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Characterization and Developmental Expression of the Amphioxus Homolog of *Notch* (*AmphiNotch*): Evolutionary Conservation of Multiple Expression Domains in Amphioxus and Vertebrates

Linda Z. Holland,* Laurent Abi Rached,† Richard Tamme,‡ Nicholas D. Holland,* Hidetoshi Inoko,§ Takashi Shiina,§ Carola Burgtorf,¶ and Michael Lardelli‡

**Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0202; †INSERM Unité 119, 27 Boulevard Leï Roure, 13009 Marseille, France; ‡Department of Molecular Biosciences, Discipline of Genetics, Adelaide University, South Australia 5005, Australia; §Department of Genetic Information, Division of Molecular Life Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan; and ¶Max Planck Institut für Molekulare Genetik, D-14195 Berlin, Germany*

Notch **encodes a transmembrane protein that functions in intercellular signaling. Although there is one** ***Notch*** **gene in** ***Drosophila,*** **vertebrates have three or more with overlapping patterns of embryonic expression. We cloned the entire 7575-bp coding region of an amphioxus** ***Notch*** **gene (*AmphiNotch*), encoding 2524 amino acids, and obtained the exon/intron organization from a genomic cosmid clone. Southern blot and PCR data indicate that *AmphiNotch* is the only *Notch* gene in amphioxus. *AmphiNotch,* like *Drosophila Notch* and vertebrate *Notch1* and *Notch2,* has 36 EGF repeats, 3 Notch/lin-12 repeats, a transmembrane region, and 6 ankyrin repeats. Phylogenetic analysis places it at the base of all the vertebrate genes, suggesting it is similar to the ancestral gene from which the vertebrate *Notch* family genes evolved. *AmphiNotch* is expressed in all three embryonic germ layers in spatiotemporal patterns strikingly similar to those of all the vertebrate homologs combined. In the developing nerve cord, *AmphiNotch* is first expressed in the posteriormost part of the neural plate, then it becomes more broadly expressed and later is localized dorsally in the anteriormost part of the nerve cord corresponding to the diencephalon. In late embryos and larvae, *AmphiNotch* is also expressed in parts of the pharyngeal endoderm, in the anterior gut diverticulum, and, like *AmphiPax2/5/8,* in the rudiment of Hatschek's kidney. A comparison with *Notch1* and *Pax5* and *Pax8* expression in the embryonic mouse kidney helps support homology of the amphioxus and vertebrate kidneys. *AmphiNotch* is also an early marker for presumptive mesoderm, transcripts first being detectable at the gastrula stage in a ring of mesendoderm just inside the blastopore and subsequently in the posterior mesoderm, notochord, and somites. As in sea urchins and vertebrates, these domains of *AmphiNotch* expression overlap with those of several *Wnt* genes and *brachyury*. These relationships suggest that amphioxus shares with other deuterostomes a common mechanism for patterning along the anterior/posterior axis involving a posterior signaling center in which the *Notch* and *Wnt* pathways and *brachyury* interact.** **© 2001 Academic Press**

Key Words: **Brachyury; pattern formation; Notch; amphioxus; lancelet.**

INTRODUCTION

Notch genes encode single-pass transmembrane receptors which mediate intercellular communication. The extracellular domain includes up to 36 EGF repeats and 3 Lin/Notch repeats, while the intracellular domain includes 6 ankyrin repeats. Notch undergoes proteolytic cleavage at three sites during maturation and signaling (reviewed in Annaert and De Strooper, 1999; Weinmaster, 2000). Notch proteins function during embryogenesis in cell fate deci-

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sions in the neuroectoderm and other tissues as well as in formation of borders as in the *Drosophila* wing and eye, vertebrate limbs, and somitic mesoderm (Beatus and Lendahl, 1998; Jiang *et al.*, 1998; Lewis, 1998; Irvine, 1999). The core Notch signaling pathway (reviewed in Bray, 1998; Jiang *et al.*, 1998; Kimble *et al.*, 1998; Fleming, 1998; Weinmaster, 2000) is highly conserved between the ecdysozoans (*Drosophila* and *Caenorhabditis elegans*) and higher deuterostomes (vertebrates), although there is evidence that Notch can act in an alternate pathway(s) (Rusconi and Corbin, 1998). The Notch signaling pathway is modulated at multiple levels by interaction with proteins such as Wingless, Dishevelled, Big Brain, Numb, and Hairless (reviewed in Panin and Irvine, 1998; Wu and Rao, 1999), Fringe (Munro and Freeman, 2000), and Scute (Cooper *et al.*, 2000). Several downstream targets of the Notch signaling pathway have been identified, including *Brachyury* in the notochord of ascidian tunicates (Corbo *et al.*, 1997, 1998), *sticks-and-stones*, involved in myoblast fusion in *Drosophila* (Bour *et al.*, 2000), *vestigial* and *wingless* in the *Drosophila* wing (Rulifson and Blair, 1995; Axelrod *et al.*, 1996; Artavanis-Tsakonas *et al.*, 1999), and *HES1* and *her1* in presomitic mesoderm in vertebrates (Takke and Campos-Ortega, 1999; Jouve *et al.*, 2000).

