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Notch2: a second mammalian *Notch* gene

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Summary

Notch is a cell surface receptor that mediates a wide variety of cellular interactions that specify cell fate during *Drosophila* development. Recently, homologs of *Drosophila* Notch have been isolated from *Xenopus*, human and rat, and the expression patterns of these vertebrate proteins suggest that they may be functionally analogous to their *Drosophila* counterpart. We have now identified a second rat gene that exhibits substantial nucleic and amino acid sequence identity to *Drosophila* Notch. This gene, designated *Notch2*, encodes a protein that contains all the structural motifs characteristic of a Notch protein. Thus, mammals differ from *Drosophila* in having more than one *Notch* gene. Northern and in situ hybridisation analyses in the developing and adult rat identify distinct spatial and temporal patterns of expression for Notch1 and Notch2, indicating that these genes are not redundant. These results suggest that the great diversity of cell-fate decisions regulated by Notch in *Drosophila* may be further expanded in vertebrates by the activation of distinct Notch proteins.

Key words: second mammalian *Notch*, cDNA cloning, mouse embryogenesis, in situ hybridization.

Introduction

Notch is a large transmembrane protein that mediates a set of cellular interactions required for correct cell-type differentiation during *Drosophila* development (reviewed in Artavanis-Tsakonas et al., 1991; Campos-Ortega and Jan, 1991; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). Although genetic studies first identified *Notch* as a gene whose activity directly affects the choice between neural and epidermal cell fates in the neurogenic region of the developing *Drosophila* embryo (Poulson, 1937; Lehman et al., 1983), subsequent studies have also demonstrated roles for *Notch* in eye (Cagan and Ready, 1989) and sensillum development (Hartenstein and Posakony, 1990), in mesoderm differentiation (Corbin et al., 1991) and in oogenesis (Ruohola et al., 1991). In all of these areas, the loss of *Notch* leads to the expansion of one cell type at the expense of another cell type. Notch is widely expressed during embryonic and adult development (Hartley et al., 1987; Markopoulou and Artavanis-Tsakonas, 1989; Johansen et al., 1989; Kidd et al., 1989; Fehon et al., 1991) and different mutant alleles of the *Notch* gene affect various tissues during different periods of *Drosophila* development (Hartley et al., 1987; Kelley et al., 1987; Markapoulou et al., 1989; Xu et al., 1990). Taken together, these results suggest that Notch mediates a large number of different cell-cell interactions during normal fly development.

While there is compelling evidence implicating Notch in processes that determine cell-fate decisions in *Drosophila*, the mechanism by which this protein exerts its widespread effects remains unclear (Greenspan, 1990; Simpson, 1990; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). Results from genetic (Hartley et al., 1987; Kelley et al., 1987; Markopoulou et al., 1989; Xu et al., 1990), biochemical (Fehon et al., 1990; Rebay et al., 1991) and genetic mosaic studies (Hoppe and Greenspan, 1990; Simpson, 1990; Heitzler and Simpson, 1991) are all consistent with the hypothesis that Notch functions as a receptor for a signal ligand that regulates the phenotype of the receiving cell. It is thought that Notch might interact with multiple ligands (Rebay et al., 1991), given its wide expression during embryonic and imaginal development, the presence of multiple repeats related to epidermal growth factor (EGF) in its ligand-binding domain and the pleiotropic nature of Notch mutations. Two strong candidates for such ligands are the proteins encoded by the *Delta* (Vässin et al., 1987; Kopcyznski et al., 1988) and *Serrate* genes (Fleming et al., 1990; Thomas et al., 1991). *Delta* has been implicated as a potential Notch ligand during neuroblast segregation, since loss-of-function mutations in *Delta* produce the same neurogenic phenotype seen in *Drosophila* embryos carrying loss-of-function mutations in *Notch* (Lehman et al., 1983; Vässin and Campos-Ortega, 1987). Furthermore, imaginal epithelium cells mutant for *Delta* behave non-autonomously in genetic mosaics, suggesting that Delta could be the signal for the cell sorting that occurs there (Heitzler and Simpson, 1991). The most

convincing data supporting a direct interaction between Notch and these putative ligands comes from cell aggregation studies (Fehon et al., 1990; Rebay et al., 1991). Cultured *Drosophila* S2 cells expressing Notch form aggregates, in a calcium-dependent manner, specifically with cells expressing either Delta or Serrate. Consistent with the direct protein-protein interaction seen in cell aggregation studies, Notch and Delta proteins colocalise in multi-vesicular structures in Notch-expressing cells (Artavanis-Tsakonas and Simpson, 1991) and are found complexed together in immunoprecipitates from *Drosophila* embryo extracts (Fehon et al., 1990).

Notch cDNA clones have been isolated from a number of vertebrate species and the proteins that these cDNAs encode are closely related to the *Drosophila* protein (Wharton et al., 1985a; Kidd et al., 1986; Coffman et al., 1990; Ellisen et al., 1991; Weinmaster et al., 1991). Furthermore, the temporal and spatial distributions of Notch transcripts in murine embryos suggest that the function of mammalian Notch may be analogous to its *Drosophila* counterpart (Weinmaster et al., 1991). The vertebrate and invertebrate *Notch* genes also share sequence homology with the *C. elegans glp-1* and *lin-12* genes, which also direct cell-fate decisions (Yochem et al., 1988; Yochem and Greenwald, 1989; Austin and Kimble, 1989; Greenwald and Rubin, 1992) and with the recently isolated mouse *int-3* oncogene (Robbins et al., 1992). This relatedness is evidenced both in the extracellular and cytoplasmic domains of the predicted proteins (Greenwald and Rubin, 1992). The relationship between vertebrate members of this gene family has been established only through sequence identity, since a functional role has yet to be demonstrated for any of these genes. However, it is notable that both TAN-1, the human Notch homolog (Ellisen et al., 1991), and *int-3* (Robbins et al., 1992; Jhappan et al., 1992) were identified as mutated sequences that interfered with cell differentiation and resulted in neoplastic transformation.

It has been suggested that interactions between specific numbers or combinations of the highly conserved 36 EGF-like repeats in *Drosophila* Notch with different ligands could account for the great diversity of cell-cell interactions regulated by Notch (Rebay et al., 1991). If the Notch signaling system regulates mutiple cell-cell interactions in vertebrates, then such diversity might also be generated through the activation of different Notch-like receptors. In this report, we present direct evidence that supports this second hypothesis. Here we describe the cloning, sequencing and expression of a second rat *Notch* gene, designated *Notch2*. Although Notch2 has retained all the structural motifs that are hallmarks of a Notch protein, it is expressed in a distinct set of cells and at different times in development than the previously isolated rat *Notch* gene.

