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Cloning and Characterization of Porcine Common γ Chain Gene

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ABSTRACT

The common γ chain, which was originally identified as a component of interleukin-2 receptors (IL-2R), plays a key role in differentiation of T lymphocytes and natural killer (NK) cells. In the present study, cDNA of the porcine common γ chain gene and its genomic DNA were molecularly cloned and characterized. The porcine common γ chain gene was found to consist of 8 exons, spanning approximately 3.7 kb, and to encode a 368-amino acid polypeptide. The amino acid sequence showed 82.4%, 71.1%, 86.1%, and 84.8% similarities with that of human, murine, bovine, and canine chains, respectively. The common γ chain gene was assigned to swine chromosome Xq13 by FISH analysis and was consistent with the result of radiation hybrid (RH) mapping. When various porcine tissues were examined for the expression of this gene, the expression was observed in lymphocytes and lymphocyte-related tissues. Since GATA, T cell factor-1 (TCF-1), Ets-1, activated protein-2 (AP-2), and Ikaros2 binding motifs were demonstrated in the 5′ upstream region of this gene, promoter activity was investigated using luciferase gene as a reporter. The results indicate that the Ets-1 binding motif in the segment from −95 to −59 (major transcription initiation site: +1) was an essential *cis*-acting regulatory element for the common γ chain gene in lymphoid cells.

INTRODUCTION

THE INTERLEUKIN-2 (IL-2) RECEPTOR (IL-2R) is a multicomponent complex consisting of three subunits, α, β, and γ polypeptide chains, and different combinations of these subunits show different affinity to IL-2. The α chain functions primarily in binding IL-2, whereas the β and γ chains contribute to IL-2 binding and are also essential to IL-2-induced activation of signaling pathways leading to T lymphocyte growth. Because the γ chain and the β chain have conserved cysteine residues and a WSXWS motif, these chains are members of the cytokine receptor superfamily.[1] The γ chain was shown to interact with Janus family tyrosine kinase 3 (Jak3) in the cytoplasmic domain and transduce ligand signals intracellularly by phosphorylation of Jak3.[2–4] It has also been demonstrated that the γ chain is a component of IL-4, IL-7, IL-9, IL-15, and IL-21 receptors.[5–10] Therefore, the γ chain is also called common cytokine receptor γ chain and is abbreviated as common γ chain or γ c (we refer to it as the common γ chain in the present report). The common γ chain is found in almost all lymphocytes, including T lymphocytes, B lymphocytes, natural killer (NK) cells, monocytes, and granulocytes.[11]

In humans, an X chromosome-linked severe combined immunodeficiency (SCID-X1) was located on a region of chromosome X by linkage analysis,[12,13] to which the common γ chain gene has been assigned.[14] When the common γ chain gene was examined as a candidate gene for genetic disease, it was revealed that SCID-X1 patients had mutations or deletions in this gene, leading to truncations of the common γ chain and possibly to loss of its function.[14] This indicated that a deficiency in the common γ chain gene was responsible for SCID-X1. In addition, knockout (KO) mice missing this gene demonstrated similar phenotypes to humans with SCID-X1, preventing the formation of T and B lymphocytes and NK cells,[15–17] which provided evidence for this indication. IL-7, IL-7R, and Jak3 KO mice also have demonstrated similar phenotypes to common γ chain-deficient mice,[18–21] whereas IL-2-deficient mice had normal lymphocytes.[22,23] These find-

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ings taken together indicate that the common γ chain-Jak3 pathway mediated by IL-7R is essential for differentiation and proliferation of lymphocytes.

Pigs frequently are used as models for human disease and are also considered to be source animals for xenotransplantation to humans because of their anatomic and physiologic similarities to humans.[24] The complement system, organization of the swine leukocyte antigen (SLA) locus, and sequence of constant regions of immunoglobulins (Ig) are similar to those of humans.[25] On the other hand, differences between pigs and humans are observed, such as the much higher ratio of γδ T lymphocytes in peripheral blood in pigs than that in humans.[26] Therefore, understanding the porcine immune system is prerequisite to extending the findings of previous studies. Because knowledge of the porcine immune system is still limited, however, in the present study, we molecularly cloned and characterized the common γ chain gene in order to understand the porcine immune system through the function of this gene.

MATERIALS AND METHODS

Preparation of porcine spleen

To prepare the spleen, a 1-month-old female pig was used. The animal received humane care as described in the Guidelines for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences Care Committee, Japan). The process for preparation of the spleen followed the guidelines of animal ethics at the National Institute of Agrobiological Sciences. The pig was killed by intravenous injection of 10 ml sodium pentobarbital. Immediately after respiration and heartbeat stopped, the spleen was excised for preparation of cDNA.

cDNA preparation and sequence analysis of common γ chain cDNA

RNA was extracted from the spleen by the guanidinium thiocyanate acid-phenol-chloroform method.[27] Using the RNA thus obtained as a template, cDNA was prepared with AMV reverse transcriptase (RT) (Toyobo, Osaka, Japan) with oligo-$d(T)_{20}$ primer flanked by CGCCAGGGTTTTCCCAGTCACGAC at the 5′-end, and a rapid amplification of cDNA ends (RACE) template was prepared with a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). In order to amplify DNA fragments from the common γ chain cDNAs supposed to be produced in the procedure, a primer pair (Table 1, primer 1 and primer 2) was designed based on the sequence conserved among human, murine, and bovine common γ chain cDNAs. PCR reactions were performed using the AmpliTaq Gold system (Perkin-Elmer Biosystems, Foster City, CA) with the cDNA as a template and using primers 1 and 2. After an initial denaturation and enzyme activation step at 95°C for 9 min, 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec were performed. The PCR product was confirmed by 2% agarose gel electrophoresis, then sequenced by the primer-walking method using BigDye terminators and an ABI DNA sequencer (Perkin-Elmer Biosystems). In order to sequence the 5′ and 3′ terminal regions of the common γ chain cDNAs, RACE primers for the terminal regions were designed based on the sequence obtained in the analysis. The 5′ and 3′ RACE analyses were performed using the Marathon cDNA Amplification Kit, following the manufacturer's instructions.

For the transcription initiation site, 5′ RACE was performed using the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen Co., San Diego, CA), and the DNA fragments thus obtained were cloned in pBluescript KS(−). The >50 fragments were then sequenced to determine major transcription initiation sites.

TABLE 1. PCR PRIMERS FOR PORCINE COMMON γ CHAIN GENE

Primer	*Sequence (5′ → 3′)*[a]
cDNA cloning and BAC library screening	
Primer 1	AGTCTGCAGCCAGACTACAG
Primer 2	TTTAGGGTGTAACATGGGGG
Primer 3	TCTGCAGCCAGACTACAGTGAACGGCTCTGCCACG
Primer 4	GGGGAAATCTCACTGACGTGGCAGAGCCGTTCACTG
Primer 5	GACGTGGCAGAGCCGTTCACTGTAGTCTGGCTGCAG
Primer 6	GAGAGTCCCAGGGGGTGTAGAGAGC
Primer 7	GAGGACCTTCGGGTTCAGTC
RH mapping	
Primer 8	GCGCTCAGCGTTGGAGCGACTGGAG
Primer 9	GCCAAAGACATCGGTGCTACAAGGG
Promoter analysis	
Primer 10	CCCAAGCTTTGACAAAAGGAAATGTGTGGGTGGG
Primer 11	CCCAAGCTTAGCCCTGGTTTCTAAGGTTCTTTCC
Primer 12	CCCAAGCTTCACCTAATCTCCCAGAGGATTTAGC
Primer 13	CCCAAGCTTAGACTGTATGTTTCATCTGGCCAAG
Primer 14	CCCAAGCTTCTGCTCCCACAACAGCTAAAGGTGG
Primer 15	CCCAAGCTTACCTCATGGTTCCTAGTCGGATTCG HindIII site
Primer 16	CATGCCATGGCGCTTACTCCTTGTTTCCTGGGTG NcoI site

[a]Underlined sequences indicate additional oligonucleotides encoding restriction sites.

Analysis of genomic structure

To select bacterial artificial chromosome (BAC) clones containing the common γ chain gene, a porcine genomic BAC library constructed by Suzuki et al.[28] was screened by PCR. The forward and reverse primers are described in Table 1 as primer 1 and primer 2, respectively. The procedure for screening the BAC library was the same as that described previously.[28] The DNAs of BAC clones thus obtained were cleaved by EcoRI and subjected to Southern blot analysis using ^{32}P-labeled cDNA fragment comprising the coding sequence of the common γ chain gene. Then the fragments presenting hybridization signals were subcloned into pBluescript KS(−) and sequenced by the primer-walking method using BigDye terminators and an ABI DNA sequencer. The exon/intron structure was determined by comparison with the cDNA sequence.

Mapping using IMpRH panel

IMpRH panel DNAs were kindly provided by INRA (France) and the University of Minnesota[29] for radiation hybrid (RH) mapping. Primer pairs were designed based on the genomic sequence to encompass the splicing sites and then were examined to select those that amplify only porcine genomic DNA fragments of the expected size or amplify both porcine genomic DNA fragments of expected size and Chinese hamster genomic DNA fragments of different sizes from those of the porcine fragments. The amplified porcine genomic fragment was sequenced to confirm that the fragment was amplified from the porcine common γ chain gene. PCR was then performed in duplicate using the IMpRH panel DNAs, and the PCR products were scored as described by Hawken et al.[30] Assignments of sequences to the RH map were performed with the RH2PT, a

