Int. J. Dev. Biol. 40: 1089-1096 (1996)

Original Article

Isolation of a novel chick homolog of *Serrate* and its coexpression with *C-Notch-1* in chick development

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ABSTRACT Intercellular signaling mediated by the transmembrane proteins, Notch as receptor and its ligands, Delta and Serrate, plays essential roles in the developmental fate decision of many cell types in *Drosophila*. The *Notch* genes are highly conserved both in invertebrates and in vertebrates, suggesting that Notch pathway regulates cell fate decisions during vertebrates development. *Notch, Delta* and *Serrate* homologs in chicken have been cloned (Henrique *et al., Nature 375:* 787-790, 1995; Myat *et al., Dev. Biol. 174:* 233-247, 1996). We isolated a novel chick homolog of *Drosophila Serrate,* named *C-Serrate-2,* and examined its expression patterns during the early chick development using whole-mount *in situ* hybridization. *C-Serrate-2* transcripts were detected in several tissues including the forebrain, the myotome and the apical ectodermal ridge (AER) of the limb bud of a 4-day-old chick embryo. In most of the regions where *C-Serrate-2* was expressed, *C-Notch-1* was also expressed. Our observations suggest that Serrate-2–Notch-1 signaling plays a role in a variety of morphogeneses during the chick development.

KEY WORDS: *Notch, Delta, Serrate, chick development*

Introduction

Development of multicellular organisms proceeds via the coordinated proliferation and differentiation of cells which interact with each other through a variety of signaling molecules. The products of the neurogenic genes, the Notch receptor and its proposed ligand Delta, play an essential role in the lateral specification of cell fates through cell-to-cell interactions in a variety of tissues during development in invertebrates (for review see Artavanis-Tsakonas *et al.*, 1995). In the ventral surface of the ectoderm of the *Drosophila* embryo, cell-to-cell interaction, which is called lateral inhibition, mediated by the Notch-Delta pathway contributes to singling out one cell to become a neuroblast among a group of equivalent cells by inhibiting other neighboring cells from having the same fate (Cabrera, 1990; Simpson and Carteret, 1990; Heitzler and Simpson, 1991; for a review see Campos-Ortega, 1994; Muskavitch, 1994). Notch signaling is not only required for the lateral specification of neuroblasts through Delta but also for appropriate wing margin formation via the other ligand for Notch, Serrate (Speicher *et al.*, 1994; Couso *et al.*, 1995; Diaz-Benjumea and Cohen, 1995; Kim *et al.*, 1995). Activity of Delta can be replaced functionally by expression of Serrate during neurogenesis in the artificially manipulated embryo (Gu *et al.*, 1995). *C. elegans* homologs of *Notch, glp-1* and *lin-12* function not only among equivalent groups of cells but also between distinct types of cells in a manner of inductive interactions (for a review see Greenwald and Rubin, 1992; Henderson *et al.*, 1994; Mello *et al.*, 1994).

Transmembrane proteins, Notch, Delta and Serrate, contain epidermal-growth-factor (EGF)-like repeats in their extracellular domains (Wharton *et al.*, 1985; Vassisn *et al.*, 1987; Haenlin *et al.*, 1990) and are evolutionary conserved in vertebrates (for a review see Artavanis-Tsakonas *et al.*, 1995). Three Notch homologs, Notch-1, Notch-2, and Notch-3, have been identified in human and mouse, and they were expressed in a wide variety of tissues both in the embryo and in the adult (Weinmaster *et al.*, 1991, 1992; del Amo *et al.*, 1992; Reaume *et al.*, 1992; Kopan and Weintraub, 1993; Lardelli *et al.*, 1994; Williams *et al.*, 1995). Vertebrate *Delta* homologs have been cloned in mouse (Bettenhausen *et al.*, 1995), chicken (Henrique *et al.*, 1995) and *Xenopus* (Chitnis *et al.*, 1995), and their expression patterns in the nervous system during early development suggest their roles in neurogenesis. Homologs

Abbreviations used in this paper: AER, apical ectodermal ridge; EGF, epidermal growth factor; CNS, central nervous system; bHLH, basic helix-loop-helix; D/V, dorsal-ventral.

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0214-6282/96/$03.00

Printed in Spain

of *Serrate* in the rat and chicken, *Jagged* and *C-Serrate-1*, respectively, have been cloned and their expressions in the spinal cord and the neural tube suggest their role in regulation of neurogenesis in the central nervous system (CNS) (Lindsell *et al.*, 1995; Myat *et al.*, 1996). Functional analyses of these genes in *Xenopus* (Coffman *et al.*, 1993; Dorsky *et al.*, 1995) and rat (Lindsell *et al.*, 1995) suggest that Notch activities are implicated in retardation or inhibition of cell differentiation, and further support the notion that Notch signaling is important in local cell-to-cell interactions.

In chick development, the expression pattern of *C-Delta-1* is similar to that of *C-Notch-1* in the presomitic mesoderm (Henrique *et al.*, 1995), and *C-Delta1*, *C-Serrate-1* and *C-Notch-1* are expressed in closely related patterns in the developing nervous systems (Myat *et al.*, 1996). However, in some regions like the apical ectodermal ridge (AER), only the receptor gene, *C-Notch-1*, is expressed but the ligand genes, *C-Delta1* or *C-Serrate-1*, are not expressed, suggesting the presence of the other ligand genes which are expressed in such regions.