Materials and methods

Cloning and sequencing

A rat Schwann cell cDNA library was hybridised to a ^{32}P-radiolabelled *Eco*RI fragment derived from the EGF-like repeat region of *Xenopus* Notch cDNA clone X-2 (Coffman et al., 1990) as previously described (Weinmaster et al., 1990). Briefly, Hybond-N membrane filters (Amersham) were hybridized in a solution containing 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 1.0% SDS, 5× Denhart's solution and 0.2 mg/ml salmon sperm DNA at 50°C for 24 hour. Filters were then washed in 5× SSC with 0.5% SDS at 21°C followed by washes at 50°C. Clone SN12, identified in this screen, was used to isolate cDNA clones encoding additional Notch-related sequence (Fig. 1). All of these clones were isolated under high-stringency conditions (0.2× SSC containing 1.0% SDS at 65°C). Initially a 1.5 kbp PCR fragment, sythesized from SN12 sequence that encoded 13 EGF-like repeats, was used to screen a random-primed rat forebrain cDNA library (J. Boulter, Salk Institute). This screen yielded 21 clones that provided novel sequence both 5′ and 3′ of the original SN12 sequence. The first clone, H11S-1, (1466 bp in size) contained 449 bp that extended 5′ of SN12 and the remaining sequences overlapped SN12 by 1017 bp. The 449 bp in H11S-1 that extended 5′ of SN12 contained 86 bp of untranslated sequence, a potential ATG translation start site, a putative signal peptide sequence and additional sequence that encoded approximately two EGF-like repeats. The second clone, H10, contained 3340 bp of sequence that overlapped H11s-1 by 572 bp and SN12 by 1231 bp, while the remaining 2109 bp encoded novel sequences related to the 35th EGF-like repeat of rat Notch (Weinmaster et al., 1991). A 287 bp PCR fragment from the 3′ end of H10 was used to screen the same library to obtain clones containing sequence 3′ of H10. From this screen, a number of clones were identified, of which H10-6 was the largest (2462 bp) and overlapped clone H10 by 670 bp. The 3′ end of H10-6 was found to contain sequences related to CDC10 repeats. A 458 bp PCR fragment containing this 3′ sequence was used to probe the originally screened Schwann cell library. This screen yielded three clones that all overlapped clone H10-6. The largest overlap was found with RSC-3, which shared 1161 bp in common with H10-6 and extended 3′ of H10-6 for more than 4.8 kbp. RSC-3 and was found to have a stop codon 1473 bp downstream of the 3′ end of H10-6. All three of the cDNA clones isolated from the Schwann cell library had at least 3 kbp of untranslated sequence at their 3′ ends (data not shown) of which only 361 bp, following the stop codon, are reported for the Notch2 sequence. The clones presented in Fig. 1 were sequenced using the dideoxy chain termination method (Sequenase, US Biochemicals).

Northern blot analysis

Poly (A)$^+$ RNA was isolated and northern blot hybridisation analysis performed as described previously (Weinmaster and Lemke, 1990; Lai and Lemke, 1991). Methylene blue staining was used to verify that equal amounts of RNA were transferred to nylon membranes (MSI). Random-primed ^{32}P-radiolabelled probes were prepared from cDNA inserts isolated from SN6 (Weinmaster et al., 1991) to identify rat Notch1 transcripts and from pooled cDNA inserts isolated from H10, H10-6 and RSC-3 to detect rat Notch2 mRNA.

In situ hybridisation

Preparation of rat embryo sections and in situ hybridisation analysis were carried out essentially as described previously (Roberts et al., 1991). Pregnant Sprague-Dawley female rats were anesthetised with 35% chloral hydrate and perfused transcardially with 4.0% paraformaldehyde in 0.1 M sodium tertraborate buffer, pH 9.5. The rat embryos (E16), were removed and submersion-fixed for 3-6 months at 4°C in the same fixative. Frozen sections, 20 μm thick, were cut on a cryostat, mounted on polylysine-coated slides and desiccated overnight. Preparations of adult rat brain tissue was essentially the same except that brains from perfused rats were post-fixed for 2-4 weeks and sectioned at 30 μm thick. Identical conditions were used to detect transcripts for Notch1 and Notch2 in adult rat brain and whole rat embryo sections. The

specificity of the antisense ^{35}S-radiolabelled riboprobe, synthesised from cDNA insert encoding amino acid residues 1755-1903 in the intracellular portions of rat Notch1, subcloned into Bluscript SK$^+$ (SN6-7), has been described previously (Weinmaster et al., 1991). For detection of rat Notch2 transcripts, an antisense ^{35}S-riboprobe was synthesised from the linearised SN12-79 plasmid that contained sequences encoding amino acids 351-535 of rat Notch2 (see Fig. 2). The details of the prehybridisation, hybridisation and autoradiographic localisation techniques have been published previously (Simmons et al., 1989; Roberts et al., 1991). After film exposure, slides were defatted and dipped in nuclear track emulsion (NTB-2, Kodak) and exposed for appropriate times. Following developing (Kodak D19) and fixation (Kodak, Ektaflo), brain sections were stained with bisbenzimide (Sigma Chemical Co., St Louis, Mo.), which provides a fluorescent blue ribosomal counter stain. Embryo sections were counter-stained with hematoxylin and eosin-Y. Sections were photographed using Kodak Gold 35 mm Kodacolor 100 ASA film and printed on Fuji paper.

Results

Isolation of Notch2 cDNA clones

A rat Schwann cell cDNA library was screened with a *Xenopus Notch* cDNA probe (Coffman et al., 1990) under low-stringency hybridisation conditions (see Material and methods). From this screen a number of cDNA clones were isolated, some of which were shown to encode the rat *Notch* gene (Weinmaster et al., 1991), while others contained related Notch-like sequences. One of the isolated cDNA clones, designated SN12, was found to encode 13 EGF-like repeats. These EGF-like repeats were aligned with EGF-like repeats 3 through 16 of rat Notch with 56% identity at the amino acid level (Fig. 1). This sequence analysis suggested that clone SN12 encoded a novel, yet related, *Notch* gene.

Rescreening of the rat Schwann cell library failed to yield any additional SN12-related clones. Since in situ hybridisation data indicated that SN12 was expressed in the adult rat brain (see below), we screened a random-primed rat forebrain cDNA library to obtain cDNA clones that overlapped and extended either 5′ or 3′ of the SN12 clone (for details see Materials and methods). All of the overlapping clones, shown in Fig. 1, were sequenced to obtain 7860 bp of cDNA sequence. This sequence carries a large open reading frame of 2472 amino acids, starting with an initiator methionine at nucleotide 86 and terminating with a stop codon at nucleotide 7413. The nucleotide and deduced amino acid sequence of rat Notch2 have been deposited with GenBank under the accession number M93661.

Analysis of Notch2 deduced amino acid sequences

Analysis of the aligned amino acid sequences reported for *Notch* cDNA clones isolated from *Drosophila* (Wharton et al., 1985a; Kidd et al., 1986), *Xenopus* (Coffman et al., 1990), human (Ellisen et al., 1991) and rat (Weinmaster et al., 1991) reveals a number of characteristic and highly conserved motifs. Inspection of the amino acid sequence, deduced from the nucleotide sequence of the cDNA clones described above, established the presence of all the previously identified Notch structural domains (Figs 1, 2). We have therefore designated the gene that encodes this sequence rat *Notch2* and refer to the previously published rat *Notch* gene as *Notch1*. Fig. 2 shows the alignment of the deduced amino acid sequence for rat Notch1 and Notch2. The two proteins exhibit overall amino acid sequence identity of 56%.