In *Drosophila* and lower deuterostomes (ascidian tunicates and sea urchins) there is a single Notch gene, while in vertebrates there are multiple *Notch* genes (four in the mouse). An independent gene duplication has resulted in two Notch genes in *Caenorhabditis* (Yochem and Greenwald, 1989). In vertebrates, *Notch1, 2,* and *3* are expressed in numerous tissues, and their expression domains partially overlap. These domains include the central nervous system, otic vesicle, presomitic mesoderm, pancreas, hemopoietic cells, limb bud, hair, tooth, and kidney (Coffman *et al.*, 1990; Bierkamp and Campos-Ortega, 1993; Lardelli and Lendahl, 1993; Conlon *et al.*, 1995; Williams *et al.*, 1995; Beatus and Lendahl, 1998; Lammert *et al.*, 2000; Singh *et al.*, 2000). The divergent *Notch4* has more restricted expression in maturing macrophages, the pancreas, and endothelial cells (Lewis *et al.*, 1998; Lammert *et al.*, 2000; Singh *et al.*, 2000). In contrast, the single *Notch* genes in sea urchins and ascidian tunicates have very limited expression domains. Ascidian *Notch* is expressed chiefly in the neural plate and anterior adhesive organ (Hori *et al.*, 1997), while zygotic expression of sea urchin *Notch* is limited in the late blastula to cells at the boundary of the future secondary mesoderm and endoderm and later to the secondary mesenchyme cells (Sherwood and McClay, 1997, 1999). The restricted expression in these lower deuterostomes raises the question of when the many domains of *Notch* expression arose in vertebrate evolution and how the evolution of these domains correlates with duplications of the *Notch* gene.

To address this question, we cloned the single *Notch* gene from the invertebrate chordate, amphioxus, and determined its intron/exon organization and embryonic expression. Amphioxus is the closest living invertebrate relative of the vertebrates (Wada and Satoh, 1994; Holland and Garcia-Fernàndez, 1996). Although the cephalochordate and vertebrate lineages separated about 500 million years ago, amphioxus is proving to be a relatively good proxy for their most recent common ancestor. Amphioxus development up to the gastrula stage is sea urchin-like: cleavage produces a hollow blastula, which then invaginates from the vegetal pole to form a gastrula. However, subsequent development is vertebrate-like. After gastrulation, the embryo develops a notochord, segmentally arranged somites, a dorsal hollow nerve cord, and a pharynx with gill slits. The larva develops homologs of the vertebrate thyroid gland, kidney, and pancreatic islet cells; however, a complete vascular endothelium, an otic vesicle, and paired eyes are lacking.

Our results indicate that amphioxus has a single *Notch* gene with a full complement of 36 EGF repeats. The gene has 30 exons (Table 2). The positions of several introns are conserved between amphioxus *Notch*, *Drosophila Notch*, and vertebrate *Notch4*. Thus, amphioxus *Notch* is probably representative of the ancestral deuterostome *Notch* gene. Fewer EGF repeats in *Notch* genes of lower deuterostomes and *Notch3* and *4* of vertebrates appear to be due to independent losses. Expression of amphioxus *Notch* throughout development closely parallels that of the multiple *Notch* genes of vertebrates put together, except that the vertebrate genes are also expressed in structures that are unique to vertebrates, supporting the idea that gene duplications in the vertebrate lineage may have facilitated the evolution of new structures. Comparisons of early developmental expression in amphioxus, vertebrates, and lower deuterostomes suggest that patterning along the anterior/posterior axis in the ancestral deuterostome embryo involved a posterior signaling center including the *Notch* and *Wnt* pathways.