In this study, we aimed to identify the chick ligand genes other than *C-Delta-1* and *C-Serrate-1*. We isolated a novel chick homolog of *Drosophila Serrate* which we named *C-Serrate-2*. We examined the expression patterns of *C-Serrate-2* and *C-Notch-1* using whole-mount *in situ* hybridization during early chick development, and showed that *C-Serrate-2* and *C-Notch-1* have similar expression patterns particularly in the telencephalon, the myotome and the AER.

Results and Discussion

Identification of C-Serrate-2, a novel chick homolog of Drosophila Serrate

We identified a 597-bp cDNA fragment of chick *Serrate* homolog by a PCR approach, using degenerate primers to regions of genes encoding common amino acids of rat Jagged and *Drosophila* Serrate for one primer and encoding human Serrate-like protein for another primer. The deduced amino acid sequence of

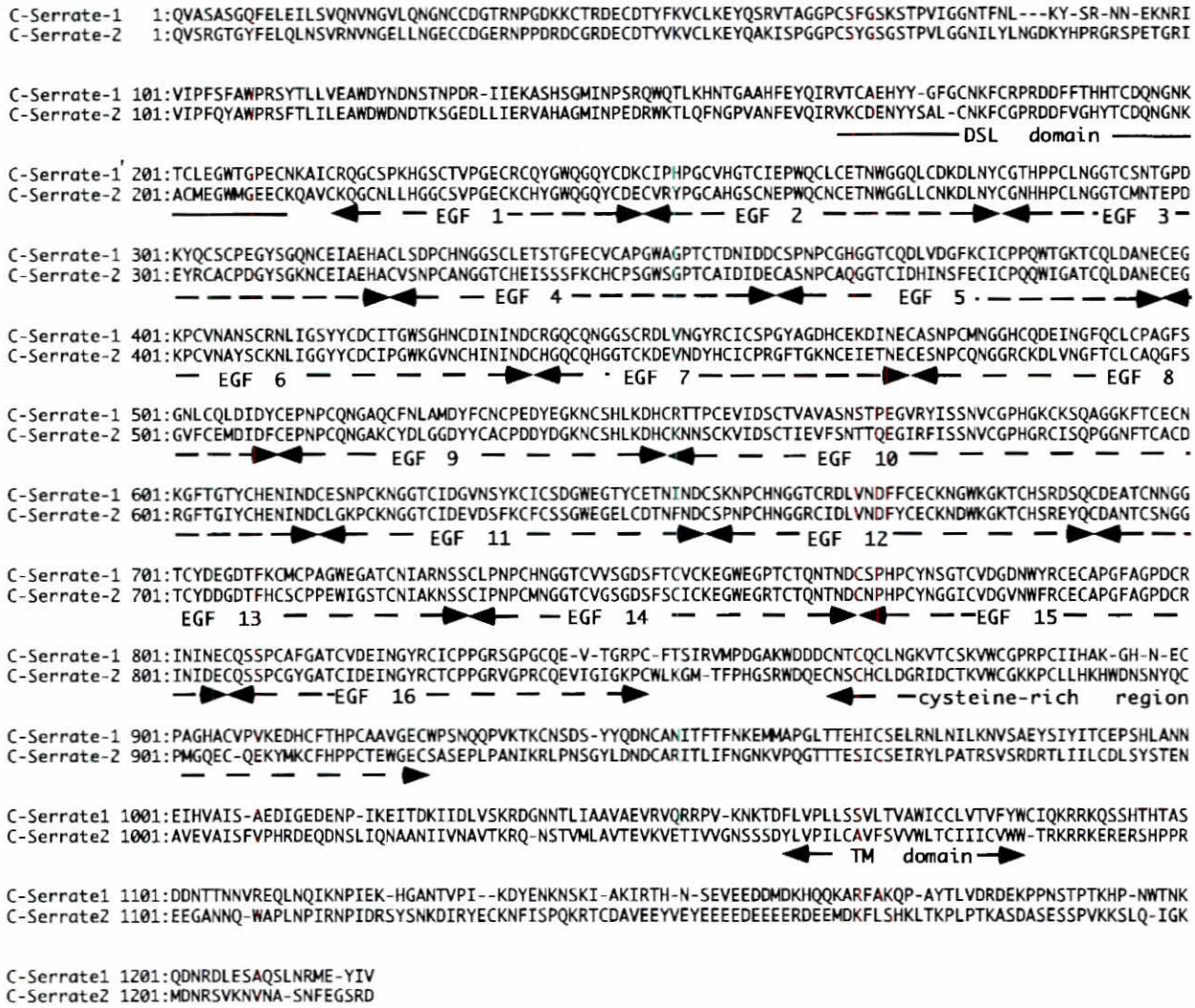

Fig. 1. Sequence of *C-Serrate-2*. *The deduced amino acid sequence of C-Serrate-2 aligned with that of C-Serrate-1(Myat et al., 1996). The C-Serrate-2 sequence encoded by cDNA clones lacks the N-terminal signal peptide compared with rat Jagged (Lindsell et al., 1995). The DSL domain is indicated by black bars below the aligned sequences, and the EGF-like repeats, the cysteine-rich region and the transmembrane (TM) domain are indicated as sequences between the arrows.*