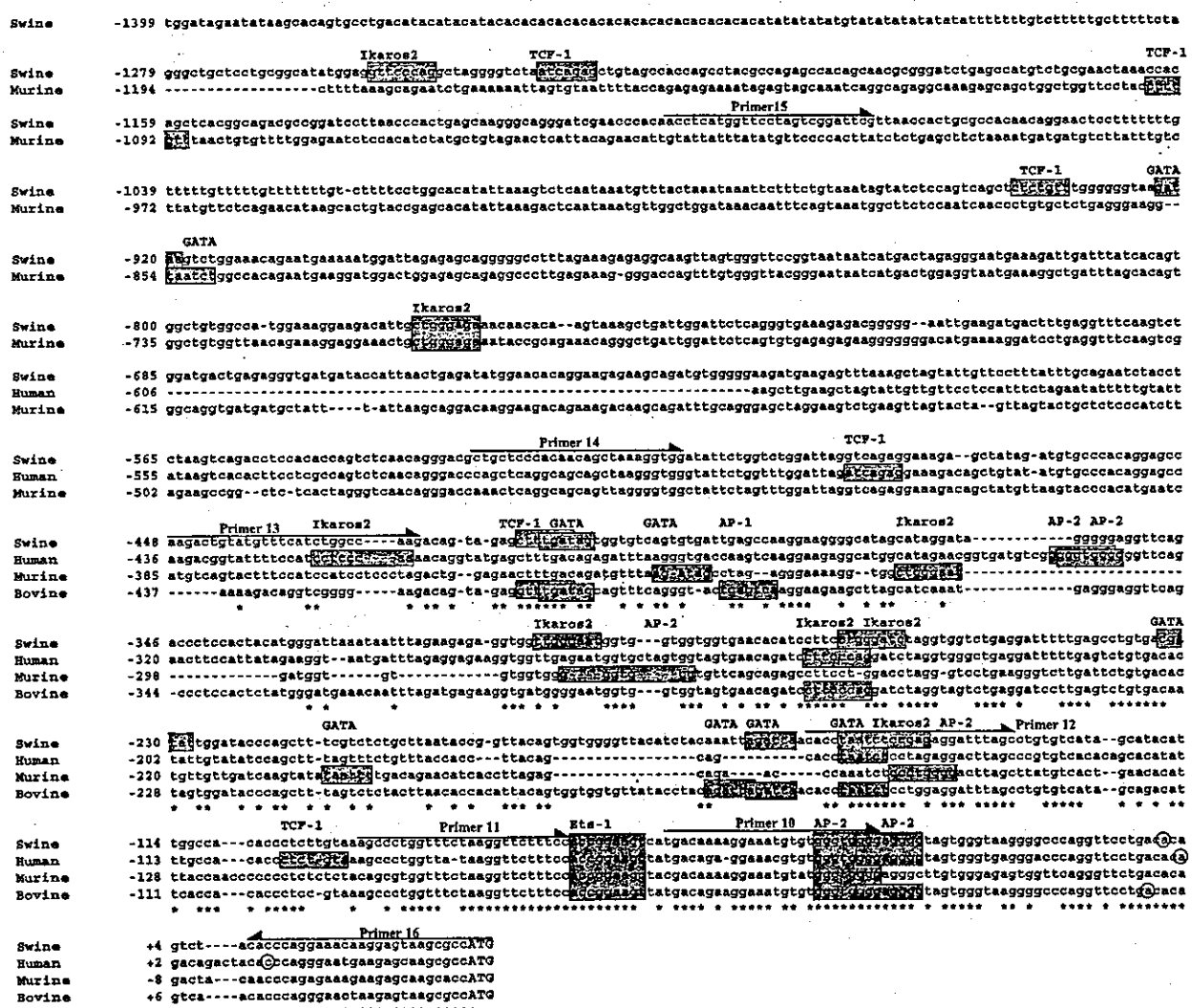

FIG. 1. Promoter region of porcine common γ chain gene. To clarify the transcriptional regulatory mechanisms of the porcine common γ chain, a region upstream of the translation initiation site was sequenced and compared with those of human, murine, and bovine promoter regions. Binding motifs for the GATA, T cell factor-1 (TCF-1), Ets-1, and activated protein-2 (AP-2) found in the upstream region are indicated by gray boxes. Major transcription initiation sites are indicated by circles. Translation initiation sites are placed at the end of the sequences and are indicated in uppercase letters. As a reference, primers 10–16 were used for the construction of a series of nested 5′ deletions in the upstream region shown in Figure 7.

part of the RHMAP3 program,[31] with PCR pattern data obtained from reference 30.

Mapping with fluorescence in situ *hybridization (FISH)*

Peripheral blood cells of male pigs were cultured and labeled with 5-bromodeoxyuridine (5-BrdU) as described previously.[32] The cultured cells were treated with hypotonic solution and fixative and then spread on glass slides as described previously.[33] The chromosome spreads thus obtained were subjected to FISH using biotin-labeled BAC DNA as a probe, following a procedure described previously.[34,35] Briefly, the BAC DNA (500 ng) containing the common γ chain gene was biotinylated with a biotin nick-translation labeling mix kit (Roche Diagnostics, Mannheim, Germany). The biotinylated DNA was dissolved in 10 μl formamide to mix with 10 μl 2 × hybridization buffer (4× SSC, 100 mM phosphate buffer, pH 7.0, 20% dextran sulfate, 2× Denhardt's solution, and 0.2% SDS) containing 5 μg porcine Cot-5 DNA (repetitive sequence-enriched DNA) and subjected to hybridization with chromosomal DNA.

Northern blot analysis

A 10-ml blood sample was collected from a 7-day-old male pig, and red blood cells (RBCs) were removed by ACK lysing buffer lysis to prepare peripheral blood lymphocytes (PBLs), following the procedure described.[36] The pig was killed by overdose injection of sodium pentobarbital into a vein. Immediately after respiration and heartbeat stopped, samples of brain, thymus, lung, heart, stomach, liver, spleen, adrenal gland, small intestine, large intestine, bladder, testis, and skin were collected from the pig. In addition, L35 and L45 line cells, purchased from the European Collection of Cell Culture (*www.ecacc.org.uk/*), were cultured in RPMI medium 1640 (Invitrogen Co., CA) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Porcine fibroblast cells prepared from the ear tissue of a gilt, in F-10 Nutrient Mixture Medium (Invitrogen), were supplemented with 20% FBS, 50 U penicillin and 50 μg streptomycin/ml at 37°C in 5% CO_2. Total RNAs were prepared from these cells and the earlier samples using the Sepasole-RNA I reagent (Nacalai Tesque, Kyoto, Japan), according to the method described by the manufacturer.

Each total RNA sample (10 μg) was electrophoresed in a

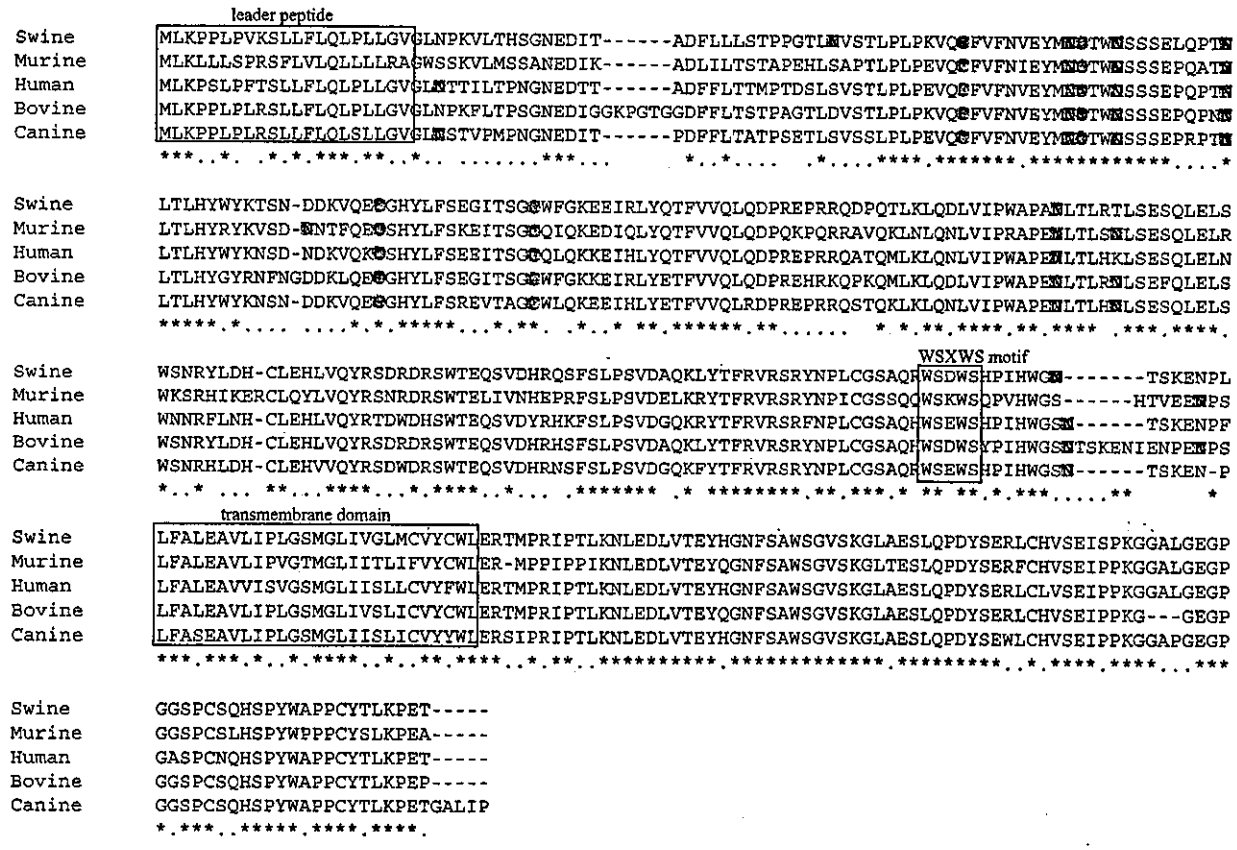

FIG. 2. Alignment of swine, murine, human, bovine, and canine common γ chain amino acid sequences. Putative leader peptide, WSXWS motif, and putative transmembrane domain are boxed. Four conserved cysteine residues and potent *N*-linked glycosylation sites are indicated by gray circles and gray boxes, respectively. The porcine common γ chain amino acid sequence showed 71.1%, 82.4%, 86.1%, and 84.8% similarities with those of murine, human, bovine, and canine common γ chains, respectively.

1% agarose gel containing 18% formalin, and the RNA in the gel was stained with ethidium bromide to examine the quality and quantity of the RNA applied on the gel. The RNA was transferred to Hybond-N nylon membranes (Amersham Biosciences, Arlington Heights, IL). The membrane thus obtained was subjected to Northern blot hybridization, following the procedure described by Church and Gilbert.[37] Briefly, for probe DNA, the DNA fragment comprising the coding sequence for the common γ chain was labeled with ^{32}P-dCTP using a random priming labeling kit (High prime) (Roche Diagnostics). The membrane was hybridized with the probe DNA at 65°C overnight and washed as indicated in the procedure. The radioactivity on the membrane was visualized using the bioimaging analyzer FLA-3000G (Fuji Film, Tokyo, Japan).

Construction of reporting vectors

DNA fragments of various lengths ranging from the translation initiation site toward the 5′ upstream sites were produced by PCR amplification using the primer pairs shown in Table 1 (primers 10–16) and Figure 1. The PCR amplification was performed using KOD DNA polymerase (Toyobo), 0.2 mM each dNTP, 1 mM $MgCl_2$, an appropriate primer pair, and buffer supplied by the manufacturer. The fragments produced were treated with restriction enzymes HindIII and NcoI to be subcloned into the HindIII/NcoI site of the multicloning site of pGV-B2 reporter vectors, which contained the firefly luciferase gene downstream of the multicloning site as a reporter gene (PicaGene Basic Vector2) (Toyo Ink, Tokyo Japan). The reporting vectors thus constructed were verified by sequencing to determine if the vectors were those expected. As a control for the transformation experiment, a reporting vector containing thymidine kinase promoter and renilla luciferase (pRL-TK) (Promega, Madison, WI) was used. For the transformation of cells, all vector DNAs were prepared through two cycles of $CsCl_2$ density gradient centrifugation just before use.

