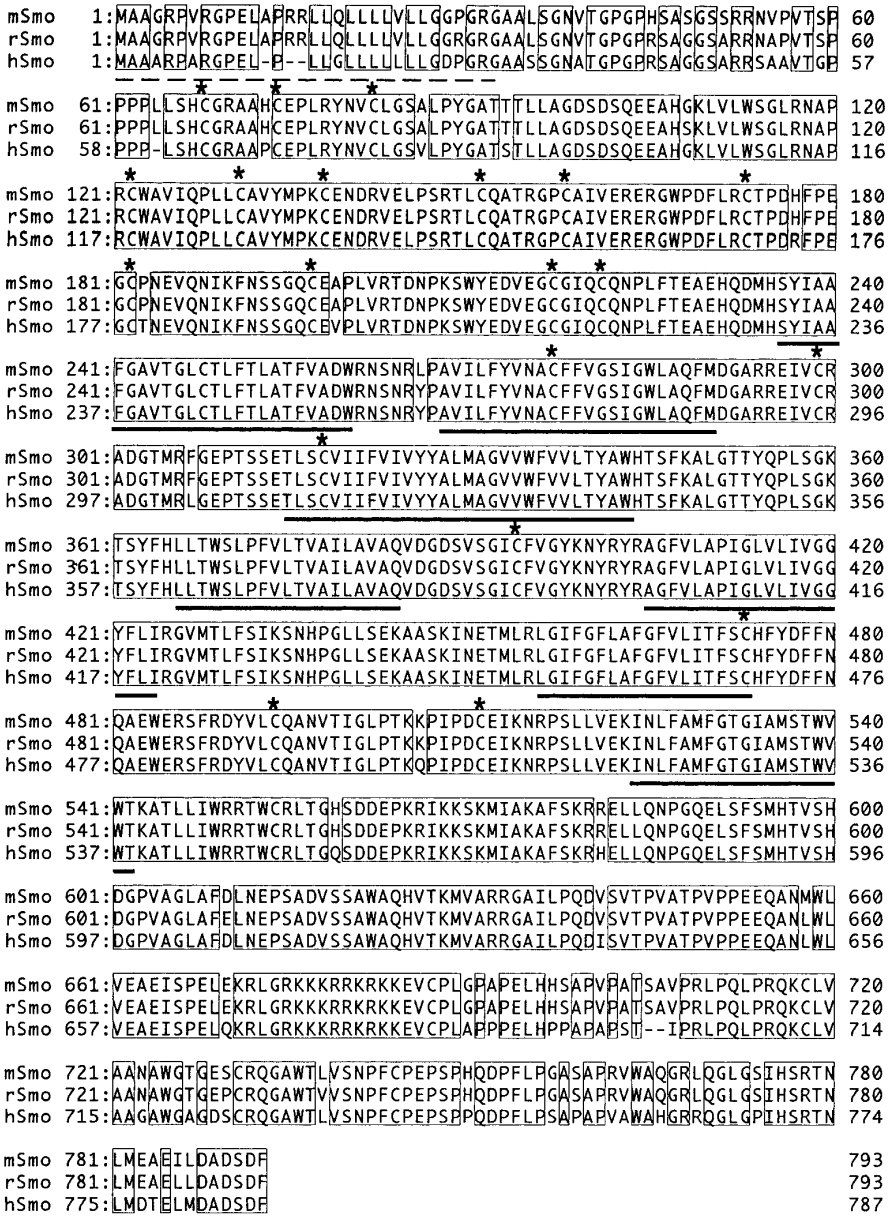

FIG. 3. Comparison of mSmo with rSmo and hSmo. Identical residues are boxed, signal peptide sequences are underlined with a dashed line, and the seven hydrophobic transmembrane domains are boldly underlined. The asterisks indicate the conserved cysteine residues.