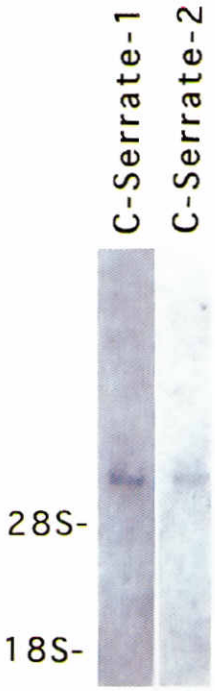

Fig. 2. Northern blot analysis of *C-Serrate-1* and *C-Serrate-2*. *Northern blot of poly(A)$^+$ RNA from 3-day-old embryos hybridized with probes of 684bp and 868bp cDNA of C-Serrate-1 and C-Serrate-2, respectively. Ribosomal RNA (18S and 28S) was used as marker.*

the amplified fragment was very similar to a corresponding region of C-Serrate-1, so the gene was named *C-Serrate-2* as the second chicken *Serrate* homolog. To clone the other regions of *C-Serrate-2* cDNA, 5'- and 3'-RACE were performed in the manner described in Materials and Methods. We cloned five fragments that were connected to give a total 4519-bp cDNA fragment which covered almost all of the coding region except for the 5' signal sequence of the rat *Jagged* gene (Lindsell *et al.*, 1995) (Fig. 1). The overall identity of the amino acid sequences of *C-Serrate-2* with *Drosophila Serrate*, rat *Jagged* and *C-Serrate-1*, is 33%, 56% and 58%, respectively, and C-Serrate-2 amino acid sequence included the EGF-like repeats and a cysteine-rich motif designated as the DSL (Delta-Serrate-Lag-2) region in the extracellular domain (Henderson *et al.*, 1994; Tax *et al.*, 1994) which is a characteristic structure common to the ligands, Delta and Serrate in *Drosophila* (Fleming *et al.*, 1990; Muskavitch and Hoffmann, 1990), and Lag-2 and Apx-1 in *C. elegans* (Henderson *et al.*, 1994; Mello *et al.*, 1994; Tax *et al.*, 1994). To identify the size of the transcript of *C-Serrate-2* and that of *C-Serrate-1*, we probed Northern blots containing 1 μg of poly(A)$^+$ RNA from 3-day-old whole chick embryos with *C-Serrate-2* and *C-Serrate-1* cDNA, respectively. Using both probes, single transcripts of approximately 6.0 kb were detected (Fig. 2).

The isolation of *C-Serrate-2* and *C-Serrate-1* (Myat *et al.*, 1996) indicates the existence of a *Serrate* gene family in the chicken. Since both *C-Serrate-1* and *C-Serrate-2* show approximately equal homology to *Drosophila Serrate*, both *C-Serrate-1* and *C-Serrate-2* may have arisen early in vertebrate evolution by gene duplication.

While the product of previously cloned *C-Serrate-1* (Myat *et al.*, 1996) shows 85% amino acid identity with the product of rat *Jagged*, the product of *C-Serrate-2* shows a lower degree of identity, 56%, with the rat Jagged at amino acid levels. Thus it is suggested that *C-Serrate-2* belongs to a group of the *Serrate* family other than that of rat *Jagged* and *C-Serrate-1*.

Expression of C-Serrate-2 in the telencephalon

We analyzed the spatial expression patterns of *C-Serrate-2*, and compared them with the expression of *C-Notch-1*, *C-Delta-1* and *C-Serrate-1*, using whole-mount *in situ* hybridization of chick 1.5-day-old (stage 11) to 4.5-day-old (stage 24) embryos. The expression of *C-Serrate-2* was initiated at stage 11 in the primary forebrain and midbrain (Fig. 3A), before the morphological differences between the telencephalon and diencephalon became clear, and was restricted to the telencephalon at later stages (Fig. 3B,C and D). On the other hand, the expression of *C-Serrate-1* was reported to be initiated in the prospective diencephalon in the forebrain at stage 11 and then to be restricted to the prospective neuromere D2 domain in the diencephalon at stage 14 (Myat *et al.*, 1996). These observations together with that regarding *C-Notch-1* expression in the forebrain suggest that Serrate-2–Notch-1 and Serrate-1–Notch-1 signaling are involved in the formation of forebrain, and/or in the regulation of a region-specific cell fate decision of the telencephalon, and the diencephalon, respectively.

C-Serrate-2 and *C-Notch-1* were coexpressed in the telencephalon of 3.5-day-old embryos (stage 20) (Fig. 3F and I). In the telencephalon of 4- to 5-day-old embryos, sections after whole-mount *in situ* hybridization revealed that both *C-Notch-1* and *C-Serrate-2* were diffusely expressed in most of cells (Fig. 4A and B; Myat *et al.*, 1996), while *C-Delta-1* and *C-Serrate-1* were expressed only in scattered cells (Fig. 4C and D; Myat *et al.*, 1996). Because the scattered patterns of *C-Serrate-1* and *C-Delta-1* expression were also observed in 3-day-old embryos (our unpublished data), these scattered patterns of expression are not transient ones and seem to be qualitatively different from the diffused patterns of *Notch-1* and *C-Serrate-2* expression.