Transformation of cells with reporting vectors and measurement of luciferase activity in the transformants

The vector DNA constructs were introduced together with the control vector, pRL-TK, into porcine T lymphocyte line cells L45[38] and primary cultured porcine fibroblast cells. For transformation of L45 cells, 1 μg appropriate vector DNAs and

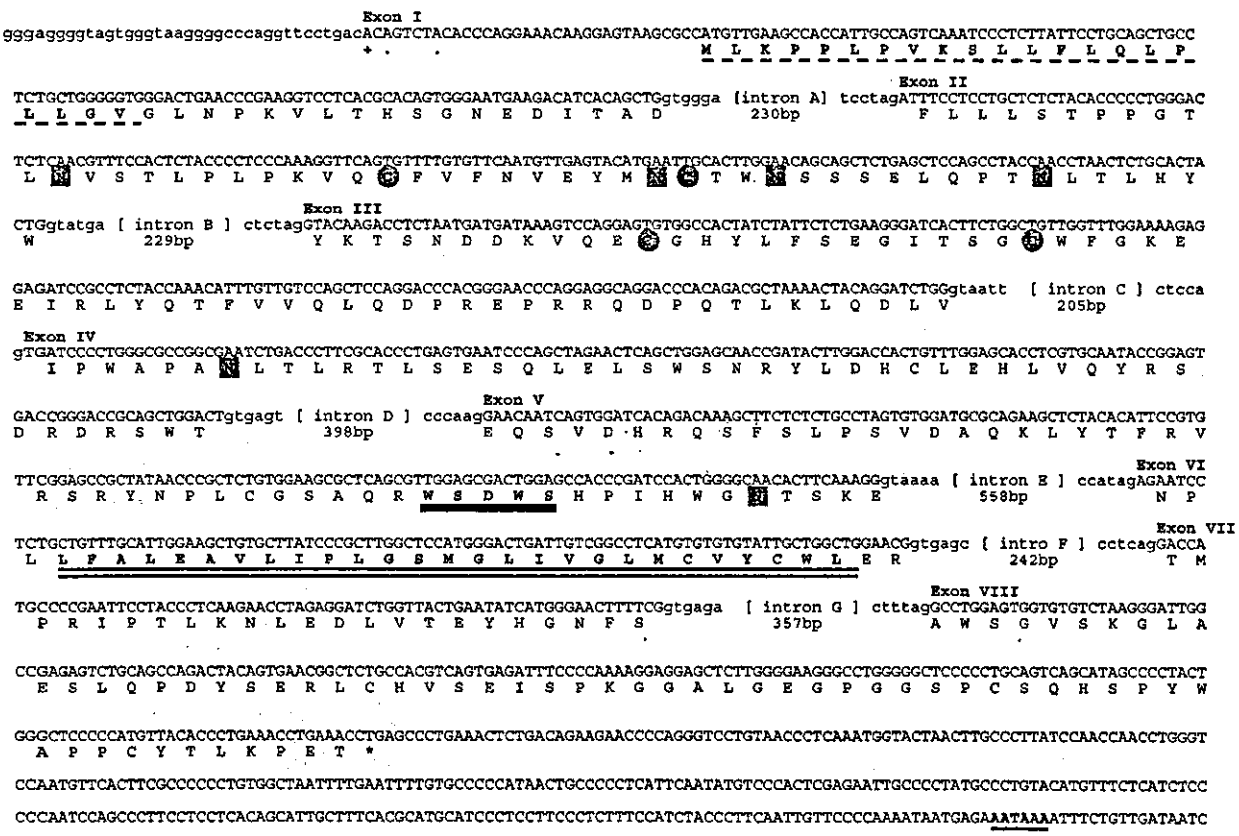

FIG. 3. Genomic organization of porcine common γ chain gene (DDBJ/GenBank/EMBL database, Accession Number: AB092652). The structure of porcine common γ chain gene is shown by base sequence. The cDNA sequence of common γ chain is indicated in uppercase letters. The single-letter amino acid sequence is shown below the corresponding triplets. The putative leader peptide is marked by a broken underline, and the possible polyadenylation signal is indicated by an underline. Four conserved cysteine residues and potent *N*-linked glycosylation sites are indicated by gray circles and gray boxes, respectively. The WSXWS motif is boldly underlined, and the putative transmembrane domain is double underlined. The major transcription initiation site is indicated by +, and two minor transcription initiation sites are indicated by dots.

100 ng pRL-TK were mixed with DMRIE-C reagent (Invitrogen) and placed in each well in 24-well plates. Subsequently, L45 cells were placed into each well at a concentration of 5×10^5 cells per well, according to the method described by the manufacturer. For transformation of the fibroblast cells, 100 ng appropriate vector DNA and 10 ng pRL-TK were mixed with Lipofect Amine Plus (Invitrogen) and placed in each well in 24-well plates, each of which contained 1×10^4 fibroblast cells, according to the method described by the manufacturer. Six wells were used for the transformation with each construct. The resulting cells were cultured in the medium for an additional 48 h and subjected to luciferase assay.

Firefly and renilla luciferase activities in the cells of each well were measured using the Dual-Luciferase Assay System (Promega) and luminometer TD-20/20 (Turner Designs, Sunnyvale, CA), following the protocols described by the manufacturers. To normalize the transformation efficiency, firefly luciferase activity was divided by renilla luciferase activity in each transformation, and the resulting value was regarded as promoter activity for the firefly luciferase gene in each construct.

RESULTS AND DISCUSSION

cDNA cloning and sequence

Total RNA was extracted from the spleen of a 1-month-old female pig and processed to obtain cDNA of the transcripts from the common γ chain gene. The cDNAs thus obtained were subjected to sequencing, demonstrating that the porcine putative common γ chain consisted of 368 amino acid (Fig. 2). When the amino acid sequence of the porcine putative common γ chain was compared with that of common γ chains of other species reported[1,39–41] (Fig. 2), the porcine putative common γ chain was revealed to have 82.4%, 71.1%, 86.1%, and 84.8% similarities with that of human, murine, bovine, and canine chains, respectively. This indicated that this putative common γ chain was indeed the porcine common γ chain.

Based on this comparison, a 21-amino acid sequence from the N-terminus of porcine common γ chain was indicated to be a leader peptide, a 234-amino acid sequence following the preceding sequence to be an extracellular domain, a subsequent 27-amino acid sequence to be a transmembrane domain, and the remaining 86 amino acids of the C-terminus to be an intracellular domain. The porcine common γ chain was found to contain features of the cytokine receptor superfamily, that is, 4 conserved cysteine residues and a WSXWS motif (Fig. 2), as are commonly observed in the common γ chain of other species reported.[1,39–41] In addition, 6 potent *N*-linked glycosylation sites (N-X-S/T) were found in the extracellular domain, and 4 of them were conserved in the common γ chains of the other four species (Fig. 2). When the similarities in amino acid sequences among swine, human, murine, bovine, and canine were examined from the standpoint of functional domain, the highest similarities ranging from 84% to 95% were observed in the intracellular domain (unpublished data).This indicates that the intracellular domain possesses evolutionally common role(s) in these animal species.

To determine the transcription initiation site of the porcine common γ chain gene, 5′-RACE was performed as described using the primers (primer 6 and primer 7) shown in Table 1, and the DNA fragments thus obtained were cloned into plasmid vectors. Fifty-seven clones containing fragments were subjected to sequencing, revealing the following: 17 clones showed

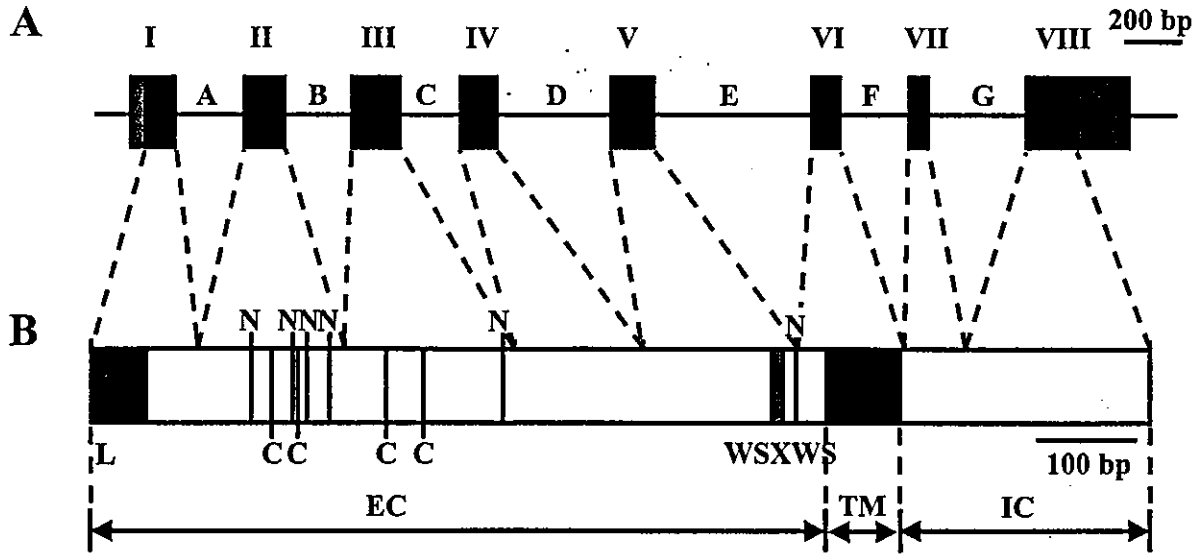

FIG. 4. Schematic presentation of porcine common γ chain gene structure. (A) Genomic structure of porcine common γ chain gene. Black boxes represent exons and are numbered accordingly, and gray boxes represent 5′-UTR and 3′-UTR (untranslated regions). (B) Porcine common γ chain cDNA. IC indicate L, putative leader peptide; C, conserved cysteine residues; N, potent *N*-glycosylation sites; WSXWS, WSXWS motif; EC, putative extracellular domain; TM, putative transmembrane domain; IC, putative intracellular domain. The correspondences between exons and the regions of the cDNA are shown by broken lines.

a transcription initiation site at the position 34 bp upstream of the translation start site, 11 clones at the position 32 bp upstream of the translation start site, and 8 clones at the position 27 bp upstream of the translation start site. The remaining clones showed various sites, including sites located in the protein coding region. These findings indicated that the major transcription initiation site was at the position 34 bp upstream of the translation start site and that minor initiation sites were at the positions 32 bp and 27 bp upstream of the translation start site.(Hereafter, the major transcription initiation site numbered +1 in the genomic sequence of the porcine common γ chain gene, and this numbering is used in the text and the figures in the present study.)

Genomic structure of the common γ chain gene

In order to investigate the genomic structure of the porcine common γ chain gene, a porcine BAC DNA library was screened to obtain a BAC clone containing at least a part of the common γ chain gene. The BAC DNA was then subjected to Southern blot analysis, demonstrating that 2.5-kb, 6.0-kb, and 15.0-kb EcoRI fragments contained sequences of the common γ chain gene. Therefore, those fragments were subcloned into the plasmid vector and sequenced by the primer-walking method. In total, a 5355-bp sequence was determined to elucidate the genomic structure of the porcine common γ chain gene (Accession Number AB092652) (Fig. 3). The porcine common γ chain was revealed to consist of 8 exons, spanning approximately 3.7 kb (Fig. 4A). The translation start site is coded in exon I, the extracellular domain is from exon I to exon VI, the transmembrane domain is in exon VI, and the intracellular domain is from exon VI to exon VIII (Fig. 4B). The exon/intron structure of the porcine common γ chain was the same as that of human, mouse, and bovine chains. Even the sequence of introns showed a high similarity between swine and bovine, which may be expected in that both species are classified in the same order, Artiodactyla.