CCRTCRTG-3′ (reverse direction) (See FIG. 1). The first PCR reaction with 5 μM each of FD1 and RD primers was cycled once at 94 °C for 5 min, followed by 30 rounds at 94 °C for 30 sec, 40 °C for 30 sec and 72 °C for 30 sec, and finally an extension step at 72 °C for 5 min. The reaction products were separated on 3 % NuSieve 3:1 agarose gels (FMC BioProducts, Rockland, ME), and the DNA fragments of approximately 230-bp were excised and purified using QIAquick Gel Extraction kit (Qiagen Inc., Chatsworth, CA). Aliquots (10 μl) of the extracted solution were used for reamplification by the nested-FD2 and RD primers under the same condition. The amplified PCR products, approximately 200-bp in size, were subcloned into pCRII (Invitrogen, San Diego, CA), and their sequences were confirmed with a ALFred DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden).

cDNA library construction and isolation of a full-length cDNA. Oligo(dT) primed cDNA library from undifferentiated ATDC5 poly(A)$^+$ RNA was constructed in λZAP Express vector (Stratagene, La Jolla, CA), and 1×10^6 plaques were screened with the nested-PCR fragment as a probe. Plaques were transferred to the membranes (137-mm nylon membrane, DuPont NEN, Boston, MA), the PCR fragment was ^{32}P-labeled (BcaBEST labeling kit, Takara, Otsu, Japan), and hybridization was performed in 6× SSPE, 0.2 % BSA, 0.2 % Ficoll 400, 0.2 % polyvinylpyrrolidone, 0.1 % SDS, 100 μg/ml denatured salmon sperm DNA and the ^{32}P-labeled probe for 16 h at 42 °C. The membranes were washed to a final stringency of 0.1× SSPE and 0.1 % SDS at 55 °C.

Northern analysis. ATDC5 cells were plated in six-multiwell plastic plates and cultured for a total of forty two days as described

above. Total RNA was isolated at the indicated time points and analyzed by northern analysis as previously described [12]. Briefly, total RNA (20 μg) was denatured, separated by 1 % agarose gel electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell, Dassel, Germany). The following cDNA fragments were used for hybridization: a 2.4-Kb cDNA fragment containing full coding sequence as a probe for *mouse (m) Smo* mRNA; a 0.9-Kb cDNA fragment as a probe for *mIhh* mRNA; a 0.4-Kb cDNA fragment as a probe for *mPtc* mRNA; a 1.4-Kb EcoRI fragment of pKT1180 as a probe for α1(II) collagen mRNA; and a 0.65-Kb Hind III fragment of pSAm10h as a probe for α1(X) collagen mRNA. After hybridization, the membranes were exposed to X-Omat films (Kodak, Rochester, NY) at −80 °C with Cronex lightening plus intensifying screens (DuPont, Boston, MA).

RESULTS AND DISCUSSION

To isolate a cDNA clone encoding *mSmo,* we first used the nested-PCR with degenerate oligonucleotide primers to obtain a 200-bp cDNA fragment of a partial *mSmo* sequence, which was then used as a probe to screen at high stringency a mouse cDNA library generated from chondrogenic undifferentiated ATDC5 cells. Fifteen cDNA clones were obtained from 1×10^6 independent plaques. Ten out of the fifteen clones contained a full-length cDNA encoding a mouse Smo protein of 793 amino acids (FIG. 2). Partial sequencing of the other five cDNAs revealed that they encoded the same Smo homologue. Analysis of the entire coding sequence showed that mSmo possessed a 28 % amino acid identity with the *Drosophila* Smo. Moreover, mSmo was highly homologous to the rat and human Smo; a 98 % identity with the rSmo and 93 % identity with the hSmo (FIG. 3). Furthermore, it is worth noting that there is a 100 % amino acid identity among mouse, rat and human Smo in their seven hydrophobic transmembrane domains [8]. The strict conservation of the transmembrane domains of Smo in higher vertebrates suggests the possibility that Smo protein has yet unknown biological functions.

We next assessed by northern and RT-PCR analyses the expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in ATDC5 cells. We extracted total RNA on the days 3, 5, 7, 9, 12, 15, 18, 21, 32 and 45, and analyzed the levels of *Ihh*, *Ptc, Smo* and *Gli* transcripts. We also examined the mRNA levels of type II collagen and type X collagen mRNAs, that are phenotypic markers of chondrocytes and hypertrophic chondrocytes, respectively [15,16] (FIG. 4). The expression of type II and type X collagen mRNAs were undetectable in undifferentiated ATDC5 cells on day 3 (FIG. 4 (A), lane a). Induction by insulin of chondrogenesis resulted in cartilage nodule formation and the expression of type II collagen mRNA on day 7 and thereafter (FIG. 4 (A), lanes c-j). The expression of type X collagen mRNA, although at a low level, was first detected on day 15 (FIG. 4 (A), lane f). Medium replacement with the calcification medium on day 21 was associated with the marked increase in the steady state levels of type X collagen mRNA on days 32 and 45 (FIG. 4(A), lanes i and j). The expression of *Ihh* mRNA was first observed on day12 (FIG. 4(A), lane e), exhibiting the time course similar to that of type X collagen mRNA expression. Our observation that *Ihh* was expressed at cartilage differentiation stage in vitro compares with the observation by Vortkamp et al. found *Ihh* expression in vivo in prehypertrophic chondrocytes in chick limb buds [3]. An only *mSmo* transcript, an approximately 4.4-Kb, was found in ATDC5 cells. *Ptc, Smo,* and *Gli* tran-