The extracelluar domain of *Drosophila* Notch contains two cysteine-rich regions (Wharton et al., 1985; Kidd et al., 1986; Yochem et al., 1988). The most N-terminal of these regions contains 36 tandemly arranged repeats that share homology with EGF. Both Notch1 and Notch2 have retained each of these EGF-like repeats in the exact arrangement described for *Drosophila* Notch. In the EGF-like repeat domain, Notch1 and Notch2 exhibit 58% amino acid sequence identity. Of these 36 EGF-like repeats, only

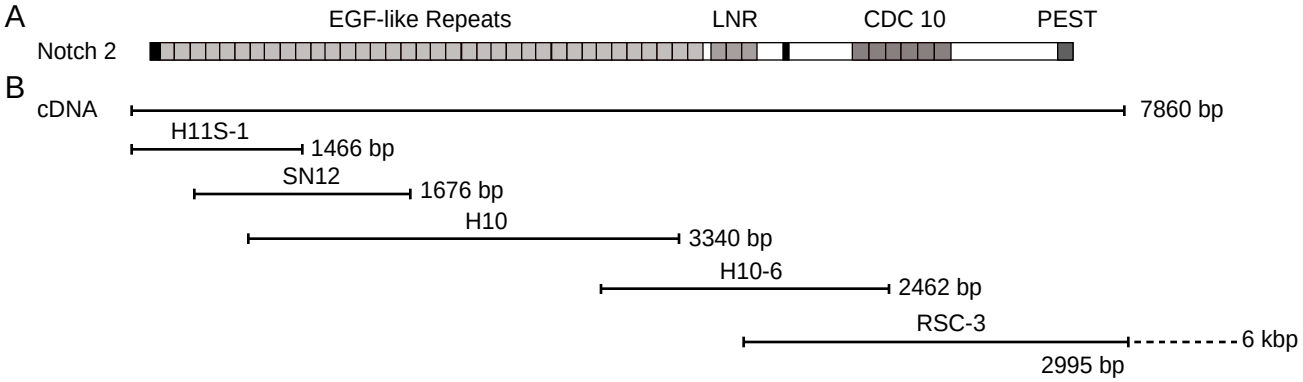

Fig. 1. Alignment of Notch2 cDNA clones relative to Notch structural motifs. (A) Structural amino acid motifs identified in the Notch proteins: EGF-like repeats for the 36 cysteine-rich repeats related to the epidermal growth factor; LNR for the 3 repeats related to *C. elegans* lin-12 and *Drosophila* Notch sequences; TM for the hydrophobic transmembrane domain sequences; CDC10 for the 6 repeats first described for certain yeast transcription factors and subsequently identified in ankyrin and thus sometimes referred to as ankyrin repeats, and the C-terminal region containing a PEST sequence. (B) Overlapping Notch2 cDNA clones were aligned relative to the published rat Notch sequence (Weinmaster et al., 1991), using the University of Wisconsin GAP program. These overlapping clones can be conceptually spliced together to produce a 7860 bp cDNA. For a description of the various cDNA clones see Materials and methods. The dotted line indicates unsequenced DNA.

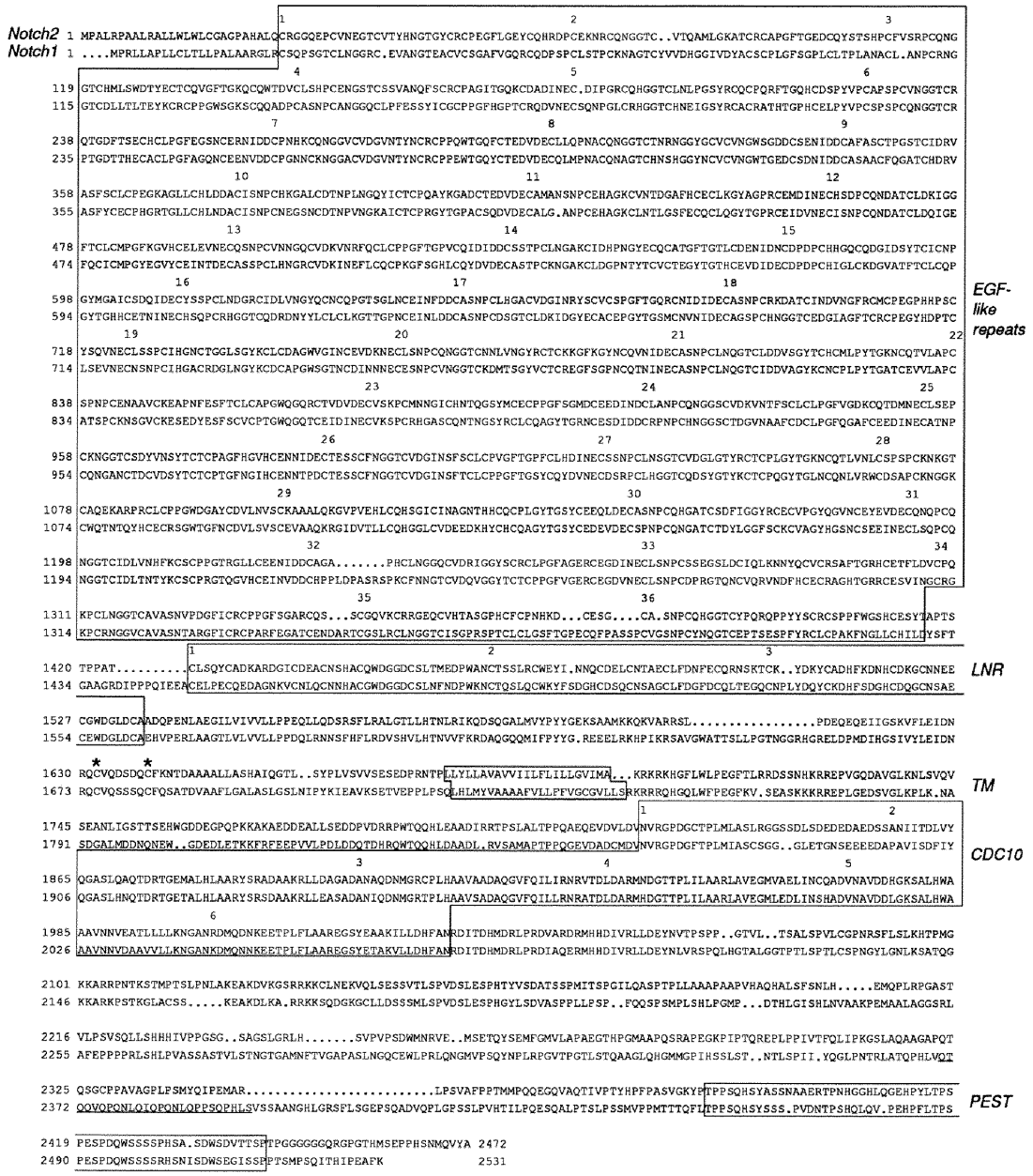

Fig. 2. Alignment of the deduced amino acid sequence for rat Notch1 and rat Notch2. The amino acid sequence of rat Notch2 was deduced from the nucleotide sequence of cDNA clones shown in Fig. 1B and aligned with the published sequence of rat Notch (Weinmaster et al., 1991) using the University of Wisconsin GAP program, with modifications according to Feng and Doolittle (1987). Characteristic structural motifs following the signal peptide are boxed and indicated as: EGF-like repeats, LNR, TM, CDC10 and PEST. The following degrees of amino acid identity were determined for the two Notch proteins: overall, 56%; EGF-like repeats, 58%; LNR, 58%; CDC10, 76% and PEST, 79%. Asterisks (*) indicate the conserved cysteines discussed in the text. The individual repeats are numbered and the residual OPA region is underlined. The single-letter abbreviations for amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr.