When a 1399-bp upstream region of the major transcription initiation site was examined for regulatory elements shown to have functions in blood cells, motifs for GATA binding pro-

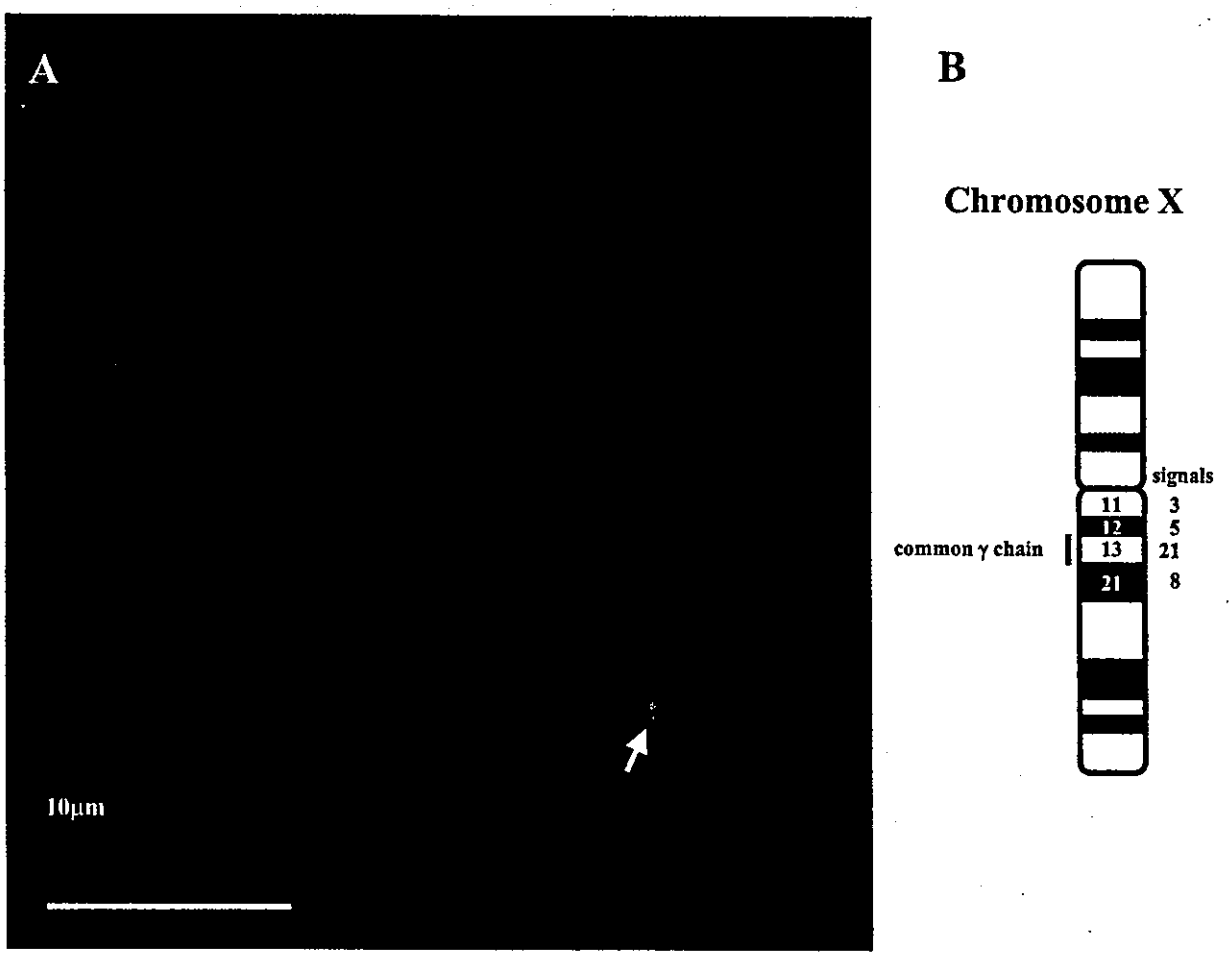

FIG. 5. Chromosomal location of porcine common γ chain gene. Swine chromosome spreads were subjected to FISH using the BAC DNA containing the porcine common γ chain gene as a probe. (A) A representative chromosome spread presenting hybridization signals is shown. The arrow indicates the chromosomal position of the common γ chain gene on chromosome Xq13. (B) Thirty-seven chromosome spreads presenting hybridization signals were scored for their localizations. An ideogram of swine chromosome X is shown with the score of the signals at the respective chromosomal regions.

tein (WGATAR, AGATTA),[42,43] T cell factor-1 (TCF-1) (WWCARAG),[44] Ets-1 (RCCGGAWGY),[45] enhancer binding protein AP-2 (CCCMNSSS),[46] and lymphoid-restricted zinc-finger transcription factor Ikaros2 (YTGGGANN)[47] were detected. As shown in Figure 1, although these motifs were commonly observed in the upstream regions of human, mouse, and bovine, the only elements having a positional correspondence were the Ets-1 binding motif and one AP-2 binding motif, both of which were located in the upstream region proximal to the major transcription initiation site (−95/+1). Neither the TATA box nor the CCAAT box was detected in the porcine regulatory region examined, as is the case for the other species.

Chromosomal location of porcine to common γ chain gene

To determine the chromosomal location of the porcine common γ chain gene, RH mapping was first performed using IMpRH panel DNAs using primer 8 and primer 9 (Table 1) as described, suggesting that the common γ chain gene was linked to Sw1835 with a lodscore of 4.59, which was indicated to localize in the region of swine chromosome (SSC) Xcen-p21 (*sol.marc.usda.gov/*). However, as the lodscore was not greater than the threshold of significance[30] and the chromosomal position of SW1835 was not accurately indicated, FISH analysis was performed using biotinylated BAC DNA containing common γ chain gene as a probe. A representative chromosome spread having hybridization signals is shown in Figure 5A. Thirty-seven chromosome spreads presenting hybridization signals were scored for their localizations, revealing that the common γ chain gene resides on SSCXq13 (Fig. 5B). The finding from FISH analysis was consistent with that of RH mapping, and localization of the common γ chain gene on chromosome X was conserved in human, mouse, and bovine genomes.

In the additional analysis of the BAC DNA containing the common γ gene, exon III of the AFX1 gene was fortuitously found about 5 kb downstream of the common γ chain gene in the opposite direction from the common γ chain gene (unpublished data). This observation is the same as that for humans[48] (*www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=4303*) and mice (*www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=54601*).These findings indicate that the common γ chain gene, including its neighboring region, has been conserved in various animal species.

Expression of common γ chain gene in various porcine tissues

Expression levels of the common γ chain gene in various porcine tissues were examined by Northern blot analysis. As shown in Figure 6, high-level expressions were observed in thymus, spleen, lymphocytes, L35 cells, and L45 cells. Low-level expressions were observed in lung and small and large intestines, and no or little expression was observed in brain, heart, stomach, adrenal gland, bladder, testis, muscle, skin, and fibroblasts. A major transcript from the gene was detected at the position of 2 kb, the size of which was estimated from the cDNA sequence described in the preceding section. In addition, two minor transcripts were detected at positions 4 kb and 6 kb. As the common γ chain gene was indicated to be a single copy gene in swine by Southern blot analysis (data not shown), these

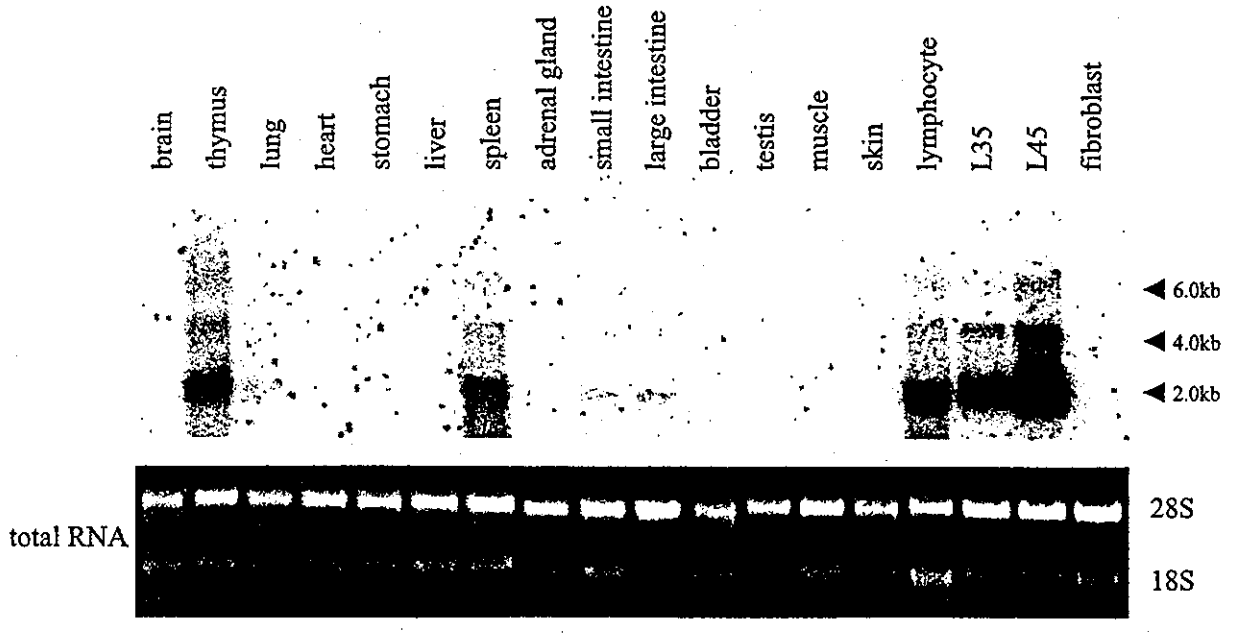

FIG. 6. Northern blot analysis of RNAs obtained from various porcine tissues using common γ chain cDNA as a probe. Each total RNA sample (10 μg) prepared from various tissues and cells was electrophoresed in a 1% agarose gel containing 18% formalin, and the RNA in the gel was stained with ethidium bromide to examine the quality and quantity of the RNA applied on the gel. The RNA was transferred to Hybond-N nylon membranes. The membrane thus obtained was subjected to Northern blot hybridization following the procedure described by Church and Gilbert.[37] For probe DNA, the DNA fragment comprising the coding sequence for the common γ chain was labeled with ^{32}P-dCTP. The membrane was hybridized with the probe DNA at 65°C overnight and washed as indicated in the procedure. The radioactivity on the membrane was visualized.

transcripts were generated from additional transcription initiation sites located far upstream of the major transcription initiation sites or ended downstream of the polyadenylation signal indicated in Figure 3. When the signal ratios of these three transcripts detected were calculated, the ratios were found to be similar in samples subjected to the calculation (data not shown).