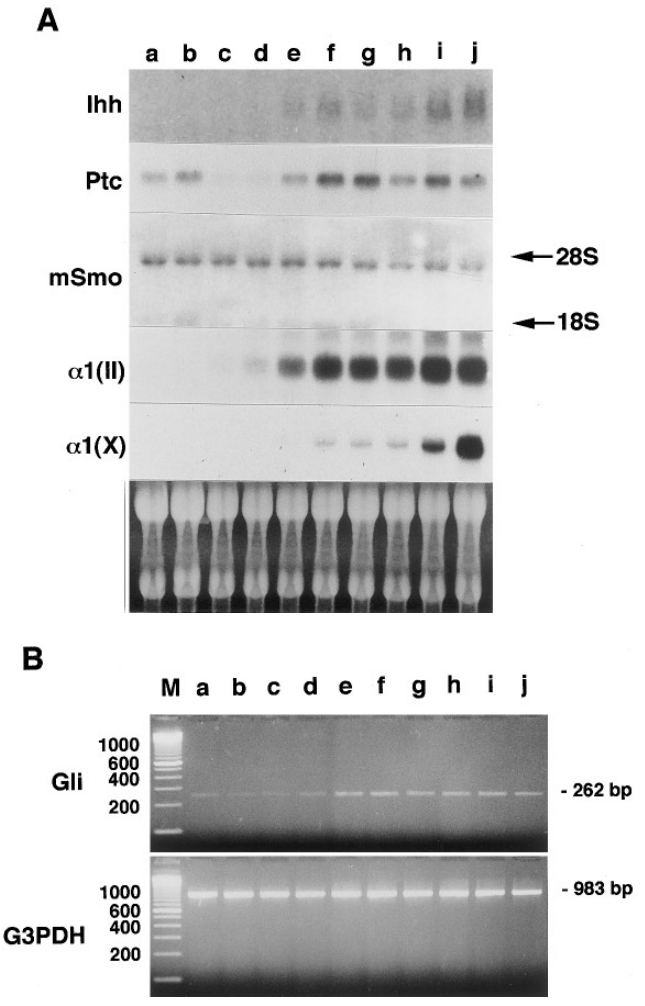

FIG. 4. The mRNA expression patterns of *Ihh, Ptc, Smo, Gli* mRNAs, type II collagen, and type X collagen during chondrogenesis and cartilage differentiation in ATDC5 cells by northern (A) and RT-PCR (B) analyses. Total RNA was prepared from ATDC5 cells on days 3 (a), 5 (b), 7 (c), 9 (d), 12 (e), 15 (f), 18 (g), 21 (h), 35 (i), and 42 (j). (A) 20 μg of total RNA was used per lane. The positions of the 28S and 18S ribosomal RNAs are marked. The integrity of the RNA analyzed was confirmed by ethidium bromide staining. (B) Aliquots (10 μl) of the PCR products were resolved on 3% agarose gels alongside markers (M).

scripts were constitutively expressed over the culture period in ATDC5 cells (FIG. 4(A) and (B)). Interestingly, *Ptc* mRNA level showed transient and substantial decreased at the cellular condensation stage (FIG. 4(A), lane c and d). Relevance of the down-regulation of *Ptc* mRNA at the cellular condensation stage in ATDC5 cells remains unknown.

The expression of hh signalling molecules in ATDC5 cells throughout chondrogenesis and the subsequent cartilage differentiation processes, together with the *Ihh* expression in maturing chondrocyte stages, provide indirect evidence for the hypothesis that hh signalling molecules are involved in cartilage differentiation. Verification of this hypothesis needs further study.

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