MATERIALS AND METHODS

Cloning of AmphiNotch cDNAs

Adult amphioxus (*Branchiostoma floridae*) were obtained by shovel and sieve from Old Tampa Bay, Florida. Spawning was induced by electric shock and embryos were raised at 23°C as previously described (Holland and Holland, 1993). Total RNA was isolated from 2- to 4-day larvae by the method of Chomczynski and Sacchi (1987) and used for cDNA synthesis by random priming. cDNAs encompassing EGF and lin-12/Notch (LN) repeat sequences of amphioxus *Notch* were amplified by PCR with the degenerate primers Mila3 [5′ TG(T/C)CA(A/G)AA(T/C)GIGGIACITG 3′] and Mila4 [5′ (A/G)CA(T/C)TCIGC(A/G)TT(A/G)CAICC 3′] as previously described (Westin and Lardelli, 1997). cDNA from a 200-μl PCR was purified on a Wizard PCR preps column (Promega, Inc., Madison, WI), precipitated with ethanol, and redissolved in 50 μl of 25 mM Tris–Cl (pH 8.0), 20 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml BSA, and 0.1 *S*-adenosyl methionine. Possible *Srf*I sites were methylated to prevent cleavage by *Srf*I during subsequent ligation by incubation with 45 units of *Hpa*II methylase (Fermentas AB, Vilnius, Lithuania) at 37°C for 1 h. To "polish" the cDNA ends, after methylation the NaCl concentration was increased to 50 mM, dNTPs to 70 μM each, and 2 units

TABLE 1
PCR Primers and Conditions

Oligo name	Oligo sequence (5′–3′)	Binding site	Paired with	T (°C) Annealing	Extn. time (s)
Mila190	TTG ACG ATG TCA GAG TGC	ANK7	Mila191	63	180
Mila191	AAC TGT GAC CAG CAG TGC	LNR2	Mila190	63	180
Mila207	ATC ATC GCC AGT GGA CC	A-T	Mila190	63	180
Mila227	TAG CTC GCA GTT GTC TCC ACC	EGF3,4	Mila281	50	270
Mila269	GAC GAT GTC AGA GTG CAT GC	ANK4	Mila270	60	60
Mila270	CTC AAC TCG CAC GCT GAT GC	ANK7	Mila269	60	60
Mila281	TAT GCA GTC CCC AAA CAT CTG C	EGF8	Mila227	62	270
Mila339	TTC CGC AGT TCA AGC AGA TGT TTG GG	EGF8	(Self)[a]	[a]	[a]
Mila379	GAT TCG CAC GTC CCT CCG TGT T	EGF6	Mila381	64	120
Mila381	TGT GCG GAG GAA AGG CGT CGC	5′UTR	Mila379	64	120

Note. PCR cycling was 35 cycles of 94°C/30 s, annealing temperature for 30 s, then ramp of +0.5°C/s to 72°C, then 72°C/(extension (Extn.) time). Binding sites are EGF, EGF repeat; LNR, LN repeat; ANK, ankyrin repeat; T-A, between transmembrane domain and ankyrin repeat 1.

[a] See Tamme *et al.* (2000).

of T4 DNA polymerase (Boehringer Inc., Mannheim, Germany) was added, and the mixture was incubated at 25°C for 30 min. The reaction was heated to 70°C for 10 min to inactivate the enzyme, cooled, and ligated to pCR-Script SK(+) (Stratagene Inc., La Jolla, CA). Thirty-five clones containing inserts of 0.6 kb or greater were sequenced (Westin and Lardelli, 1997).

To isolate DNA corresponding to the ankyrin repeats of the amphioxus *Notch* gene, a genomic library constructed in the Lawrist 7 vector (Lawrence Livermore Laboratory, Livermore, CA) was screened with the insert of one of the cDNA clones (Amph26) obtained by PCR. High-density colony filters on Hybond N^+ membrane (Amersham Life Sciences, Inc., Arlington Heights, IL) were hybridized with 10^6 cpm/ml of the probe labeled with ^{32}P by random priming in 6.95% SDS, 1 mM EDTA, 0.1 mg/ml tRNA, 0.5 M sodium phosphate buffer (pH 7.2) at 65°C. Washes were 3× 20 min in 1× SSC, 0.1% SDS at 60°C.

One of the cosmid clones obtained (E1080) was restricted with *Hinc*II and the resultant fragments were subcloned into the *Sma*I site of the pBluescript SK(+) vector (Stratagene). To detect clones containing *Notch* exons, DNA from these subclones was screened on Southern blots with cDNAs spanning the entire open reading frame of mouse *Notch3* using low-stringency hybridization and washes in 6× SSC at 65°C. Two subclones approximately 1 kb long were identified and sequenced at their termini. Alignment of these sequences to vertebrate *Notch* genes revealed exons encoding regions corresponding to *Notch1* EGF repeats 7 to 10 and *Notch1* ankyrin repeats 3 to 7. Oligonucleotides (see Table 1) corresponding to these sequences and to the initially isolated cDNA region were designed and used in reverse transcriptase PCR on mRNA from 2-day-old larvae to amplify additional regions of amphioxus *Notch* cDNA.