The porcine common γ chain gene was found to be expressed exclusively in lymphocytes and lymphocyte-related tissues, and this expression pattern was similar to that reported in the mouse.[24] However, a 6-kb transcript from the gene in swine was not observed in humans and mice, which may suggest that transcription control for the porcine common γ chain gene is slightly different from that in humans and mice.

Regulatory elements in the upstream region of porcine common γ chain gene

In order to specify the region containing the functional regulatory elements in the upstream sequence of the porcine common γ chain gene, a series of nested 5′ deletions were introduced into the upstream sequence of the common γ chain gene in the reporting construct described. Based on the fact that the regulatory elements for the human common γ chain gene were indicated to be confined to the segment from −177 to 34 (+1 corresponds to the major transcription initiation site)[49] and the fact that the regions having positional correspondence of regulatory elements (Ets-1 and AP-2 binding motifs) were located within the −95 to +1 segment (Fig. 1), 6 deletion constructs were produced (Fig. 7). The resulting constructs were introduced into L45 cells and fibroblast cells together with pRL-TK DNA as a control for transformation.

As described in Materials and Methods, the activity of firefly luciferase was normalized in each transformation and is presented as promoter activity in Figure 7. The promoter activity was observed in the segment from −95 to +34 and in the segments longer than the preceding segment, but it was not observed in the segment from −59 to +34. The activity of the segment from −95 to +34 was found to be two thirds of the maximum activity observed in the transformation experiments, and the activity was found to increase with increases in the

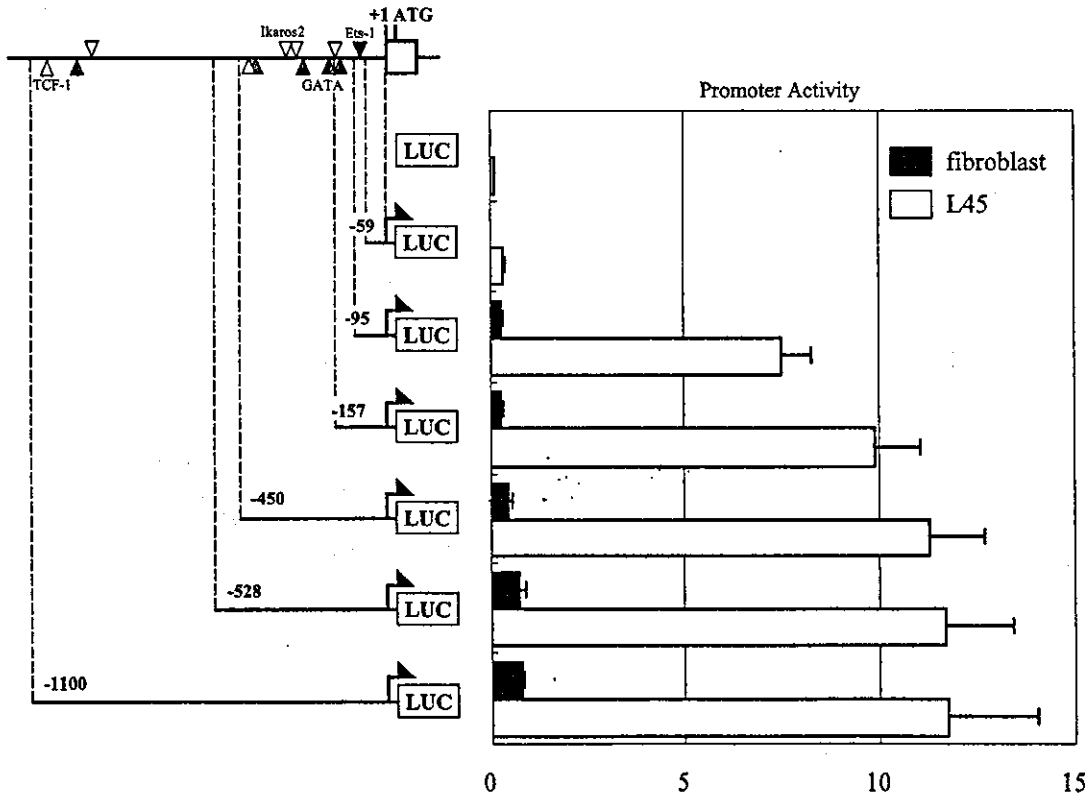

FIG. 7. Promoter activity of the 5′ upstream region of the porcine common γ chain gene. DNA fragments with various lengths ranging from the translation initiation site toward the 5′ upstream sites were produced by PCR amplification using primer pairs shown in Table 1 and Figure 1 (primers 10–16). The fragments produced were treated with restriction enzyme HindIII and NcoI to be subcloned into the HindIII/NcoI site of the multicloning site of pGV-B2 reporter vector, which contained the firefly luciferase gene downstream of the multicloning site as a reporter gene. The vector DNA constructs were introduced together with the control vector, pRL-TK, into porcine T lymphocyte line cells, L45,[38] and porcine fibroblast cells as described in Materials and Methods. Six wells were used for the transformation with each construct. The resulting cells were cultured for an additional 48 h and subjected to luciferase assay. Firefly and renilla luciferase activities in the cells of each well were measured using the Dual-Luciferase Assay System and luminometer TD-20/20 following the protocols described by the manufacturers. To normalize the transformation efficiency, firefly luciferase activity was divided by renilla luciferase activity in each transformation, and the resulting value was regarded as the promoter activity for the firefly luciferase gene in each construct.

length of the segment to attain a plateau with the segment from −450 to +34. On the other hand, little promoter activity, if any, was observed in any of the transformations using fibroblast cells. When these results are taken together, the Ets-1[45] binding motif located in the segment from −95 to −59 is strongly indicated to be an essential *cis*-acting regulatory element for the porcine common γ chain gene in lymphoid cells, and some or all of GATA,[42,43] TCF-1,[44] AP-2,[46] and Ikaros2[47] binding motifs located between −450 and −95 are indicated to serve as additional, *cis*-acting regulatory elements for the common γ chain gene expression in those cells.

Similar observations were reported in human.[49,50] Additionally, in the analysis of the human common γ chain gene, destruction of the corresponding Ets-1 binding motif rendered the promoter functionless. These findings indicate that the Ets-1 binding motif, which has regional correspondence among species, is a common essential *cis*-acting regulatory element.

In conclusion, the observations obtained to date demonstrate that the common γ chain gene has structural and functional similarities in the mammalian species examined. The similarities of whole amino acid sequences between swine and human are higher than those between mouse and human (data not shown). This is additional support for the opinion that pigs are preferable model animals for human disease and may also be considered source animals for xenotransplantation to humans. As there is a difference in lymphocyte subsets in peripheral blood lymphocytes between swine and human/mouse, however, more comprehensive study, such as expression of the gene in subsets of porcine lymphocytes, is required.

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Original articles

Suppression of differentiation and proliferation of early chondrogenic cells by Notch

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Abstract Notch is a transmembrane protein involved in cell fate determination. In the present study, we observed temporally and spatially restricted expression of Notch1 in developing cartilage. Notch1 was localized starting from the mesenchymal condensation stage of embryonic mouse forelimbs. Interestingly, although localization could not be detected in the proliferating chondrocytes, obvious immunoreactivity indicating its expression was retained in the perichondrial region. Next, we investigated the expression of Notch1 and related molecules in a chondrogenic cell line, ATDC5 cells. Notch1, Delta-like (Dll)1, Deltex2, and Deltex3 were coexpressed after 6-day insulin treatment. Expression of Hairy and Enhancer of split homologue (HES)-1 followed thereafter. These results suggest that Notch may have a role in the early stage of chondrogenesis. To assess the effect of Notch activation, we cultured ATDC5 cells with a myeloma clone constitutively expressing Dll1, a ligand of Notch. We also used an adenovirus vector to express the constitutively active Notch1 intracellular domain (NIC). Activating either the endogenous or exogenous Notch receptor dramatically inhibited chondrogenic cell differentiation of ATDC5 cells, as assessed by Alcian blue staining of the cells and chondrocyte differentiation markers. Last, we investigated the effect of NIC on the proliferation of the ATDC5 cells. Expression of NIC by the adenovirus strongly suppressed thymidine incorporation. These results indicate that Notch is expressed in the initial stage of chondrogenic cell differentiation and has a strong inhibitory effect on both differentiation and proliferation of the cells when activated. The expression of Notch decreases as chondrogenic differentiation proceeds; however, a population of the cells with sustained expression of Notch1 become perichondrial cells. Considering that the perichondrium acts as a stem cell source of osteoblasts and chondrocytes, Notch1 may have a role in the formation of these cells by suppressing both differentiation and proliferation.

Key words Notch · chondrocyte · perichondrium · ATDC5 · Deltex

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Introduction

Notch signaling is an evolutionarily conserved mechanism that mediates cell–cell interaction required for cell fate determination [1]. The Notch signaling pathway is activated when a ligand–receptor interaction induces a proteolytic cleavage of the intracellular domain of the Notch receptor (NIC). Hairy and enhancer of split homologue (HES)-1 has been shown to transduce Notch signals in mammals [2,3]. Activation of HES-1 is dependent on the translocation of the intracellular domain of Notch into nuclei in association with CSL (CBF-1/Suppressor-of-Hairless/LAG-1) DNA-binding proteins, such as RBP-J [4]. Recently, the presence of other signaling pathways has also been suggested. One of these involves the binding of Deltex to the ankyrin repeat of Notch and plays an important role in the suppression of neuronal cell differentiation independent of RBP-J and HES-1 [5].

Jagged and Delta are membrane-tethered Notch ligand proteins. Notch receptors and ligands play roles in development and differentiation of various types of tissues and cells. For example, Notch1 and Jagged2 are expressed in the developing sensory epithelium of the cochlea, and a deficiency of Jagged2 resulted in a significant increase in the numbers of sensory hair cells [6]. Notch signaling is also involved in somitogenesis

Knockout mice for Notch1, Delta like (Dll)-1, Presenilin, RBP-J, HES-7, and Lunatic Fringe showed similar defects in somite segmentation [7–12]. Among them, Presenilin1-deficient mice showed a significant defect in vertebral bone. In these mice, the vertebral bones were underossified and a significant part of the bones consisted of an unsegmented cartilaginous mass, suggesting that Notch signaling is involved in the transition from cartilage to bone tissue as well as in the segmentation of vertebral bones. A similar defect in the axial skeleton was also reported in human spondylocostal dysostosis, in which mutations were found in DLL3, a human Delta homologue gene [13]. The fact that Serrate2 was mutated in the mouse limb mutant *syndactylism*, as well as deformation of cranyofacial and limb cartilages in the Jagged2 knockout mice, also pointed out specific roles for these genes in cartilage morphogenesis [14,15].

Endochondral ossification involves the formation of cartilage tissue from aggregated mesenchymal cells and subsequent replacement of the cartilage tissue by bone. In the developing chick embryo, expression of exogenous Delta-1 resulted in a significant malformation of limb cartilage, and thus a negative effect of Notch activation on hypertrophic differentiation of chondrocytes was suggested [16].