Drosophila Delta is expressed in scattered subsets of populations of neural precursor cells, and Delta plays its role by inhibiting neighboring *Notch*-expressing cells from becoming neural cells by lateral inhibition (Haenlin *et al.*, 1990; Kooh *et al.*, 1993). In the chick brain, the patterns of expression of both *C-Delta-1* and *C-Serrate-1* in the scattered neural precursor cells are similar to the pattern of expression of *Drosophila Delta* (Fig. 4C and D; Henrique *et al.*, 1995; Myat *et al.*, 1996), and the former publication suggested that the product of *C-Delta-1* and *C-Serrate-1* also mediate signaling like lateral inhibition in the same manner as the *Drosophila Delta* product (Henrique *et al.*, 1995; Myat *et al.*, 1996). On the other hand, *C-Serrate-2* was diffusely expressed in most of the cells (Fig. 4B), therefore C-Serrate-2 may be involved with the cell fate decision at the level of cell population.

Expression of C-Serrate-2 in the myotome

C-Serrate-2 was expressed in the somites of 3.0- to 4.5-day-old (stage 18-24) embryos (Fig. 3E,F and G). In whole-mount sections, *C-Serrate-2* transcripts were detected in the myotome of a 4-day-old embryo (Fig. 5B). *C-Notch-1* transcripts were also detected in the myotome, the neural tube and the dorsal root ganglia of a 4-day-old embryo (Fig. 5A). The coexpression of *C-Serrate-2* and *C-Notch-1* in the myotome suggest that Serrate-2–Notch-1 signaling is involved in the development of the myotome.

In cell culture assays, rat Jagged, a homolog of C-Serrate-1, prevents differentiation of myoblast cells expressing the rat Notch-1 (Lindsell *et al.*, 1995), and activation of mouse Notch-1 inhibits