Additional PCR with primers and conditions listed in Table 1 yielded much of the remainder of the Notch cDNA sequence. To obtain the ankyrin repeat and 3′ region, cDNA from an oligo(dT)-primed cDNA library of amphioxus gastrula–neurula stages in Lambda ZAP II (Stratagene) was amplified with oligonucleotide primers Mila269 and Mila270 corresponding to ankyrin repeat sequences. One clone was obtained containing Notch cDNA, which was then used to screen the same library. A single clone containing the remaining 3′ extent of the open reading frame of amphioxus *Notch* was obtained. To clone cDNA sequences corresponding to EGF repeats 4 to 8, we used primer Mila339 in the nonspecifically primed RT-PCR technique of Tamme *et al.* (2000). Sequence of EGF repeats 8 through 34 was obtained by PCR with primer pairs Mila227 and Mila281 from first-strand cDNA synthesized from total RNA of 36-h larvae with primer Mila280 (5′-TGA GGA TGT GGA TGA ATG TAT GC-3′). Finally, the complete sequencing of cosmid E1080 permitted the identification of a putative translational start codon.

Genomic Sequencing

Cosmid E1080 was subsequently sequenced in its entirety by the shotgun strategy (Deininger, 1983; Wilson, 1993; Rowen *et al.*, 1996). Five micrograms of cosmid DNA was sheared by sonication, repaired with the large fragment of DNA polymerase I (Klenow fragment) to generate blunt ends, and size fractionated on Chroma Spin-1000 columns (Clontech, Inc., Palo Alto, CA). Fragments larger than 1.0 kb were ligated into the *Sma*I site of pUC19 and transformed into *Escherichia coli* strain DH5α. Approximately 400 recombinant pUC19 clones were sequenced. Individual sequences were minimally edited to remove vector sequences, transferred to a SPRAC station (Sun Microsystems, Palo Alto, CA) on the TCP/IP protocol, and assembled into contiguous sequences with the GENETYX-S/SQ software (SDC: Software Development Co., Tokyo). Remaining gaps or areas of ambiguity were analyzed either by sequencing PCR amplification products or by sequencing the clones in pUC19 with custom primers.

Sequence Comparisons

Sequence alignments were done with the ClustalW program [written by Des Higgins (e-mail: Des.Higgins@ebi.ac.uk)] and manually adjusted. The parameters for the comparison were pairwise similarity parameters—*K*-tuple length, 1; gap penalty, 3; number of diagonals, 5; diagonal window size, 5; scoring method—percentage; multiple alignment parameters—gap penalty (fixed), 10.00; gap penalty (varying), 0.05; gap separation penalty range, 8; percentage identity for delay, 40%; list of hydrophilic residues, GPSNDQEKR; protein weight matrix—blosum.

The Notch cDNA sequence has been deposited in the EMBL and GenBank databases under Accession No. Y12539.

Phylogenetic Analysis

Phylogenetic analysis by the neighbor-joining method was based on the 114-amino-acid sequence of the Lin/Notch domain. Sequences were aligned with the ClustalX program and only conserved portions were used for the phylogenetic analysis. The distance measure was estimated with the Protdist program (categories model George Hunt/Barker categorization of amino acids) of the Phylip program (v3.5c). The reliability of clustering was tested by bootstrapping (100 samples). Only values greater than 49 are shown. The tree was either unrooted or rooted with the *Drosophila* and blowfly Notch sequences with the assumption that the duplications giving rise to the vertebrate *Notch* genes occurred after the deuterostome/protostome split. Sequences used and their accession numbers are *Drosophila Notch* (K03508), blowfly (*Lucilia cuprina*) *SCL* (U58977), sea urchin (*Lytechinus variegatus*) *Notch* (AF000634), zebrafish *Notch1* (Y10352), zebrafish *Notch5* (Y10353), goldfish (*Carassius auratus*) *Notch3* (U09191), *Xenopus Notch1* (M33874), chicken *Notch1* (AF159231), mouse *Notch1* (Z11886), mouse *Notch2* (D32210), mouse *Notch3* (X74760), mouse *Notch*4 (U43691), rat *Notch2* (M93661), human *Notch*1 (M73980), human *Notch*3 (NM_000435), and human *Notch*4 (D63395).