However, the role of Notch in the early stage of cartilage development has not been well investigated. Here, we show that Notch1 is expressed in condensed mesenchymal cells and in the perichondrial region in the developing mouse limb and that Notch activation has a strong suppressive effect on both cell differentiation and proliferation of ATDC5 cells, which are commonly used as an in vitro model of chondrogenic cell differentiation [17].

Materials and methods

Cells and reagents

ATDC5 mouse chondrogenic cell lines were obtained from Riken Gene Bank (Tsukuba, Japan), and cultured in a maintenance medium, Dulbecco's modified eagle medium nutrient mixture F-12 (Sigma, St. Louis, MO, USA), containing 5% fetal bovine serum (FBS; Boehringer Mannheim, Mannheim, Germany), 10 μg/ml human transferrin, and 3×10^{-8} M sodium selenite. For induction of chondrogenesis, the cells were cultured in a differentiation medium, maintenance medium plus 10 μg/ml bovine insulin, as described before [17]. The medium was replaced every other day. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

D10, a myeloma clone constitutively expressing mouse Dll1, and a vector-transfected control clone (SE1) were cultured in RPMI 1640 (Sigma) containing 10% FBS and 0.3 mg/ml G418 (Gibco-BRL, Rockville, MD, USA) at 37°C under 5% CO_2 in air [18].

Oligonucleotides were obtained from Amersham-Pharmacia Biotech Japan (Tokyo, Japan). Chemicals were purchased from Wako Chemicals (Osaka, Japan). Taq polymerase and sequencing reagents were obtained from Perkin Elmer (Branchburg, NJ, USA) and restriction enzymes from Takara Shuzou (Otsu, Japan).

Histology and immunohistochemistry

Alcian blue staining of the cells was performed as follows. ATDC5 cells were grown in culture dishes or two-well slides coated with collagen. After 2 days in culture in maintenance medium, the cells became confluent and were then cultured in differentiation medium. The cells then were washed with phosphate-buffered saline (PBS, pH 7.2), fixed with 2% paraformaldehyde for 5 min or methanol for 1 h, and stained with 1% Alcian blue in 3% acetic acid for 30 min to estimate the formation of cartilage nodules.

For immunocytostaining, cells were washed with PBS and fixed with methanol for 1 h. After having been washed with PBS, the cells were air dried, and immunostaining was carried out by the immunohistochemical procedure described next.

C57BL/6 mouse embryos were used for this study. Forelimbs were collected from 12.5-day-old embryos (E12.5) or 16.5-day-old embryos (E16.5). These specimens were fixed with methanol at 4°C overnight, embedded in Tissue-Tec O.C.T. (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and cryosectioned at an 8-μm thickness. Four serial sections were made, two for immunohistochemistry, one for Alcian blue staining (pH 2.5), and one for hematoxylin and eosin staining. Immunohistochemistry was performed by the avidin-biotin peroxidase complex method with a slight modification. Endogenous peroxidase activity was depleted with methanol containing 3% hydrogen peroxide for 30 min at 4°C. After having been washed twice in PBS and once in 0.1% Triton X-PBS for 8 min each, the specimens were incubated with 0.1% Triton X-PBS containing 2% normal rabbit serum (Vector, Burlingame, CA, USA) for 30 min at room temperature to block nonspecific antibody binding. To eliminate nonspecific avidin binding, sections were blocked by using an avidin/biotin blocking kit (Vector). The sections were then incubated with the specific Notch1 antibody (1:75 dilution, C-20: goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C plus 1 h at room temperature in a humidified chamber. This polyclonal antibody has previously been used in many studies [19,20]. Washing with 0.1% Triton X-PBS three times for 8 min each time was followed by incubation

with biotinylated rabbit antigoat IgG antibody (1:150) and avidin-biotin peroxidase complex (1:100) (Vector) in a humidified chamber for 1h at room temperature, respectively. All dilutions were made in 0.1% Triton X-PBS. Peroxidase activity was visualized with a 3,3′-diaminobenzidine substrate kit (Vector). No counterstaining was performed after the immunostaining. The sections were then dehydrated and mounted. For a negative control, the same concentration of normal goat IgG (Santa Cruz Biotechnology) was used instead of the primary antibody.

Construction of adenovirus vectors

Adenovirus expression vector for enhanced green fluorescent protein and Notch-IC (EGFP-NIC) fusion protein was prepared as described previously [21]. Rat HES-1 cDNA fragment [22] was kindly provided by Dr. Kageyama (Kyoto University Faculty of Medicine), and was inserted downstream of the CGA promoter of the pAxCAwt cosmid vector [23]. Recombinant adenovirus was prepared by the COS-TPC method using an Adenovirus Expression Vector kit (Takara Shuzou) [24].

Northern blot analysis

Total RNA was prepared by using an RNA extraction kit (Amersham-Pharmacia Biotech Japan). PolyA$^+$ RNA was purified with an mRNA extraction kit (Amersham-Pharmacia Biotech Japan), separated by agarose gel electrophoresis, and blotted onto a nylon membrane (Hybond-N; Amersham-Pharmacia Biotech Japan). Hybridization was performed under the standard conditions described previously [25]. Probes for aggrecan, Scleraxis, and Sox9 were kindly provided by Dr. Kimura (Toyama Medical and Pharmaceutical University), Dr. Shukunami (Kyoto University), and Dr. Koopman (University of Queensland), respectively.

Western blot analysis

ATDC5 cells were infected with adenovirus expressing EGFP-NIC or β-galactosidase. Total cell lysates were prepared 48h after infection, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto an Immublotin-P membrane (Millipore, Bedford, MA, USA). Blots were probed with rabbit anti-HES-1 polyclonal antibody at 0.6μg/ml [26]; this probing was followed by detection with horseradish peroxidase-conjugate antirabbit immunoglobulin G(IgG)F(ab′) fragment (1:1000) and enhanced chemiluminescence (Amersham-Pharmacia Biotech Japan). The anti-HES-1 antibody was kindly provided by Dr. Sudo (Toray).

Cell proliferation assay

ATDC5 cells were allowed to become confluent in 96-multiwell plates in DME/F12 culture medium containing 5% FBS for 3 days, and further maintained for an additional 2 days without replacement of medium until they had become quiescent. Then, the cells were infected with adenovirus expressing EGFP-NIC, HES-1, or β-galactosidase and cultured for 48h [27]. Proliferation was assessed by measuring the incorporation of ^{3}H-thymidine (1μCi/well) added 4h before the end of the culture period.

Results

Expression of Notch1 in developing cartilage

The aim of our study was to understand the function of Notch1 during early chondrogenesis. To elucidate the role of Notch1, we examined the localization of an immunoreactive Notch1 using an immunohistochemical method. Mouse limbs taken of the time of appearance of condensations of chondrogenic precursor cells (12.5-day-old mouse embryo; E12.5) and during the maturation stage of chondrogenesis (16.5-day-old mouse embryo; E16.5) were investigated. In the forelimb of E12.5 embryos, Notch1 immunoreactivity was observed in the region of condensed mesenchymal cells, which was destined to become cartilage tissue (Fig. 1). Strong immunoreactivity for Notch1 was found in the periphery of the developing cartilage and the developing tendon of the forelimb in E16.5 embryos (Fig. 2). Notch1-positive cells were also observed in prehypertrophic and hypertrophic zones of these embryos. Therefore, Notch1 is expressed in the developing cartilage from a very early stage of differentiation, subsequently decreased in the proliferating chondrocytes, and temporally expressed again in the prehypertrophic stage.

Expression of Notch signaling molecules in chondrogenic ATDC5 cells

Next, we investigated the expression and function of Notch-related molecules in a series of stages of chondrogenic cell differentiation by using ATDC5 cells. These cells rapidly proliferate and cease to grow at confluence. In the presence of insulin, the cells reenter the growth state and form cellular condensates that become cartilage nodules [17]. Expression of Notch1 mRNA was observed as early as 3 days after the start of the insulin treatment (Fig. 3A). The expression of Dll1 was observed from day 6, when the cells began to express type II collagen. Both Notch1 and Dll1 were at their highest level at day 6, and thereafter their levels

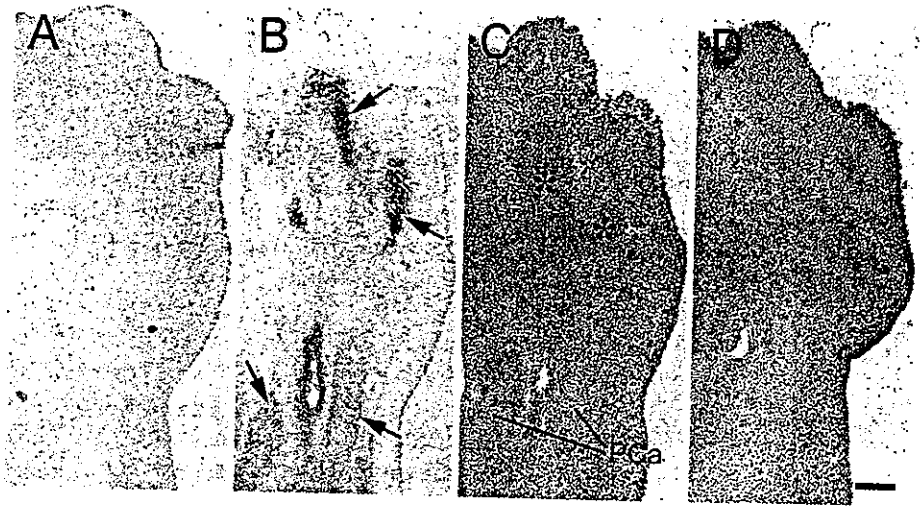

Fig. 1. Immunolocalization of Notch1 in the forelimb of E12.5 mouse embryos. Four serial horizontal sections were stained with (**A**) normal goat IgG as control, (**B**) anti-Notch1 antibody, (**C**) Alcian blue, and (**D**) hematoxylin and eosin. **A** No specific localization is observed in the negative control. **B–D** Immunoreactive cells (*arrows*) are observed in and around the region of mesenchymal condensation (*) supposed to become cartilage. Notch1 immunoreactivity is also found in the area of precartilage (*PCa*). *Bar* 100 μm