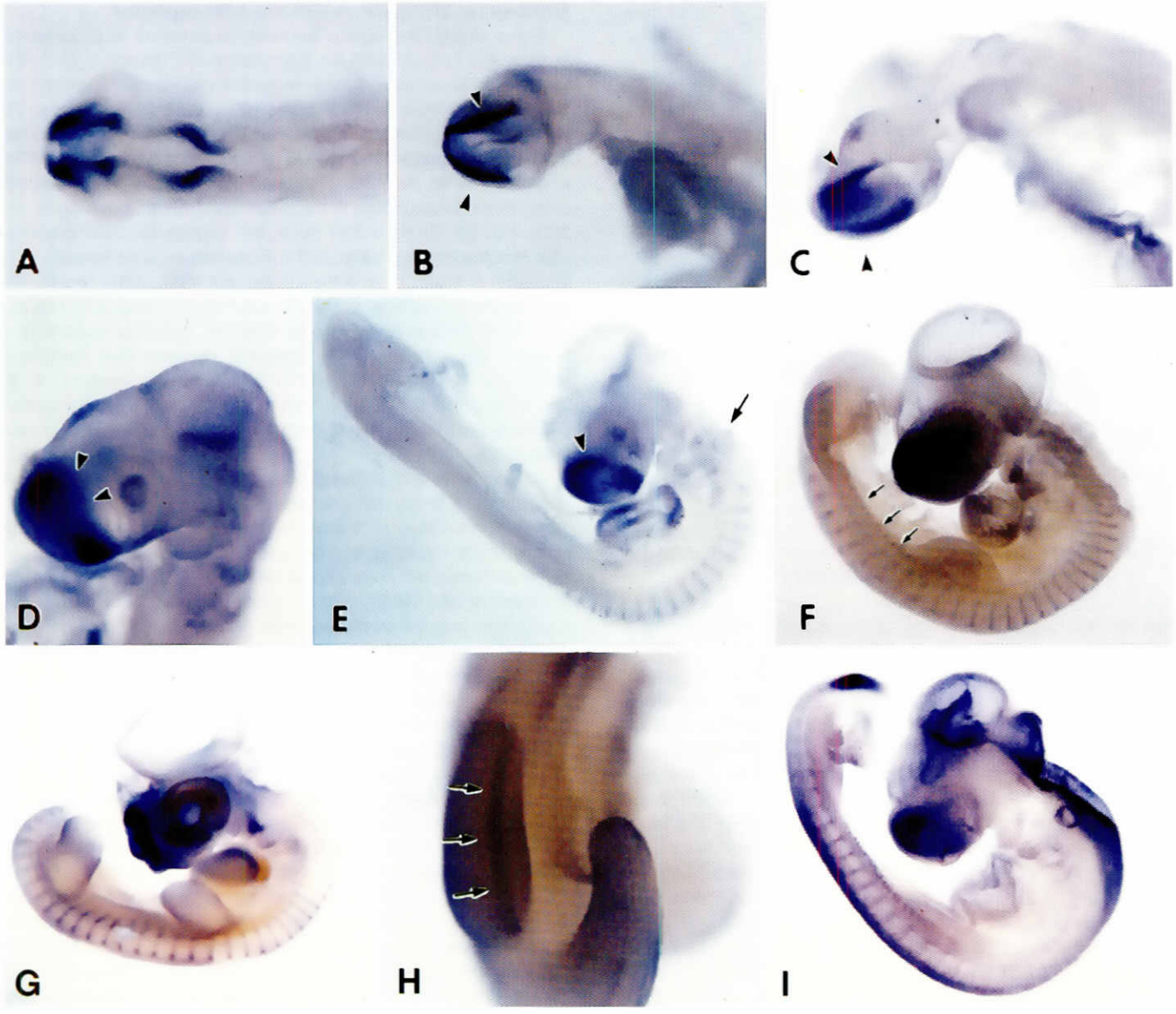

Fig. 3. Expression of *C-Serrate-2* (A-H) and *C-Notch-1* (I) in the early embryo as detected by whole-mount *in situ* hybridization. (A) *C-Serrate-2: head of a stage 11 embryo, dorsal view. Note the expression in the presumptive forebrain and midbrain.* **(B,C)** *C-Serrate-2: head of a stage 13* **(B)** *and stage 14* **(C)** *embryo, frontal view. Note the expression in the telencephalon (arrowheads).* **(D)** *C-Serrate-2: head of a stage 16 embryo, lateral view. Note the expression in the telencephalon (arrowheads) and lens.* **(E)** *C-Serrate-2: stage 18 (3-day-old embryo), lateral view. C-Serrate-2 was expressed in the telencephalon (arrowhead), lens, heart, somite, visceral arches, and otic cup (arrow).* **(F)** *C-Serrate-2: stage 21 (3.5-day-old embryo), lateral view. Note the expression in mesonephric tubules (arrows).* **(G)** *C-Serrate-2: stage 24 (4.5-day-old embryo), lateral view.* **(H)** *C-Serrate-2: trunk of a stage 21 (3.5-day-old embryo) embryo, ventral view. Note the expression in the AER of the limb bud (arrows).* **(I)** *C-Notch-1: stage 20 (3.5-day-old embryo), lateral view. The staining in the mesencephalon of brain was an artefact resulting from non-specific staining of the basement membrane of the brain.*

myogenesis regulated by the basic-helix-loop-helix type (bHLH) myogenic genes (Kopan *et al.*, 1994). Cells in the chick myotome are not the terminally differentiated myotubes but express such bHLH myogenic genes, suggesting that their fates are determined to become muscle cells (Ott *et al.*, 1991; Pownall and Emerson, 1992). Our results that *C-Notch-1* and *C-Serrate-2* are coexpressed in the myotome may indicate that Serrate-2–Notch-1 signaling play a role in keeping the myotome cells in committed but undifferentiated state by counteracting the function of myogenic genes.

Expression of C-Serrate-2 in limb buds

In the AER of limb buds of 4- to 5-day-old embryos, *C-Notch-1* (Fig. 6A; Myat *et al.*, 1996) and *C-Serrate-2* (Figs. 3H and 6B) transcripts were both detected, while *C-Serrate-1* was expressed in the mesenchyme underlying the AER of a 4-day-old embryo (Fig. 6C; Myat *et al.*, 1996). We observed that another *Notch* family member, *C-Notch-2*, was expressed in the mesenchyme of the limb (our unpublished data). Thus it is suggested that Serrate-2–Notch-1 and Serrate-1–Notch-2 signaling may play roles in AER development