Southern Blot Analysis

DNA was extracted from adults in guanidinium isothiocyanate and purified according to methods in Holland *et al,* (1996). Fifteen 10-μg aliquots of genomic DNA were each digested with a different restriction enzyme, subjected to electrophoresis on an 0.7% agarose gel in 1× TAE buffer, and transferred to Hybond N^+ (Amersham Life Sciences, Cleveland, OH) according to L. Z. Holland *et al.* (1995). Probes were labeled to a specific activity of 1×10^8 cpm/μg by random priming and used at a concentration of 1×10^6 cpm/ml. For low-stringency hybridization to determine the number of *Notch*-related genes in amphioxus, the probe was an 850-bp Mila207–Mila190 clone of the ankyrin repeat region of amphioxus *Notch* (Table 1). Hybridization was in 10× Denhardt's, 0.1 mg/ml tRNA, 0.2% SDS, 6× SSC, 1 mM EDTA at 50°C. Washes were at 55°C in 2× SSC, 0.1% SDS. For a high-stringency blot to determine the specificity of the riboprobe, the Southern blot was stripped and rehybridized with the 850-bp insert of a clone in pCR-Script (Stratagene) containing EFG and LN repeats. Hybridization was as above with the temperature raised to 65°C; washes were 3 × 20 min in 1× SSC, 0.1% SDS at 65°C (L. Z. Holland *et al.,* 1995). Since the probe hybridized with a single band of amphioxus genomic DNA cleaved with 7 of 10 enzymes (data not shown), we concluded that the riboprobe is specific for *Notch* mRNA.

In Situ Hybridization

Expression of amphioxus *Notch* was determined by *in situ* hybridizations on developmental stages of *B. floridae* fixed at intervals during the first 2 days of development. Fertilization envelopes were removed from prehatching stages to facilitate penetration of reagents. The same 850-bp clone used to probe the Southern blot was used as a template for a reverse-sense riboprobe. Methods of fixation, probe synthesis, and hybridization were according to Holland *et al.* (1996). After photography of hybridized embryos as whole mounts, they were counterstained with 1% Ponceau S in 1% acetic acid, dehydrated in ethanol, embedded in Spurr's resin, and sectioned at 3 μm.

RESULTS

Amphioxus Has a Single Notch Gene

Our PCRs with first-strand cDNA of larval *B. floridae* as a template yielded overlapping clones that constituted the entire 7575-bp reading frame of an amphioxus *Notch* gene, which we call *AmphiNotch* (Fig. 1). This sequence corresponded with minor polymorphisms to the open reading frame deduced from the sequence of genomic cosmid clone E1080 that contained the entire *Notch* gene. Thus, there appears to be only one *Notch* gene in amphioxus, since all 21 *Notch* clones obtained from PCR with degenerate primers to the highly conserved EGF region represented the same gene. In addition, on a low-stringency Southern blot probed with the most conserved domain of *Notch* (the ankyrin repeat region) 6 of 10 enzymes resulted in one major hybridizing band (Fig. 2). Since probing a similar blot under somewhat more stringent conditions with the 3′UTR of one of the two muscle actin genes in amphioxus gave multiple bands in all lanes (Kusakabe *et al.*, 1997), the very weakly hybridizing bands on the *Notch* blot are likely due to the hybridization of the probe with the ankyrin repeats of distantly related genes such as *ankyrin* and not to the presence of a second *Notch* gene. Indeed, when probed under higher stringency with a longer probe (1000 bp) to the EGF and Notch/Lin-12 repeat region, 7 of 10 enzymes revealed a single band, and no weakly hybridizing bands were detected (data not shown).

Structure of the AmphiNotch Protein

The AmphiNotch protein (Fig. 1) is 2524 amino acids long and includes 36 EGF repeats, 3 Notch/lin-12 repeats, a transmembrane region, a RAM23 domain, 6 ankyrin repeats (Fig. 1), and an additional highly conserved domain just C-terminal of the ankyrin repeats. There are also S1 (furin), S2 (TACE), and S3 sites for proteolytic cleavage. All of these domains are conserved among AmphiNotch, *Drosophila* Notch, and mouse Notch1 proteins. In addition, most of the EGF repeats have sites for residues of O-linked fucose residues or glucose residues or both (Fig. 3A). These sites are highly conserved among *Drosophila,* amphioxus, and mouse *Notch* proteins. Conserved sites for Ca^{2+} binding and for Asx hydroxylation also occur on most of the EGF repeats (Fig. 3B). With one or two exceptions, these sites cooccur on the same EGF repeats, which is not surprising because some of the Asx residues in the hydroxylation sites are also part of the Ca^{2+}-binding sites.