repeats 11 and 12 are necessary and sufficient for *Drosophila* Notch to bind Delta or Serrate proteins, when measured in an in vitro aggregation assay (Rebay et al., 1992). The residues between the first and second cysteine of EGF-like repeat 11 appear crucial for this interaction, since a leucine-to-proline change in this region of *Xenopus* Notch obliterates Delta and Serrate binding (see Fig. 3). At this site, Notch2 has one and two additional amino acids, respectively, relative to vertebrate Notch and *Drosophila* Notch sequences (see Fig. 3). The second cysteine-rich region consists of three contiguous lin12/Notch repeats (LNR), which are also present in the *C. elegans* lin-12 and glp-1 proteins (Yochem et al., 1988; Yochem and Greenwald, 1989; Austin and Kimble, 1989). Notch1 and Notch2 also have these repeats, which are related to each other with 58% amino acid sequence identity.

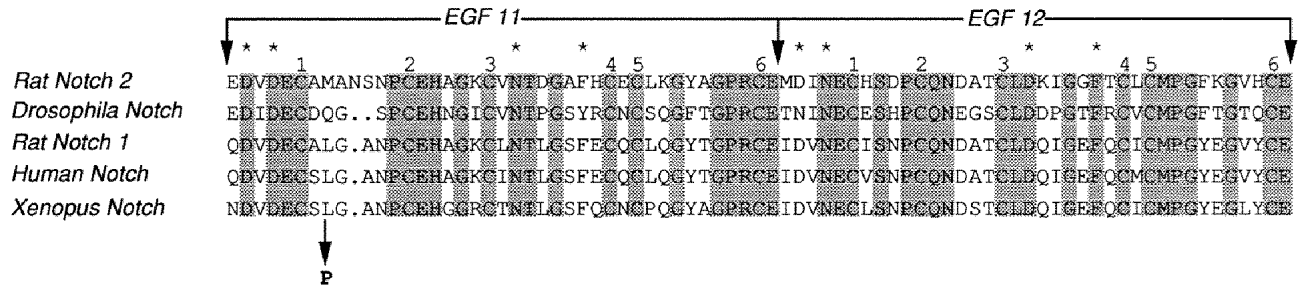

Fig. 3. Comparison of vertebrate and invertebrate EGF-like repeats sufficient for binding either Delta or Serrate in *in vitro* aggregation assays. The rat Notch2 sequence encoding the 11th and 12th EGF-like repeats was aligned to the equivalent published sequence from *Drosophila* Notch (Wharton et al., 1985a; Kidd et al., 1986), rat Notch (Weinmaster et al., 1991), human Notch (Ellisen et al., 1991) and *Xenopus* Notch (Coffman et al., 1990), (modified from Rebay et al., 1991). The cysteines are numbered and the conserved putative calcium-binding residues (Handford et al., 1991) are marked with an asterisk (*). Amino acid residues conserved between the Notch proteins are shaded. The leucine (L) to proline (P) change between the first and second cysteine of EGF repeat 11 from *Xenopus* Notch is indicated by the arrow (Rebay et al., 1991). See text for discussion of this missense mutation in *Xenopus* Notch, the apparent additional residues in Notch2 relative to the other Notch proteins and the relavence of these changes to ligand binding.

Hydropathy analysis of the Notch2 amino acid sequence identified two hydrophobic domains. One region, 27 residues in length, located at the extreme N terminus, probably corresponds to a cleaved signal peptide sequence. The second hydrophobic region, located between amino acids 1677-1699 (see Fig. 2), is a putative transmembrane domain. Between this putative transmembrane domain and the LNR are 2 cysteines that are conserved in all previously described Notch proteins as well as *C. elegans* lin-12 and glp-1. In *C. elegans*, these highly conserved cysteines are thought to promote dimerization of lin-12 following ligand binding (Greenwald and Seydoux, 1990). They are found in the Notch2 amino acid sequence at positions 1632 and 1639 (Fig. 2).

The intracellular region of *Drosophila* Notch contains CDC10 repeat elements that were first described in yeast cell cycle control proteins (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989) and have subsequently been identified in a diverse set of proteins including ankyrin (Lux et al., 1990) and a number of transcription factors (Thompson et al., 1991; LaMarco et al., 1991; Hoffman, 1991). Notch2 was found to have six of these CDC10 repeats, a number that is characteristic of the Notch family of proteins. Notch1 and Notch2 show 76% amino acid sequence identity in this region. Immediately carboxy-terminal to the CDC10 repeats Notch1 and Notch2 have several short areas of amino acid homology. However, the homology between Notch1 and Notch2 begins to break down near residues 2210 and 2171, respectively, which is also an area where *Drosophila* Notch has a large insert of approximately 100 residues relative to the vertebrate Notch proteins (Weinmaster et al., 1991). The amino acid sequence of Notch2 from residues 2171 to 2385 shows no signifigant homology with either *Drosophila* Notch or rat Notch1 protein sequences from the equivalent region.

The OPA region of *Drosophila* Notch contains a stretch of 30 glutamine residues (Wharton et al., 1985b). These glutamines are only partially conserved in Notch1 (10 glutamines) and in Notch2 only 3 glutamines were detected in the general OPA area (Fig. 2). It is possible that the OPA repeats are similar to the unstable CAG repeats associated with mutations in the androgen receptor gene, which are thought to underly to X-linked spinal and bulbar muscular atrophy (Spada et al., 1991).

The amino acid sequences near the C termini of the *Drosophila* and vertebrate Notch proteins are highly conserved and contain a number of putative phosphorylation sites (Pearson and Kemp, 1991) nested in a PEST consensus sequence (Rogers et al., 1986). The Notch2 protein sequence also contains a PEST sequence near its C terminus, as well as a number of putative phosphorylation sites. In this region, the Notch1 and Notch2 proteins exhibit 79% amino acid sequence identity. Finally, 12 out of the last 14 amino acids in the human, *Xenopus* and rat Notch1 proteins are identical, while the terminal 20 residues in *Drosophila* Notch appear unrelated to these sequences. Interestingly, the Notch2 protein sequence ends with 28 amino acids that have no sequence homology with any of the previously described Notch proteins.