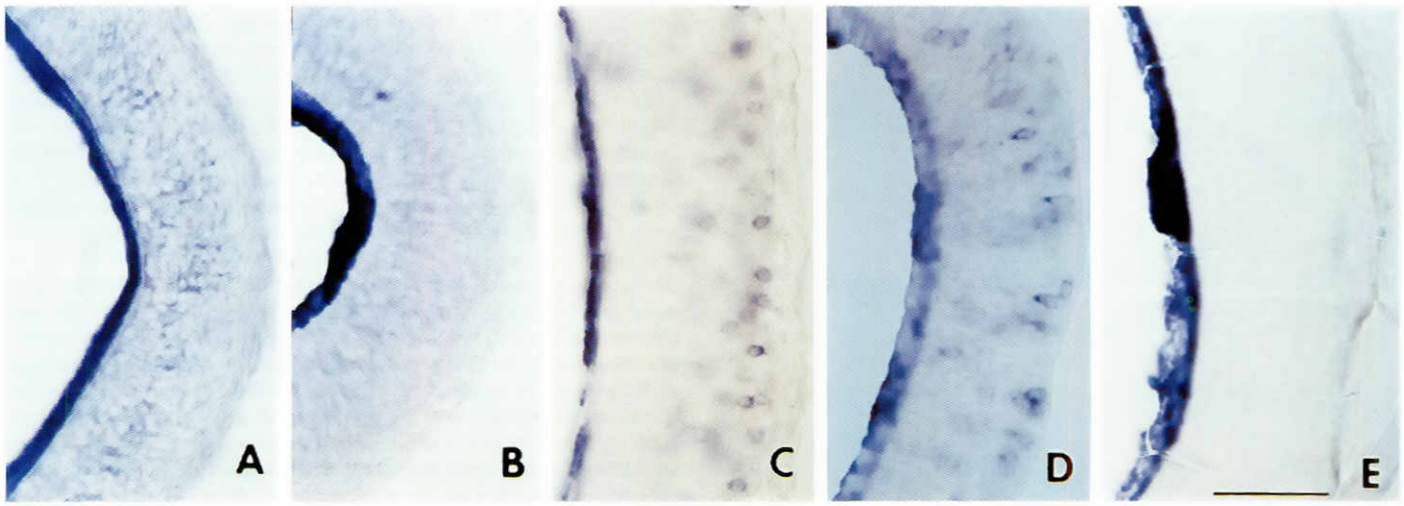

Fig. 4. Expression of *C-Notch-1* (A), *C-Serrate-2* (B) and *C-Delta-1* (C) in the telencephalon and *C-Serrate-1* (D) in the diencephalon in 4- to 5-day-old embryos as shown in sections after whole-mount *in situ* hybridization. *C-Notch-1* **(A)** *and C-Serrate-2* **(B)** *were expressed in all cells, while C-Delta-1* **(C)** *and C-Serrate-1* **(D)** *were expressed in scattered cells. The outer side of the brain is facing right and the inner side is facing left.* **(E)** *Control section stained using a C-Serrate-2 sense probe.* **(A-E)** *The inner side of the brain that was stained strongly is due to an artefact resulting from the staining of the basement membrane of the brain. Scale bar, 50 μm.*

and mesenchymal growth, respectively. However, as Baker and Schubiger presented evidence that Notch can non-autonomously function across germ layers from the mesoderm to the epidermis (Baker and Schubiger, 1996), it is possible that different combinations of these receptors and ligands, that is, Serrate-2–Notch-2 and Notch-1–Serrate-1, may mediate the interaction between the AER and the mesenchyme.

Serrate–Notch signaling is required for the specification of the cells in the dorsal-ventral (D/V) boundary of the wing imaginal disc of *Drosophila* (Speicher *et al.*, 1994; Diaz-Benjumea and Cohen, 1995; Kim *et al.*, 1995; de Celis *et al.*, 1996; Doherty *et al.*, 1996). The D/V boundary of *Drosophila* in the wing imaginal disc is considered to correspond to the AER of the vertebrate limb bud and some common mechanisms regulated by homologous genes are employed (for a review see Blair, 1995). In vertebrate, *C-Notch-1* is expressed in the AER of chick limb bud (Myat *et al.*, 1996), but expression of a ligand for Notch in the AER has yet not been reported. Thus the coexpression of *C-Serrate-2* and *C-Notch-1* in the AER suggests that Serrate-2–Notch-1 signaling may play a role in the establishment of the AER and may be involved with the specification of D/V positional identity.

Expression of C-Serrate-2 in other regions of chick embryos

C-Serrate-2 was also expressed in the lens, visceral arches, nasal placode, otic placode, heart, and mesonephric tubules of 1.5- to 4.5-day-old chick embryos (Fig. 3A-H). *C-Notch-1* expression was also detected in the above tissues (Fig. 3I). The overall structural homology of the *C-Serrate-2* product to the other characterized ligands and the coexpression of *C-Serrate-2* and *C-Notch-1* in several tissues described above suggest that the C-Serrate-2 protein may be a ligand for C-Notch-1.

The expression patterns of the three ligand genes, *C-Delta-1*, *C-Serrate-1*, *C-Serrate-2* and the receptor gene, *C-Notch-1*, are summarized in Table 1. The expression patterns of these ligand genes are not identical but partially overlapped. Therefore *C-Delta-1*, *C-Serrate-1*, and *C-Serrate-2* may play redundant roles in overlappingly expressed regions.

Materials and Methods

Embryos

Eggs of White Leghorn chicken were incubated in a humidified atmosphere at 37.6°C. Developmental stages of embryos were identified according to the Hamburger-Hamilton Stages (Hamburger and Hamilton, 1951). Late stages of development were described in terms of days of incubation.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from 3-day-old chick whole embryos using a solution of phenol and guanidine isothiocyanate, TRIzol™ reagent (Gibco BRL) and poly(A)$^+$ RNA was isolated from the total RNA using oligo(dT)

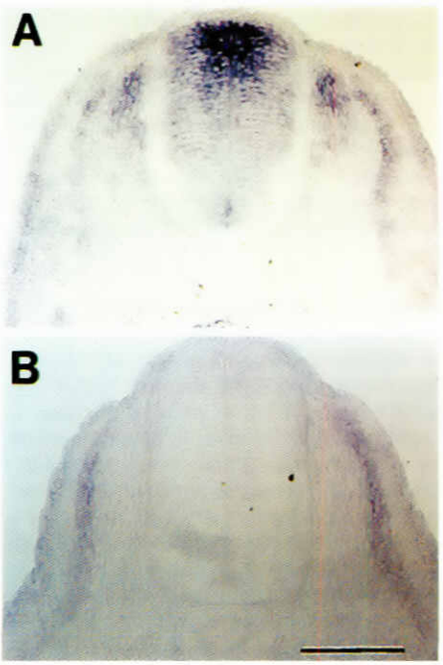

Fig. 5. Expression of *C-Notch-1* (A) and *C-Serrate-2* (B) in the myotome in 4-day-old embryos. (A) *C-Notch-1 was expressed in the myotome, the ventricular zone of the neural tube, and in the dorsal root ganglions.* **(B)** *C-Serrate-2 was expressed in the myotome. Scale bar, 100 μm.*