Exon/Intron Structure of AmphiNotch

Analysis of the genomic clone shows that the entire translated sequence of *AmphiNotch* is contained in 30

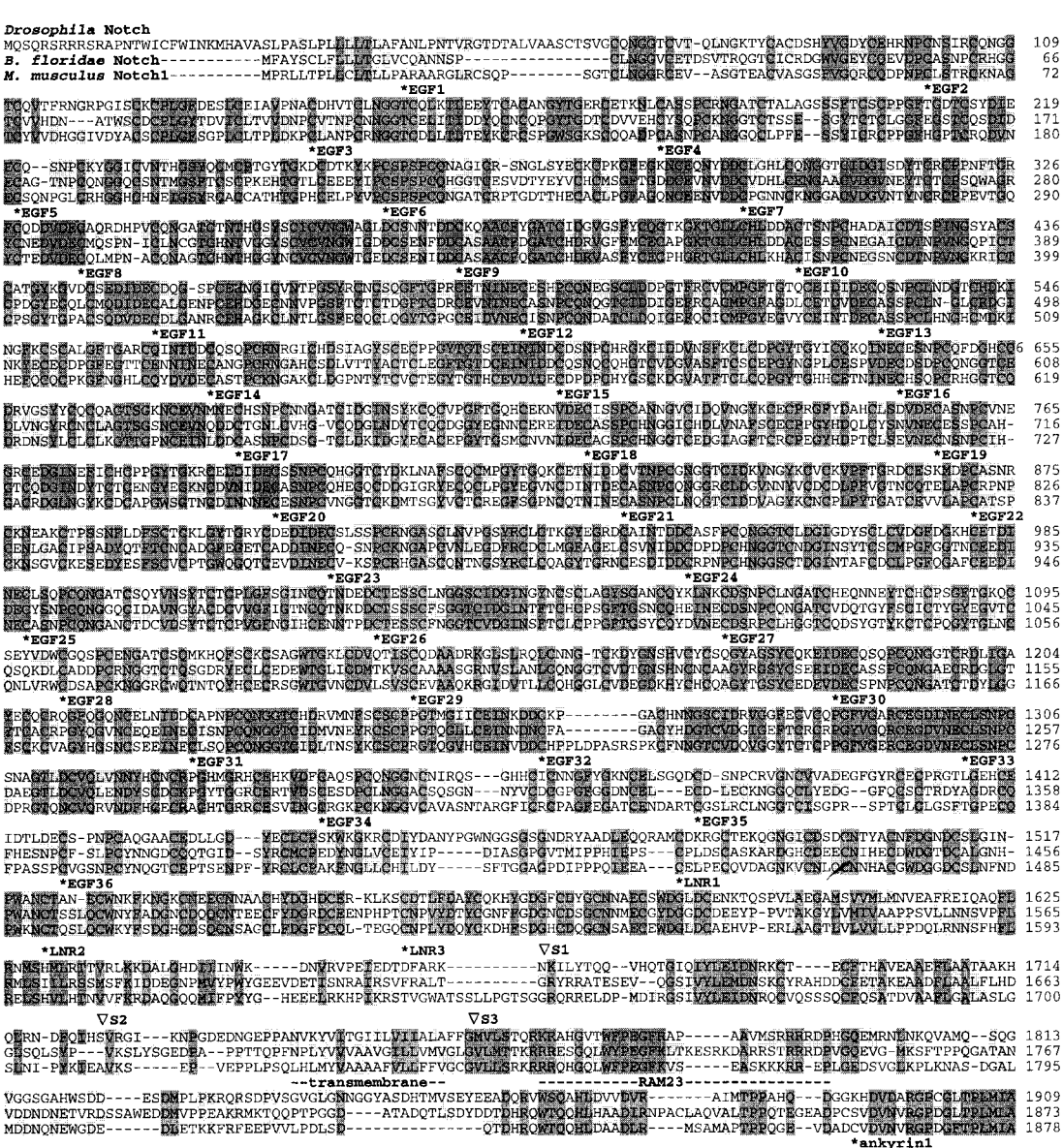

FIG. 1. Alignment of the AmphiNotch, *Drosophila* Notch, and mouse Notch1 proteins. Asterisks indicate the starting residues for EGF, Notch/Lin-12 (LNR), and ankyrin repeats. Shading indicates identities or conserved substitutions among all three (dark gray) or two (light gray) of the homolog proteins. There are three proteolytic cleavage sites, S1 (furin), S2 (TACE), and S3 (dependent on PS/γ-secretase activity); a transmembrane region; the RAM23 domain [which binds effectors of Notch signaling such as Su(H), CBF1, and lag-1]; and a PEST domain.