A phylogenetic tree for the *Notch* gene family was obtained as described by Feng and Doolittle (1987) (Fig. 4). This representation of the *Notch* gene family suggests that rat Notch1, human Notch and *Xenopus* Notch are homologs and that they are more closely related to each other than to either rat Notch2 or *Drosophila* Notch. Moreover, since the amino acid sequences of rat Notch1 and Notch2 are equally related to *Drosophila* Notch, it is difficult from sequence alone to discern which of the two rat genes is the true homolog of *Drosophila* Notch (see Discussion). The *C. elegans* proteins, lin-12 and glp-1, and mouse int-3 are more distantly related to the vertebrate and *Drosophila Notch* genes and may represent distinct subfamilies.

Northern blot hybridsation analyses of Notch1 and Notch2 gene expression

Fig. 5 illustrates the amount of Notch1 and Notch2 mRNA present in embryonic (E14 and E17), postnatal (P10 and 2 mos) and adult rat brain. As previously reported, Notch1 expression was high in embryonic brain and decreased thereafter (Weinmaster et al., 1991). In contrast, the level of Notch2 expression in the rat brain remained nearly con-

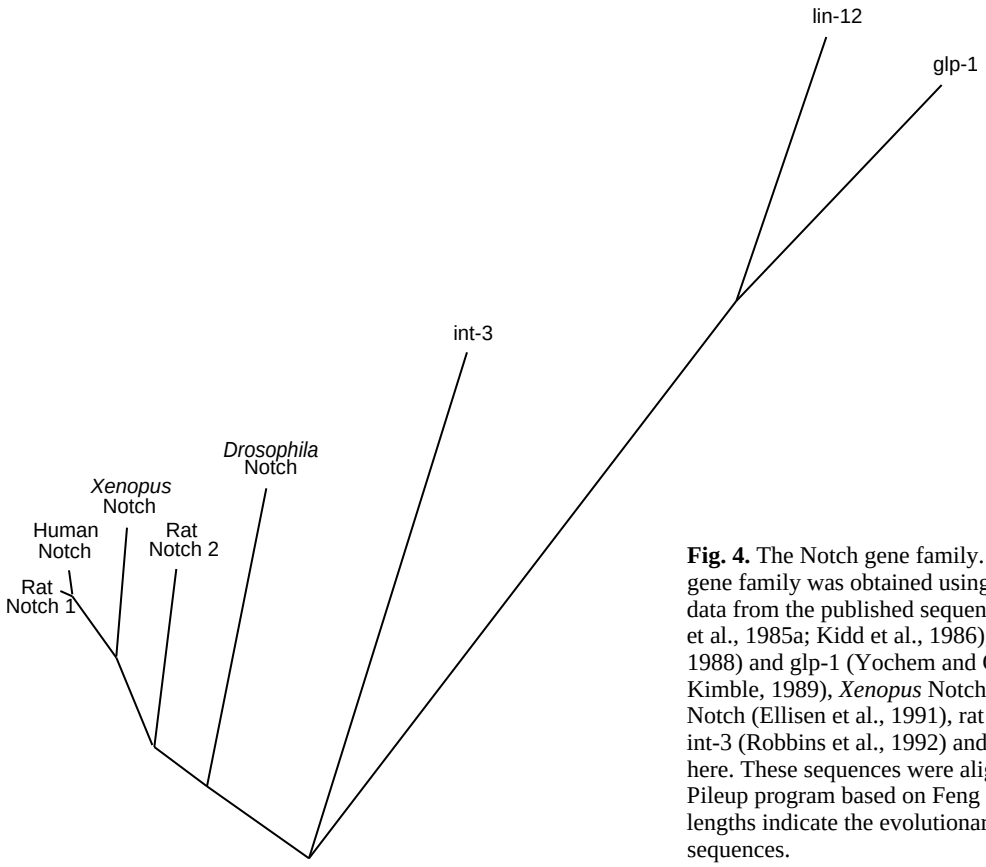

Fig. 4. The Notch gene family. A phylogenetic tree for the Notch gene family was obtained using deduced amino acid sequence data from the published sequences of *Drosophila* Notch (Wharton et al., 1985a; Kidd et al., 1986), *C. elegans* lin-12 (Yochem et al., 1988) and glp-1 (Yochem and Greenwald, 1989; Austin and Kimble, 1989), *Xenopus* Notch (Coffman et al., 1990), human Notch (Ellisen et al., 1991), rat Notch (Weinmaster et al., 1991), int-3 (Robbins et al., 1992) and the rat Notch2 sequence reported here. These sequences were aligned and analysed using the GCG Pileup program based on Feng and Doolittle (1967). The branch lengths indicate the evolutionary distance between the different sequences.

stant throughout this same period (Fig. 5). To determine if the two rat Notch genes were expressed outside the nervous system, we examined RNA isolated from various tissues obtained from a 27-day-old rat. The results presented in Fig. 5 indicate that both Notch1 and Notch2 are expressed in a number of postnatal tissues; however, the level of RNA expressed by the two Notch genes in the various tissues examined was markedly different. Most notable is the signal obtained for Notch2 in the spleen, which was approximately 50-fold higher than that observed for Notch1. A similar ratio of mRNA expression for Notch2 versus Notch1 was also found with IG-positive mouse splenocytes (G. W. and J. Lesley, unpublished data).

Comparison of Notch1 and Notch2 transcripts in E16 rat embryos

The rat Notch1 gene is transcribed in both neural and non-neural cells (Weinmaster et al., 1991). To determine if the related Notch2 gene had a similar pattern of expression, we used in situ hybridisation. This analysis revealed both overlapping as well as differential expression for the two Notch genes. Table 1 summarises the sites of Notch1 and Notch2 gene expression identified in adjacent sections of E16 rat embryos.

Both genes were expressed in the developing central nervous system (CNS) and peripheral nervous system (PNS), but in different cell types. A strong signal for Notch1 was clearly visible around the ventricles in the developing rat brain (Fig. 6A,B), with the signal restricted to the periventricular zone (Fig. 7B). In contrast, no signal above background was detected for Notch2 in the ventricular linings (Fig. 6C,D). Conversely, a strong Notch2 signal was detected in the epithelial cells of the choroid plexus (Fig. 7D), although no Notch1 signal was detected in this tissue (Fig. 7B).

Table 1. *Distribution of Notch 1 and Notch 2 mRNA expression in E16 rat embryos determined by in situ hybridisation*

Site	Notch 1	Notch 2
Neuroepithelium (ventricular zone)	+	−
Choroid plexus	−	+
Eye	+	+
Trigeminal ganglion	+	+
Dorsal root ganglion	+	+
Neural canal	+	+
Nasal cavity	+	−
Cochlear duct	+	−
Thymus	+	−
Salivary gland	+	−
Aorta	+	−
Lung	+	−
Kidney	+	+
Adrenal	+	+
Stomach	+	+
Intestine	+	−
Pancreas	+	−
Skin	+	−
Vibrissae	+	−

Symbols characterize mRNA signal: −, not detected; +, positive.