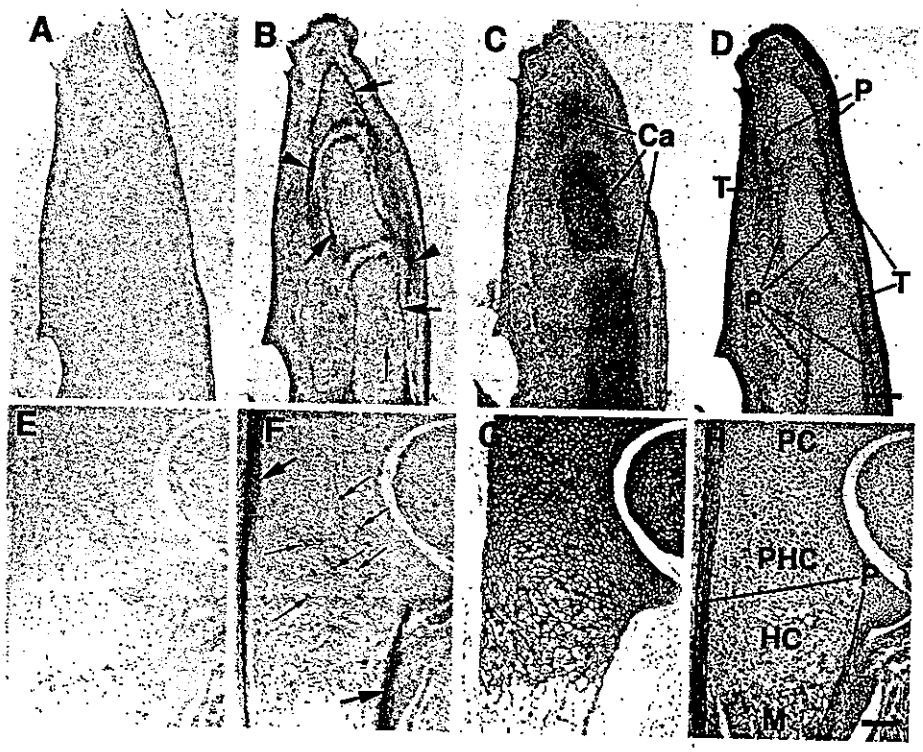

Fig. 2. Immunolocalization of Notch1 in sagittal sections of the forepaw (**A–D**) and in horizontal sections of the ulna (**E–H**) of an E16.5 embryo. Each four serial sections were stained with (**A,E**) normal goat IgG for control, (**B,F**) anti-Notch1 antibody, (**C,G**) Alcian blue, and (**D,H**) hematoxylin and eosin. **A,E** Control sections show no immunostaining. **B–D,F–H** *Large arrows*, immunoreactivity to Notch1 antibody in the perichondrium or periosteum (*P*); *small arrows*, positive reactions in the prehypertrophic chondrocytes (*PHC*) and hypertrophic chondrocytes (*HC*) of the developing cartilage (*Ca*); *arrowheads*, positive staining in the tendon (*T*); *M*, mineralized bone; *PC*, proliferating chondrocytes. *Bar* 100 μm

decreased. Other Notch ligands, such as Jagged1 and Jagged2, were not detected (data not shown). HES-1 was upregulated following the expression of Notch1 and Dll1, and its expression was sustained after the expression of these molecules decreased. We also found that Deltex2 and Deltex3 were expressed in ATDC5 cells in a differentiation-dependent manner (Fig. 3B).

When the ATDC5 cells were stained with anti-Notch1 antibody after 3 days of insulin treatment, a limited number of sparsely distributed positive cells were seen (Fig. 4A–C). No or scarce Alcian blue staining appeared at this stage (Fig. 4D). After 6 days of insulin treatment, cell condensates appeared (Fig. 4G), and these areas stained with Alcian blue (Fig. 4H). Cells immunoreactive with anti-Notch1 increased in number and were localized inside these areas of cell condensation (Fig. 4E–G). After 10 days of insulin treatment, cell condensation (Fig. 4K) and matrix production (Fig. 4L) became more obvious, and Notch-positive cells were observed in these area (Fig. 4I–K). Each chondrogenic nodule was a mixture of cells at various differentiation stages. Therefore, it was very difficult to determine whether the cells positive for Notch immnoreactivity corresponded to the perichondrial cells observed in

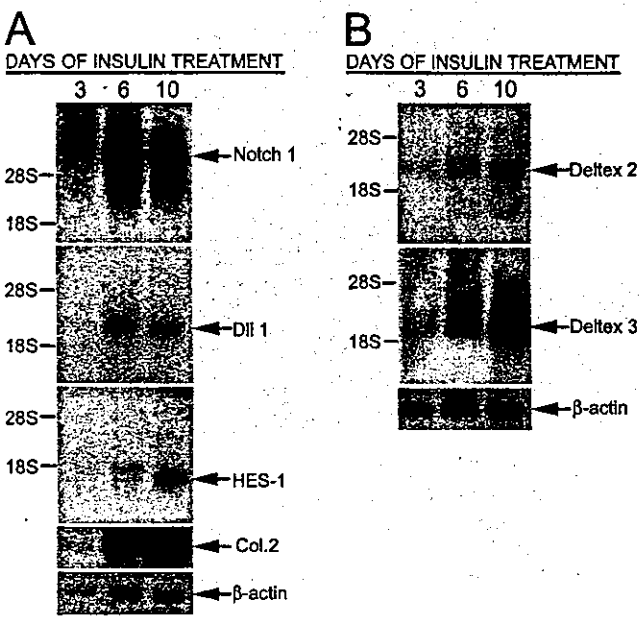

Fig. 3. Expression of Notch signaling molecules in ATDC5 chondrogenic cells. After having been cultured in maintenance medium until confluent, ATDC5 cells were cultured in differentiation medium for the indicated length of time, and RNA was prepared. **A** PolyA$^+$ RNA (4.5 μg) was separated by formaldehyde gel electrophoresis and transferred onto a nylon membrane. Radioactive probes for mouse Notch1, Dll1, HES-1 and human β-actin were used for hybridization. Migration points of 18S and 28S ribosomal RNA are indicated. **B** PolyA$^+$ RNA (4 μg) was separated by formaldehyde gel electrophoresis and transferred onto a nylon membrane. Radioactive probes for mouse Deltex 2, Deltex 3, and human β-actin were used for hybridization. Migration points of 18S and 28S ribosomal RNA are indicated at the *left*

vivo. However, the fact that Notch1 is expressed before apparent nodule formation is consistent with the histological data suggesting that Notch and related molecules are expressed and may have a role in early chondrogenic cell differentiation.

Suppression of chondrogenic cell differentiation by activation of the endogenous Notch signaling pathway

ATDC5 cells expressed Notch1 in the early stage of chondrogenic differentiation (see Fig. 3A). To examine the effect of activation of Notch on the differentiation of these cells, we cocultured ATDC5 cells with myeloma clone D10, which constitutively expresses Dll1 and can activate Notch [18,21]. When ATDC5 cells were cultured with a control myeloma clone, SE1, the cells differentiated normally and produced Alcian blue-positive nodules (Fig. 5). In contrast, ATDC5 cells cocultured with D10 cells showed a significant reduction in Alcian blue-positive nodule formation. These results indicate that activation of endogenous Notch has a negative effect on the chondrogenic nodule formation and matrix production.

Suppression of chondrogenic cell differentiation by adenovirus-mediated expression of Notch and HES-1

To confirm the suppressive effect of Notch1 activation in early chondrogenic cell differentiation, we expressed the fusion protein of Notch1 cytoplasmic domain and EGFP, EGFP-NIC, in ATDC5 cells by using an adenovirus vector (Fig. 6). Fluorescence microscopy revealed EGFP-NIC to be localized in the nucleus in ATDC5 cells, and the efficiency of infection was about 70% at a multiplicity of infection (m.o.i.) of 200 (data not shown). Upregulation of endogenous HES-1 mRNA and protein was also observed, indicating that EGFP-NIC was properly expressed and functioning in ATDC5 cells (Fig. 6A,B). Differentiation of ATDC5 cells expressing EGFP-NIC was strongly inhibited (Fig. 6C). As shown in Fig. 7, expression of type II collagen and aggrecan mRNA was also dramatically suppressed by infection with EGFP-NIC adenovirus. Similarly, but less effectively, overexpression of HES-1 suppressed these chondrogenic differentiation markers. We also observed suppression of mRNA of chondrogenic transcription factors Sox9 and Scleraxis by EGFP-NIC (Fig. 7B). HES-1 less effectively but significantly suppressed the expression of Scleraxis.

Suppression of thymidine incorporation by Notch

Notch was earlier reported to have a suppressive effect on the proliferation of B cells and to induce apoptosis in these cells [28]. To determine whether EGFP-NIC had a similar effect on ATDC5 cells, we measured thymidine incorporation after serum starvation of the cells. Quiescent ATDC5 cells were infected with EGFP-NIC or HES-1 adenovirus. EGFP-NIC strongly inhibited thymidine incorporation, whereas β-gal or HES-1 had minor effects (Fig. 8). We did not observe apparent signs of apoptosis in these cells (data not shown).

Discussion

In chick limb development, retroviral expression of Delta-1 blocked chondrocyte maturation from the prehypertrophic to the hypertrophic state [16]; however, the role of Notch in the early stages of chondrogenic differentiation has not been well investigated. We observed Notch1 localization in condensed mesenchymal cells in the expected cartilage region. Interestingly, in the later stage, distribution of Notch1 moved to the periphery of the developing cartilage, and immunoreactivity was decreased in proliferating chondrocytes.

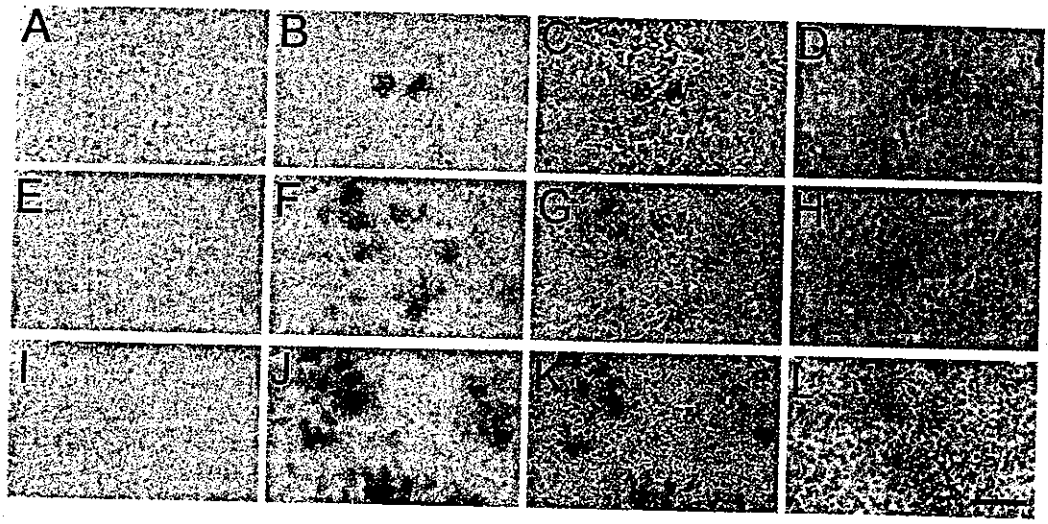

Fig. 4. Immunolocalization of Notch1 in ATDC5 cells cultured for different times in the presence of insulin. ATDC5 cells were plated at 5×10^4 cells/well in two-well slides and cultured in maintenance medium. After 2 days of culture, the cells became confluent and were then cultured in differentiation medium containing 10 μg/ml insulin for 3 days (**A–D**), 7 days (**E–H**), or 10 days (**I–L**). The medium was changed every other day. Cells were stained with normal goat IgG as control (**A,E,I**), anti-Notch1 antibody (**B,C,F,G,J,K**), and Alcian blue (**D,H,L**). **C,G**, and **K** are phase-contrast views of the same areas seen in **B,F**, and **J**, respectively. *Bar* 100 μm