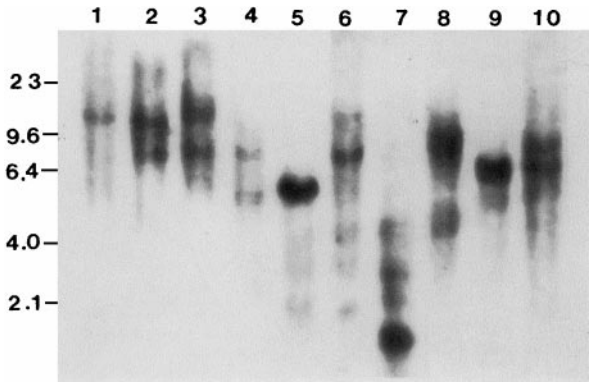

FIG. 2. Low-stringency Southern blot of genomic DNA from *B. floridae* probed with the ankyrin repeat region of *AmphiNotch*. Enzymes are (1) *Bam*HI, (2) *Bgl*II, (3) *Bst*EI, (4) *Eco*0109, (5) *Eco*RI, (6) *Hin*dIII, (7) *Kpn*I, (8) *Nco*I, (9) *Pst*I, (10) *Sal*I. Molecular size markers in kb are indicated at left.

exons (Table 2, Fig. 4). A comparison of intron positions with those of *Drosophila Notch* and human *Notch4* (a vertebrate *Notch* gene for which all intron positions have been published) (Li *et al.*, 1998) shows that three of the three most 5′ introns in amphioxus are conserved among all three organisms (Fig. 4). Furthermore, even through human *Notch4* has only 29 EGF repeats compared to 36 for both the *Drosophila* and the amphioxus *Notch* genes, there are an additional six conserved intron positions between the amphioxus and the vertebrate gene.

Phylogenetic Analysis

To determine the phylogenetic relationship between AmphiNotch and the four vertebrate Notch proteins, we constructed both unrooted (Fig. 5) and rooted (data not shown) trees by the neighbor-joining method. In both trees, AmphiNotch lies at the base of the vertebrate Notch genes, suggesting that duplications of the vertebrate *Notch* genes occurred after the split between amphioxus and the vertebrates.

Expression of AmphiNotch

In situ hybridization reveals no detectable expression of *AmphiNotch* in cleavage stages or the blastula. Transcripts of *AmphiNotch* are first detectable in the midgastrula in a ring of presumptive mesendoderm just inside the widely open blastopore and dorsally in the presumptive notochord and somites (Figs. 6A and 6B). These cells are columnar, and the transcripts of *AmphiNotch* are most abundant in the perinuclear cytoplasm near the cell apices (Fig. 6B). As gastrulation proceeds and the neural plate begins to flatten dorsally, transcripts of *AmphiNotch* (Figs. 6C and 6D) spread throughout the cytoplasm of the mesodermal cells. Dorsally there is a gradient of *AmphiNotch* expression in the presumptive somites and notochord with a high level of expression posteriorly, tapering off anteriorly. Expression progressively decreases ventrally and is undetectable in ventral endoderm (Figs. 6E and 6F). At this stage there is no ventral mesoderm. Ventral mesoderm forms at the midneurula stage as ventral extensions from the somites. In histological sections of the late gastrula/early neurula weak expression is also visible in the posterior neural plate (Fig. 6F, arrow).

During the first phase of amphioxus neurulation, the ectoderm bordering the neural plate on either side moves medially across the open neural plate and fuses in the midline, except at the extreme anterior end where the neuropore remains open to the exterior. Only after the ectoderm has covered the neural plate does the neural plate gradually roll up to form the neural tube (Fig. 6H). At the start of neural tube formation, the first four somites evaginate from the wall of the archenteron (Figs. 6G and 6H). The strongest *AmphiNotch* expression is in the posterior mesoderm and in the anteriormost three somites, especially in the dorsal portion of each (Figs. 6G and 6H). In