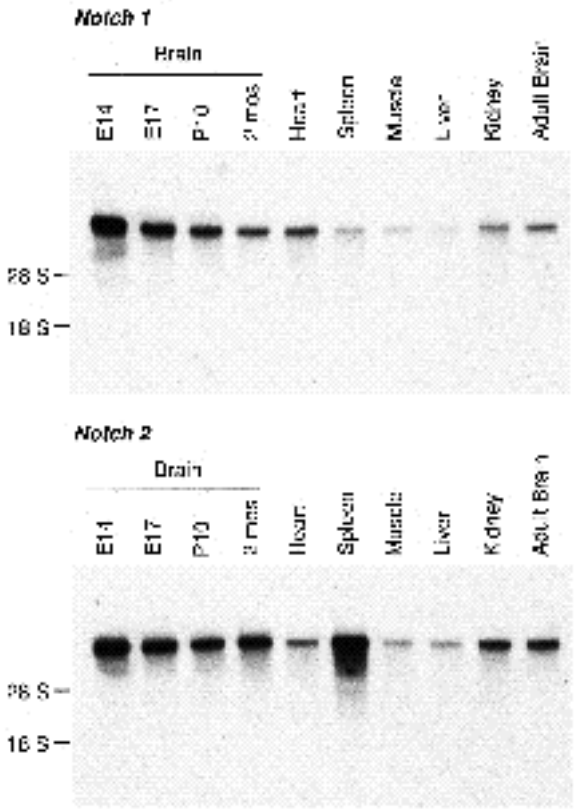

Fig. 5. Northern blot hybridisation analysis of Notch1 and Notch2 mRNAs isolated from embryonic, postnatal, and adult rat brains and various rat adult tisssues. RNA blots were prepared using 2 μg of poly(A)$^+$ RNA isolated from the brains of rat embryos (E14 and E17), postnatal day 10 (P10) and adult (27 days and 2 months) and from indicated tissues of 27-day-old rats. The blots were hybridised with either rat Notch1 or rat Notch2 probes as indicated. Both probes identify transcripts of ~10 kb. The locations of the 28S and 18S ribosomal RNAs are marked. Methylene blue staining of the membrane indicated that equal amounts of RNA are present in each lane allowing quantitative comparisons between Notch1 and Notch2 expression.

Notch1 and Notch2 also had complementary expression patterns in distinct regions of the eye and dorsal root ganglia. Notch1 was expressed in proliferating cells of the retina (Fig. 7F), while Notch2 mRNA was detected in mesenchyme external to the pigmented epithelium (presumptive choroid) and not in the retina (Fig. 7H). Examination at higher power identified silver grains over nuclei of pigmented epithelial cells for both Notch1 and Notch2 (data not shown). The entire dorsal root ganglion (DRG) appeared positive for Notch1 expression (Fig. 8F), while the Notch2 probe labelled only the perineurium surrounding the DRG (Fig. 8H). The branches of the trigeminal ganglion were positive with both Notch probes (Fig. 8B,D); however, the Notch1 signal appeared more intense than that detected for Notch2. Finally, while both genes were expressed in the glomeruli of the kidney (Fig. 9B,D), only Notch1 was expressed in the bronchiole tubes in the lung (Fig. 9F,H) and matrix cells of the developing vibrissae (Fig. 9J,L). In both of these areas, the Notch1 signal was restricted to epithelial cells.

In situ hybridisation of Notch1 and Notch2 in adult rat brain

The northern blot hybridisation analyses described above indicated that both Notch1 and Notch2 were expressed in the adult brain, but at dissimilar levels. The results presented in Fig. 10 show that the most prominent site of differential expression between Notch1 and Notch2 was the choroid plexus. A dense autoradiographic signal was found over the choroid plexus in the lateral (Fig. 10B), third and fourth ventricles (data not shown) with the Notch2 probe. In contrast to the robust Notch2 signal detected in the choroid plexus, Notch1 was not expressed at this site (Fig. 10A). Coronal sections through the hippocampal formation in the forebrain identified a second site of differential expression. A strong Notch2 signal localised over the granule cells of the dentate gyrus (Fig. 10D), yet other areas of the hippocampus appeared negative. In contrast, Notch1 expression was below the level of detection in all areas of the hippocampus examined (Fig. 10C). The leptomeninges that surround the brain were strongly positive for Notch2 expression and negative for Notch1 (Fig. 10E,F). The ependymal cells lining the third (Fig. 10G,H), lateral (Fig. 10A) and fourth ventricles (not shown) in the brain were the only cell types identified that were positive for Notch1 and negative for Notch2 expression. Finally, hybridisation to coronal sections of adult rat cerebellum identified shared expression for the two Notch genes in the Purkinje cell layer (Fig. 10E,F). Examination at higher power localised the Notch2 signal to small cells surrounding the Purkinje cells and adjacent to the granule cells, indicative of Bergmann glia (data not shown).

Discussion

We have identified a cDNA clone that exhibits substantial nucleotide and deduced amino acid sequence identity with both *Drosophila* Notch and the vertebrate Notch proteins. Based on conservation of the number, arrangement and type of amino acid sequence motifs present in the encoded protein, this gene has been designated *Notch2*. Although amino acid sequence analysis indicates that Notch1 and Notch2 are equally related to *Drosophila* Notch, the following observations suggest that Notch1 may be closer in function to the *Drosophila* protein. First, the strong expression and pronounced developmental regulation of Notch1 during neurogenesis is similar to that reported for *Drosophila* Notch. In addition, the Notch1 sequence in the region of EGF-like repeats 11 and 12 is more closely related to the sequence of the 11/12 repeats in *Drosophila* Notch than it is to the equivalent Notch2 sequence. As noted above, these repeats in *Drosophila* Notch are required for the binding of Delta and Serrate. Finally, the OPA repeats described for *Drosophila* Notch are more highly conserved in Notch1 than in Notch2.

Northern blot and in situ hybridisation analyses have indicated that Notch1 and Notch2 have partially overlapping, yet distinct, patterns of expression in the developing and adult rat. Both of these genes are expressed in developing tissues in which cell-cell interactions are critical. For