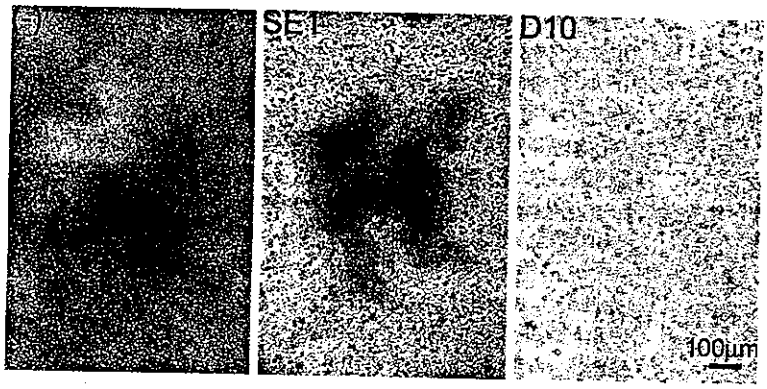

Fig. 5. Dll1-expressing cells prevent differentiation of ATDC5 chondrogenic cells. ATDC5 cells were cultured for 14 days either alone (−), together with γ-irradiated (3000 rad) control myeloma cells SE1 (*SE1*), or with γ-irradiated D10 (*D10*) in differentiation medium. Medium was changed every other day; cells were stained with Alcian blue at the end of the 14 days

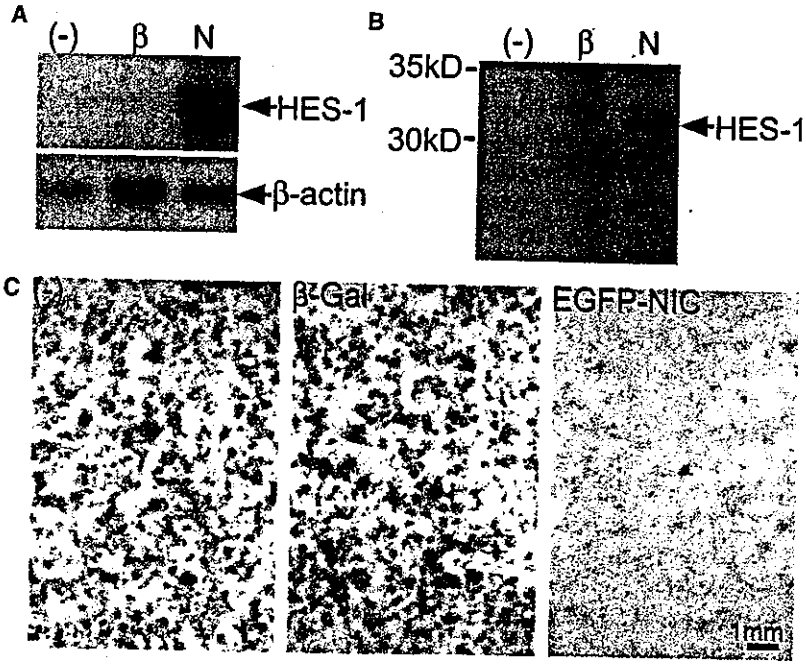

Fig. 6. Effect of EGFP-NIC expression on HES-1 in ATDC5 cells. ATDC5 cells were infected with EGFP-NIC adenovirus (*N*) or β-galactosidase adenovirus (β) at a multiplicity of infection (m.o.i.) of approximately 200 and cultured for 48 h. **A** PolyA$^+$ RNA (2 μg) from these cells was subjected to Northern blotting for HES-1 and β-actin. **B** Total cell lysate from these cells was subjected to Western blotting using anti-HES-1 antibody. **C** Effect of EGFP-NIC and HES-1 on early-phase chondrogenic differentiation of ATDC5 cells. ATDC5 cells were cultured in six-well culture plates to confluence and then infected or not with EGFP-NIC adenovirus (*EGFP-NIC*, –) or β-galactosidase (β-*Gal*) adenovirus at an m.o.i. of approximately 200. The cells were then cultured for 14 days in differentiation medium. Medium was changed every other day; cells were stained with Alcian blue at the end of the 14 days

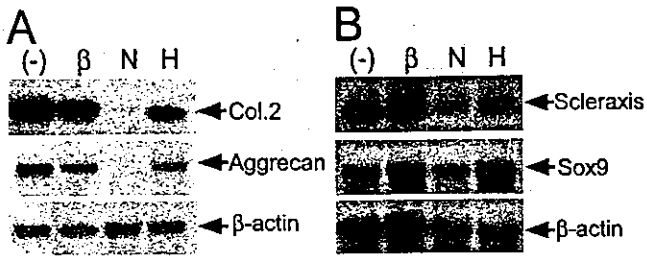

Fig. 7. Effect of EGFP-NIC and HES-1 on early-phase chondrogenic marker genes in ATDC5 cells. ATDC5 cells were infected with EGFP-NIC (*N*) adenovirus, HES-1 (*H*) adenovirus, or β-galactosidase (β) adenovirus at an m.o.i. of approximately 200. Cells were then cultured for 14 days in differentiation medium. **A** PolyA$^+$ RNA (2μg) from these cells was subjected to Northern blotting for type II collagen (Col.2), aggrecan and β-actin. **B** PolyA$^+$ RNA (2μg) from these cells was subjected to Northern botting for scleraxis, sox 9, and β-actin

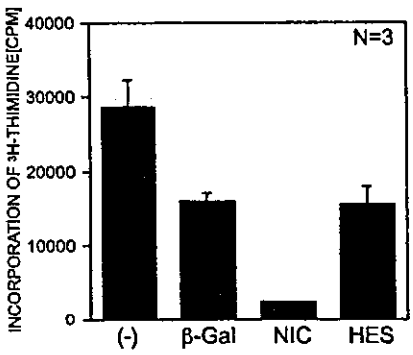

Fig. 8. Effect of EGFP-NIC and HES-1 on proliferation of ATDC5 chondrogenic cells. Cells were plated at 5×10^3 cells per well in 96-well plates and grown to confluence in differentiation medium for 3 days. After the growing period, they were further maintained for 2 days without replacement of medium until they had become quiescent. Cells were then infected with EGFP-NIC (*NIC*) adenovirus, HES-1 (*HES*) adenovirus, or β-galactosidase (β-*Gal*) adenovirus at an m.o.i. of approximately 200. After 48h, ^{3}H-thymidine (1μCi/well) was added; 4h later, the radioactivity was measured. Mean and SD of triplicate samples are shown

Immunoreactivity temporally increased again in the prehypertrophic and hypertrophic chondrocytes. These results indicate that Notch may have a dual role in chondrocyte differentiation: one is suppression of hypertrophy in the late stage of chondrocyte differentiation, as reported previously, and the other is developmental control of condensed mesenchymal cells at a very early stage of chondrogenesis.

The results of our in vitro experiments using ATDC5 cells support this hypothesis. Notch1 and Dll1 were expressed in ATDC5 cells at an early stage of cell differentiation, and activation of endogenous Notch1 resulted in significant suppression of chondrogenic differentiation of these cells. Expression of EGFP-NIC resulted in suppression of chondrogenic transcription factors and matrix production. HES-1 showed similar effects on chondrogenic cell differentiation. However, the inhibitory effects of HES-1 were weaker than those found by using Notch1. ATDC5 cells expressed Deltex2 and Deltex3, which play important roles in the Notch signaling pathway. In neural progenitor cells, Deltex1 inhibited transcriptional activation of neural-specific helix-loop-helix-type transcription factor MASH1 independently of the previously characterized Notch signaling pathway involving RBP-J and HES-1/HES-5 [5]. These results and facts suggest that the inhibitory effect of Notch on chondrogenesis may be mediated by multiple pathways in addition to HES-1. EGFP-NIC also strongly suppressed thymidine incorporation by ATDC5 cells, but this effect could not be reproduced by overexpression of HES-1, suggesting that this effect may be more dependent on the other pathways.

Sox9 and Scleraxis are transcription factors thought to be involved in cartilage differentiation. It is a high-mobility group domain transcription factor that is expressed in chondrocytes; it is known to bind to type II collagen α1 chain and type XI collagen α2 chain genes and is thought to act as one of the chondrocyte-specific enhancers [29]. By generating chimeric embryos using lacZ-marked Scleraxis-null and wild-type embryonic stem cells, Brown et al. examined the ability of the mutant cells to contribute to chondrogenesis [30]. Interestingly, scleraxis-null cells were specifically excluded from the sclerotomal compartment of somites, which forms later than the axial skeleton and rib cartilage, whereas they were able to contribute to most other regions of the embryo including other mesodermal tissues. Similarly, Sox9-null cells were excluded from cartilage in chimeric mice [31]. Suppression of these transcription factors by Notch activation may suggest a role of Notch signaling in cartilage development. The localization of Notch1 in the peripheral region of developing cartilage also raises the possibility that regulation of chondrogenic differentiation by Notch has a potential role in the formation of perichondrial tissues. Supporting this hypothesis, overexpression of one of the Notch ligands, Delta-1, in the chick limb disrupted the perichondrial structure [16]. The authors of that study described that disorganized perichondrium formation was a secondary effect of Delta-1 misexpression, because the perichondrium of some elements showed a apparently normal morphology even if they showed abnormality in the terminal differentiation of hypertrophic chondrocytes. However, our results propose another possibility, that Notch is also involved in the maintenance of the perichondrium more directly.

In brain tissues, Notch is expressed in the neural stem cells, and very recently it was suggested that Notch sig-

naling is required for maintenance but not for generation of these cells [32]. Notch and Dll1were expressed from day 6 of ATDC5 culture, and subsequent expression of HES-1 suggested activation of Notch at this stage. This result seemed contrary to the fact that these cells start to differentiate at the same stage as observed by type II collagen expression. By immunostaining, we observed weak expression of HES-1 in the periphery of the nodules of differentiating ATDC5 cells, but could not detect it in differentiating chondrocytes (data not shown). Therefore, these results indicate that the cells in which Notch was activated do not participate actively in the differentiation of chondrocytes. The perichondrium is a thin layer of cells surrounding cartilage and provides progenitor cells for both chondrocytes and osteoblasts. We earlier found Notch to be a positive regulator of osteoblastic cell differentiation [21]. Introduction of EGFP-NIC significantly stimulated osteoblastic cell differentiation in vitro. Interestingly, in MC3T3-E1 osteoblastic cells, expression of EGFP-NIC significantly upregulated calcification, but did not accelerate cell differentiation and rather delayed it. Considering the fact that perichondrium is a source of both chondrogenic and osteogenic progenitor cells, Notch may have an important role in determining when and how these cells start to differentiate.

In conclusion, we observed the expression of Notch at an early stage of cartilage development. We conclude that Notch may be one of the important factors controlling the early stage of chondrocyte differentiation or proliferation and may be involved in the maintenance of the function of perichondrium as a reservoir of osteogenic and chondrogenic stem cells.

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