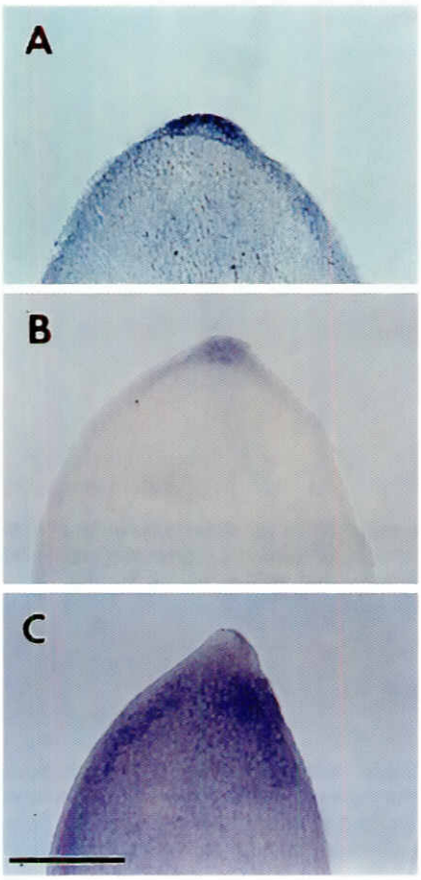

Fig. 6. Expression of *C-Notch-1* (A), *C-Serrate-2* (B), and *C-Serrate-1* (C) in the limb buds in 4- to 5-day-old embryos. *C-Notch-1* **(A)** *and C-Serrate-2* **(B)** *were coexpressed predominantly in the AER.* **(C)** *C-Serrate1 was expressed in the mesenchyme but not in the AER. Left side is dorsal and right side is ventral. Scale bar, 100 μm.*

beads, Oligotex™-dT30 (Daiichikagaku, Tokyo) according to the manufacturer's instructions. Poly(A)$^+$ RNA was transcribed into cDNA using Superscript™ reverse transcriptase (Gibco BRL) and random hexadeoxy-nucleotide primer as follows. RNA (0.1 μg) and 100 pmol of primer were incubated for 10 min at 70°C, and chilled on ice. 5xRT-buffer (4 μl) (Gibco BRL), 2 μl of 5 mM dNTPs, 2 μl of 0.1 M DDT, 50 U RNase inhibitor (Pharmacia), and 100 U reverse transcriptase were added to a final volume of 20 μl, and incubated for 1 h at 37°C. For the PCR reaction, 1 μl of cDNA was added with 60 pmol each of primers, 4 μl of 2.5 mM dNTPs, 5 μl of 10xPCR-buffer (Takara, Japan), 2.5 U Taq polymerase (Takara, Japan) and H_2O to a final volume of 50 μl. PCR reaction was carried out for 30 cycles of 40 sec at 94°C and 60 sec at 55°C, 90 sec at 72°C. This PCR product (1 μl) was used as a template for the second PCR reaction under the same conditions.

Cloning of a novel chicken homolog of Drosophila Serrate

A 597 bp PCR fragment of *C-Serrate-2* was generated as described above using the degenerate oligonucleotide primers: GGGAATTCTG(T/C)GA(T/C)CA(A/G)AA(T/C)GGIAA(T/C)AA(A/G)AC and GCTCTAGAAT(A/G)TG(A/G)CA(A/G)TT(A/G/T)ATICC(T/C)TTCCA, which correspond to the rat Jagged sequence CDQNGNKT (Lindsell *et al.*, 1995) with an *Eco* RI site added onto the 5' end for a sense primer, and the human Serrate-like protein sequence WKGINCHI (Genbank accession number H39899) with an *Xba* I site added onto the 5' end for an antisense primer, respectively. The amplified cDNAs were subcloned into *Eco* RI-*Xba* I digested pBluescript KS- (Stratagene) using standard techniques (Maniatis *et al.*, 1982) and sequenced automatically with an Applied Biosystems Sequencer A373. The other regions of *C-Serrate-2* were cloned by 5'-and 3'-RACE using the Marathon™ cDNA amplification kit (Clontech) according to the manufacturer's instructions. The following primers were used: antisense (A1) (CACTGCAACCCCCATGGAGCAAAT) and nested antisense (A2) (CAAGTTCCACCATTAGCACAGGGA) for 5'-RACE, and sense (S1) (GTGTACCTGGGGAATGCAAGTGTC) and nested sense (S2) (ATGAGTGTGTCCGCTACCCAGGCT) for 3'-RACE. Approximately 500-bp and 1600-bp PCR fragments were amplified by 5'- and 3'-RACE, respectively. The PCR products were cloned using TA Cloning™ kit (Invitrogen) and sequenced. Based on these sequence data, another antisense primer (A3) (TTCACGTACGTGTCGCACTCGTCC) for 5'-RACE and another sense primer (S3) (TGGCAGTGGAGACTCCTTTT) for 3'-RACE were produced and 5'- and 3'-RACE were again performed using these primers. Approximately 250-bp and 2500-bp PCR products were amplified by these 5'- and 3'-RACE reactions, respectively, and cloned as described above. All cDNA sequences were determined by sequencing at least three clones in the same PCR pools. The sequences of these five clones covered almost all of the coding region of *C-Serrate-2* except for a small portion of the 5' end which corresponded to the region coding for 26 amino acids of the signal sequence of rat *Jagged* (Lindsell *et al.*, 1995). Sequences were aligned and analyzed using GENETIX-MAC (Software Development). The nucleotide sequence data of *C-Serrate-2* will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession number D87558. As a probe for Northern analysis, we used an 868-bp cDNA fragment corresponding to the sequence of *C-Serrate-2* from nucleotide position 731-1599.