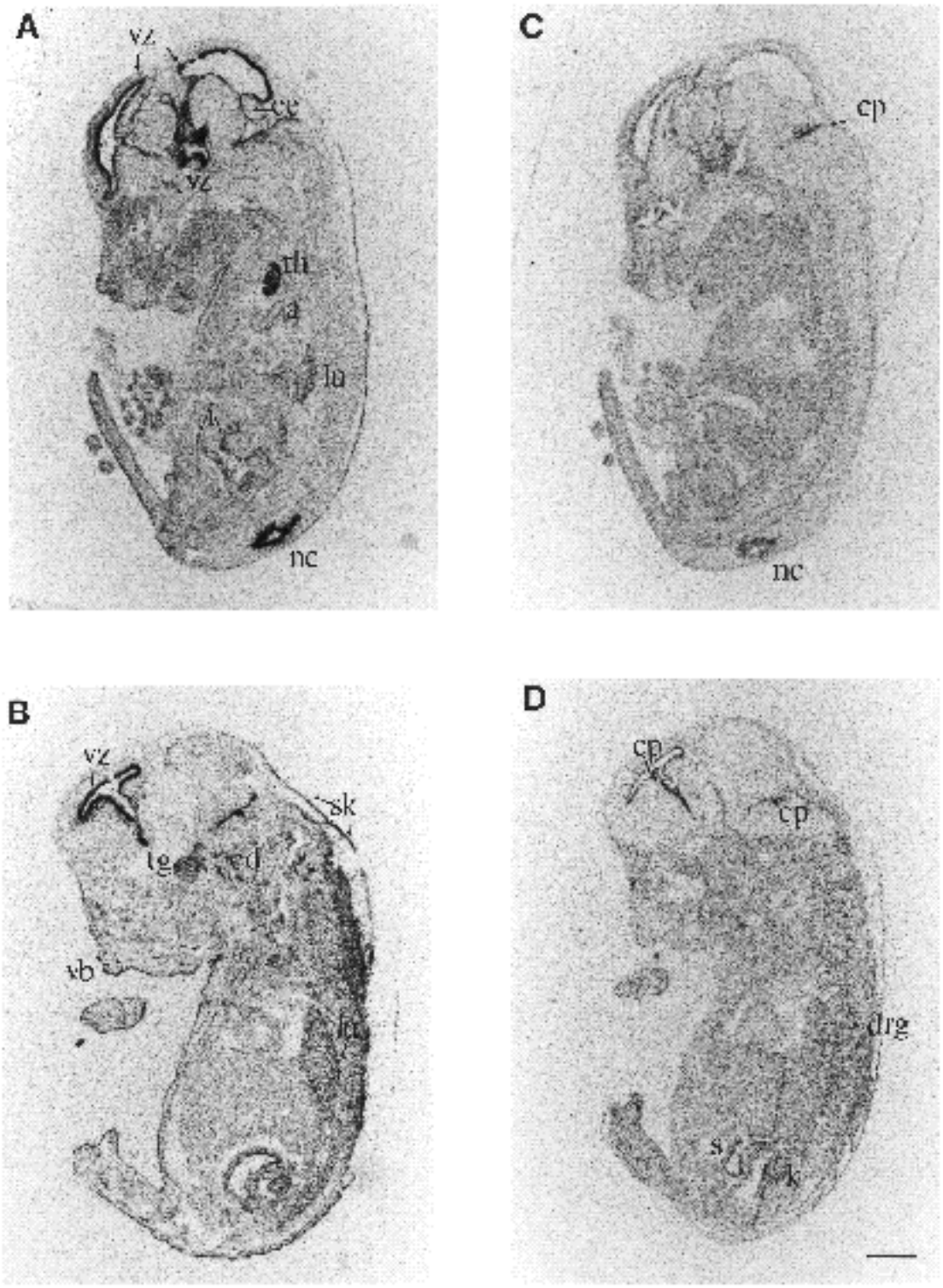

Fig. 6. Localisation of Notch1 and Notch2 gene transcripts in E16 rat embryos. In situhybridisations were performed with [^{35}S]UTP-labelled antisense riboprobes synthesised from either Notch1 or Notch2 cDNAs. Photomicrographs of autoradiagraphs are presented for adjacent sagittal sections through whole E16 rat embryos hybridised with Notch1 probe (A, B) or Notch2 probe (C, D). Tissues and organs with strong hybridisation signals (dark areas) are labelled. The weak signal lining the ventricles in C and D is attributed to the high cell density in this area (see Fig. 7C) and is therefore considered non-specific (confirmed at higher magnification) except where labelled cp. Abbreviations: a, aorta; cd, cochlear duct; ce, cerebellum; cp, choroid plexus; drg, dorsal root ganglia; i, intestines; k, kidney; lu, lung; nc, neural canal; s, stomach; sk, skin; tg, trigeminal ganglion; th, thymus; vb, vibrissae; vz, ventricular zone. The bar in the lower right corner of panel D represents 1.0 mm.

example, the nervous and immune systems require precise cell-cell interactions for their development, and both Notch1 and Notch2 were expressed in dissimilar cell types, at different times during their development. Most notable was the strong Notch1 expression in the ventricular zone of the developing brain, which contains precursor cells that are rapidly proliferating and beginning to differentiate (Fujita, 1967; Rakic, 1988; McConnell, 1988; Price and Thurlow, 1988; Gao et al., 1991). All the cell types required to form the brain originate from these precursor cells (Jacobson, 1990) and it is these cells that display strong Notch1 expression. However, in the adult rat brain the Notch1 signal was greatly reduced and restricted to the ependymal cells lining the ventricles, indicating that Notch1 transcripts are down regulated in many cell types during brain development. In contrast, Notch2 was not expressed by the precursor cells in the ventricular zone of the embryonic brain, yet the granule cell neurons in the dentate gyrus of the adult brain did contain transcripts from this gene. Therefore, Notch2 appears to be up-regulated in certain cells during brain development, unlike the down-regulation observed for Notch1 in similar cell types.

High Notch2 expression was detected both in the newly forming choroid plexus and the adult choroid plexus. This finding raises the possibility that Notch2 not only plays a role in establishing the choroid plexus, but that it may also function in its maintenance. Notch2 expression was confined to the choroid plexus epithelium, which produces the cerebrospinal fluid (Barr and Kiernan, 1988). Notch2 was also expressed by the leptomeninges surrounding the brain and by the perineurium surrounding the DRGs. It is interesting to note that each of these tissues contains special polarized cells that form an occluding epithelium and that function as diffusion barriers in the nervous system (Barr and Kiernan, 1988; Dyck et al., 1984).

Specific cell-cell interactions in the fetal thymus direct the correct differentiation of thymocytes (vonBoehmer, 1988; Shortman, 1990; Scollay, 1991). Notch1, but not Notch2, was expressed strongly in the thymus during this critical period of T-cell development. Consistent with the idea that Notch1 may function during T-cell differentiation, the human Notch gene, TAN-1, was identified through its association with a T-cell-specific acute lymphoblastic leukemia (Ellisen et al., 1991). In the adult spleen, this expression pattern was reversed; high levels of Notch2 transcripts were detected in IG-postive splenocytes that had weak Notch1 expression. It will be interesting to determine whether the expression of Notch1 or Notch2 correlates with different lymphocyte subtypes and/or their differentiated state.

During development of the kidney (Saxen et al., 1986), lung and vibrissae (Kopan and Fuchs, 1989; Hirai et al., 1992), specific interactions between mesenchymal and epithelial cells take place to direct their correct morphogenesis. In each of these cases, Notch1 is expressed by epithelial cells at a time when they are undergoing morphogenesis through receiving signals from the adjacent mesenchyme. Moreover, neither Notch1 nor Notch2 was expressed by the mesenchymal cells adjacent to the developing Notch-expressing epithelial cells. Although speculative, this spatial and temporal expression pattern is not inconsistent with a role for the *Notch* genes in pattern formation and inductive tissue interactions (Cunhan et al., 1985).

Notch mediates a diverse set of cell-cell interactions that direct cell-fate decisions in *Drosophila*. The Notch1 and Notch2 expression data described above support the hypothesis that these proteins play similar developmental roles in mammals. However, we have made the unanticipated finding that the rat differs from *Drosophila* in having more than one *Notch* gene. Furthermore, the two rat *Notch* genes are differentially expressed, suggesting that these genes are not redundant and that the two different receptors they encode may respond to different ligands. The amino acid differences between Notch1 and Notch2 in sequences critical for Delta and Serrate binding lend credence to the idea that Notch1 and Notch2 interact with different ligands. The existence of two *Notch* genes is reminiscent of the two Notch-related *C. elegans* genes, *lin-12* and *glp-1*. The lin-12 and glp-1 proteins function in the receiving cell to direct cell fate during nematode development. Each gene controls a distinct set of cell fates, yet when expressed in the appropriate cells they have been shown to be functionally redundant during embryogenesis (Mango et al., 1991; Lambie and Kimble, 1991). It has been suggested that the glp-1 and lin-12 proteins are interchangeable, but that differential gene regulation accounts for their divergent roles in development (Lambie and Kimble, 1991). We do not know whether Notch1 and Notch2 are interchangeable in the rat, or if the differences in expression reported for these two closely related genes means that they could regulate different cell-fate decisions during mammalian development. Functional analysis in transgenic animals, identification of ligands and the development of cell-culture systems designed to dissect the Notch signal transduction pathway, will help to define the role that *Notch* genes play during mammalian development.

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Fig. 7. Differential expression of Notch1 and Notch2 transcripts in the CNS of E16 rat embryos. In situhybridisations were performed with [^{35}S]UTP-labelled antisense riboprobes synthesised from either Notch1 or Notch2 cDNAs. Photomicrographs of photoemulsion-dipped sections showing morphology (bright-field) and signal (white grains in dark-field) are presented for the following organs and tissues: E16 rat brain bright-field (A) and dark-field (B) probed with Notch1 showing signal in the proliferative zone lining the 4th ventricle; the choroid plexus in the ventricle is negative. Adjacent section bright-field (C) and dark-field (D) hybridised with Notch2 probe showing a strong signal in the epithelium of the choroid plexus. The weak signal in the cells lining the ventricle was considered non-specific due to the high cell density in the region (C). E16 rat eye bright-field (E) and dark-field (F) probed with Notch1 showing signal in the proliferating cells of the retina and the ganglion cells. Adjacent section bright-field (G) and dark-field (H) hybridised with Notch2 probe showing complementary signal in the choroid, external to the pigmented epithelium and not in the retina. Abbreviations: ce, choroid epithelium; cp, choroid plexus; g, ganglion cell; pe, pigmented epithelium; vz, ventricular zone. Bar in the lower right corner of G represents 100 μm.

Fig. 8. Differential and shared expression of Notch1 and Notch2 in the PNS of E16 rat embryos. In situ hybridisations were performed with [^{35}S]UTP-labelled antisense riboprobes synthesised from either Notch1 or Notch2 cDNAs. Photomicrographs of photoemulsion-dipped sections showing morphology (bright-field) and signal (white grains in dark-field) are presented for the following tissues: Sagittal section through E16 rat embryo head in the area of the trigeminal nerve, bright-field (A) and dark-field (B), probed with Notch1 showing the signal in the maxillary branch of the trigeminal nerve. Adjacent section bright-field (C) and dark-field (D) probed with Notch2 also showing signal in this branch of the trigeminal nerve (B). Sagittal section through an E16 rat embryo near the spinal column bright-field (E) and dark-field (F) probed with Notch1 showing signal in the DRGs between the vertebrae. Adjacent section bright-field (G) and dark-field (H) probed with Notch2 showing the reciprocal signal to Notch1 in the perineurium and background signal in the DRGs. Abbreviations: drg, dorsal root ganglion; pn, perineurium; tn, trigeminal nerve. The bar in the lower right corner of C and G represents 100 μm.

Fig. 9. Differential and shared expression of Notch1 and Notch2 in tissues and organs undergoing morphogenesis requiring epithelial-mesenchymal interactions. In situ hybridisations were performed with [^{35}S]UTP-labelled antisense riboprobes synthesised from either Notch1 or Notch2 cDNAs. Photomicrographs of photoemulsion-dipped sections showing morphology (bright-field) and signal (white grains in dark-field) are presented for the following organs and tissues: E16 rat embryo kidney bright-field (A) and dark-field (B) probed with Notch1 showing signal in the developing glomeruli. Adjacent section bright-field (C) and dark-field (D) probed with Notch2 also shows signal in the kidney glomeruli. E16 rat embryo lung bright-field (E) and dark-field (F) probed with Notch1 showing signal in the epithelium of the developing lung bronchiole tubes. Note that the mesenchymal cells surrounding the bronchiole tubes are negative for Notch1 transcripts. Adjacent section bright-field (G) and dark-field (H) probed with Notch2 showing only background signal in both the bronchiole epithelium and surrounding mesenchymal cells. E16 rat embryo vibrissae bright-field (I) and dark-field (J) probed with Notch1 showing a strong Notch1 signal in the matrix cells of the bulb and the strikingly negative signal in the dermal papilla which is mesenchymal in origin. Ajacent section bright-field (K) and dark-field (L) probed with Notch2 showing only background signal thoughout the entire area. Abbreviations: bt, bronchiole tubes; dp, dermal papilla; gl, glomeruli; m, matrix cells of the whisker bulb. The bar in the lower right corner of K represents 100 μm.

Fig. 10. Expression of Notch1 and Notch2 in the adult rat brain. In situ hybridisations were performed with [^{35}S]UTP-labelled antisense riboprobes synthesised from either Notch1 or Notch2 cDNAs. Dark-field photomicrographs of emulsion-dipped sections showing signal (white grains) are presented for selected brain sections. Coronal section through the lateral ventricle probed with Notch1 (A) showing signal in the ependymal cells lining the lateral ventricle. Note the lack of signal in the choroid plexus. A matched section (B) probed with Notch2 shows a dense autoradiographic signal over the choroid plexus. Coronal sections through the hippocampus probed with Notch1 (C) or Notch2 (D) showing a strong Notch2 signal only in the granule cell neurons in the dentate gyrus (D). The remaining structures in the hippocampus are negative for both Notch1 and Notch2 transcripts. Coronal section through the cerebellar cortex probed with Notch1 (E) showing the signal in Bergmann glial cells in the Purkinje cell layer. A matched section probed with Notch2 shows a strong signal in the leptomeninges and in the Bergmann glia. Coronal section through the 3rd venticle probed with Notch1 (G) showing signal in the ependymal cells lining the ventricle. A matched section probed with Notch2 shows no specific signal (H). Abbreviations: bg, Bergmann glial cells; cp, choroid plexus; dg, dentate gyrus; ep, ependymal cells; gc, granule cell layer; lm, leptomeninges; lv, lateral ventricle; oc, optic chiasma; 3rd V, third ventricle. The bar in the lower right hand corner of B and H represents 400 μm.