C-Notch-1, C-Delta-1 and C-Serrate-1 probes

To isolate the chick *Notch-1* homolog, *C-Notch-1*, an oligo(dT)-primed chick 9-day-old pigment epithelium cDNA library (Mochii *et al.*, in preparation) was screened using mouse *Notch-1* probes (Lardelli and Lendahl, 1993). We cloned a 2481-bp cDNA fragment of the intracellular region of the chick *Notch-1* homolog which codes for the region corresponding to amino acid residues 1774-2444 of human Notch-1 (TAN-1).

A 959-bp chick *Delta* homolog, *C-Delta*, was cloned by the PCR procedure using the gene specific primers: CTTCTGCGACAAACCTGGGGAAT and AGAATAATCCCGGCGCACACT, which were previously described by Henrique *et al.* (1995).

We identified a 386-bp fragment of the chick *Jagged* homolog using the PCR procedure with the same sense primer that was used for *C-Serrate-2* and a degenerate antisense primer: GGGTCTAGACAIGC(A/G)TG(C/T)TCIGC(A/G/T)AT(C/T)TC(A/G)CA, which corresponds to the region coding the rat Jagged sequence CEIAEHAC (Lindsell *et al.*, 1995) with an *Xba* I site added onto the 5' end. This fragment sequence was identical to a partial sequence of *C-Serrate-1* (Myat *et al.*, 1996). The other regions of *C-Serrate-1* were amplified by 5'-RACE using the Marathon™ cDNA

TABLE 1

COMPARISON OF C-*Notch-1, C-Delta-1, C-Serrate-1 and C-Serrate-2* EXPRESSION AT STAGE 18-25 (3.0-4.5-day-old EMBRYO)

Regions	*C-Notch-1*	*C-Delta-1*	*C-Serrate-1*	*C-Serrate-2*
Primitive streak	+	+	-	-
Presomitic mesoderm	+	+	-	-
Brain and spinal cord	+	+	+(diencephalon)	+(telencephalon)
Dorsal root ganglia	+	+	-	-
Retina	+	+	-	-
Lens	+	-	+	+
Otic placode	+	-	+	+
Nasal placode	+	-	+	+
Visceral arches	+	-	+	+
Somites	+(myotome)	-	-	+
Mesonephric kidney	+	+	+	+
Heart	+(weak)	-	+	+
Limb buds	+(AER)	-	+(mesenchyme)	+(AER)

amplification kit with the primer GGGTTCCACAGTAGTTCAGGTCC. A 684-bp PCR product corresponding to the sequence of *C-Serrate-1* from nucleotide 145-829 was cloned.

Northern blot analysis

Poly(A)$^+$ RNA (1 μg) was separated on a 1% agarose gel, blotted onto Hybond-N+ membrane (Amersham) and baked at 80°C for 2 h. Filters were prehybridized at 65°C for 1 h with 5xstandard saline citrate (SSC)-5xDenhardt's solution containing 0.5% SDS and 0.1 mg/ml salmon sperm DNA, and hybridized at 65°C overnight with radioactively labeled DNA probes. Filters were washed finally in 0.1xSSC containing 0.1% SDS at 65°C. The autoradiogram was analyzed using a Fujix Bio-Image Analyzer BAS 2000. The labeled DNA probes were synthesized using the Oligolabeling™ kit (Amersham) .

Whole-mount in situ hybridization

Embryos were fixed overnight in 3.7% formaldehyde, 0.1% MOPS, pH 7.4, 2 mM EGTA, and 1 mM $MgSO_4$ (FMEM buffer). After fixation, embryos were rinsed briefly in 100% methanol and stored in 100% methanol at -20°C. Digoxigenin-labeled sense and antisense riboprobes were produced with the Digoxigenin RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's protocol. For *C-Serrate-2, C-Delta-1 and C-Serrate-1* antisense probes, the above described clones of PCR amplified fragments were used, and for a *C-Notch-1* antisense probe, a 1626-bp cDNA clone corresponding to the region coding for amino acids from 1902 to 2444 of C-Notch-1 was used. Whole-mount *in situ* hybridizations were performed as described by Hemmati-Brivanlou *et al.* (1990). After staining, embryos were washed twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0), fixed in FMEM buffer once again for 1 h, and stored in 100% ethanol at 4°C, and photographed on a Leica Wild M420 photomacroscope with Fuji Provia 100 film. For sections, stained embryos were washed three times with 100% ethanol, three times with xylene, embedded in paraffin, and sectioned (10 μm thickness) with a microtome. Sections were photographed under an Olympus Vanox-T microscope.

Acknowledgments

We are grateful to Ms. C. Takagi and Ms. Y. Takahashi for technical assistance. This study was supported by Grants-in-Aid from the Ministry of Education, Science and Culture to G.E.

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Accepted for publication: September 1996