TABLE 2
Exon Positions of *AmphiNotch*

	Genomic DNA		cDNA		
Exon	Start	End	Start	End	aa position
1	1592	1713	1	122	41
2	15379	15632	123	376	126
3	16024	16359	377	712	238
4	17943	18065	713	835	280
5	20613	20846	836	1068	357
6	21172	21972	1069	1870	624
7	22187	22297	1871	1981	661
8	22571	22749	1982	2160	720
9	23137	23296	2161	2320	774
10	23726	23958	2321	2554	852
11	24311	24463	2555	2707	903
12	25907	26135	2708	2936	979
13	26843	27044	2937	3138	1046
14	27631	27784	3139	3292	1098
15	28144	28461	3393	3610	1204
16	28926	29263	3621	3948	1316
17	29708	30011	3949	4252	1418
18	30256	30311	4253	4308	1436
19	30575	30768	4309	4502	1501
20	31326	31528	4503	4705	1569
21	31921	32092	4706	4877	1626
22	32404	32555	4878	5029	1677
23	33090	33327	5030	5267	1756
24	34029	34146	5268	5385	1795
25	34665	34872	5386	5593	1865
26	35270	35419	5594	5743	1915
27	35750	35901	5744	5895	1965
28	36205	36352	5896	6043	2015
29	36672	36769	6044	6141	2047
30	36895	38328	6142	7575	2524

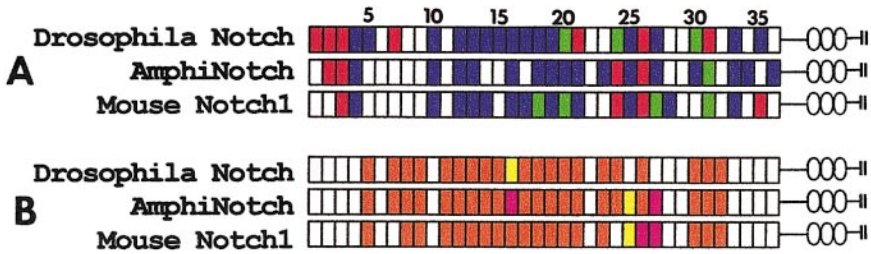

FIG. 3. Comparison of EGF repeat region of *Drosophila* Notch, AmphiNotch, and mouse Notch1 showing sites for (A) glycosylation and (B) Ca^{2+} binding. Red, sites for O-linked fucose. Blue, sites for O-linked glucose. Green, sites for both O-linked fucose and glucose. Yellow, aspartic acid/asparagine hydroxylation sites. Pink, Ca^{2+} binding sites. Orange, sites for both aspartic acid/asparagine hydroxylation and Ca^{2+} binding.

the most recently-formed somite (i.e., the most posterior), there is no detectable *AmphiNotch* expression. As successive somites are added, *AmphiNotch* transcription begins in the second-youngest somite. At this stage *AmphiNotch* is also expressed weakly throughout the neural plate and forming notochord (Fig. 6H).

The pattern of *AmphiNotch* expression within the somites changes with time. By hatching at 11 h, expression in the somites is still predominantly dorsal (Fig. 6I), but by 13 h it is also strong in the posterior half (Figs. 6J and 6L). Transcripts in the posterior mesoderm remain conspicuous in elongating embryos (Figs. 6I–6K, 6M, 6O, and 6S). Moderate expression continues in the notochord while that in the neural plate intensifies as it begins to roll up (Fig. 6L). In the late neurula the pattern of *AmphiNotch* transcripts in somites and posterior mesoderm remains unchanged (Figs. 6M and 6N), but there is a new zone of expression in the anterior pharyngeal endoderm (arrow, Fig. 6M). At this stage, the neural tube has rolled up and most of its cells still contain a low level of *AmphiNotch* transcripts (Fig. 6N). By 22 h (Figs. 6O and 6P) expression of *AmphiNotch* is downregulated in the somites and notochord but is upregulated in cells in the dorsal half of the cerebral vesicle. Transcripts remain conspicuous in the posterior mesoderm, the anterior pharyngeal endoderm, and the wall of the anterior left gut diverticulum (Hatscheck's left diverticulum) (Figs. 6O, 6P, and 6Q). In the late embryo (28 h) in which the mouth and first gill slit are forming, *AmphiNotch* is still expressed in the posterior mesoderm and in cells of the cerebral vesicle, but is downregulated in all but a few cells of the posterior nerve cord (Figs. 6S and 6T). In larvae older than about 30 h (the time the mouth opens) expression decreases in the posterior mesoderm (data not shown). Expression in the anterior pharyngeal endoderm remains strong and is also detectable in mesothelial cells that are apparently part of Hatschek's nephridium (Figs. 6S and 6T).

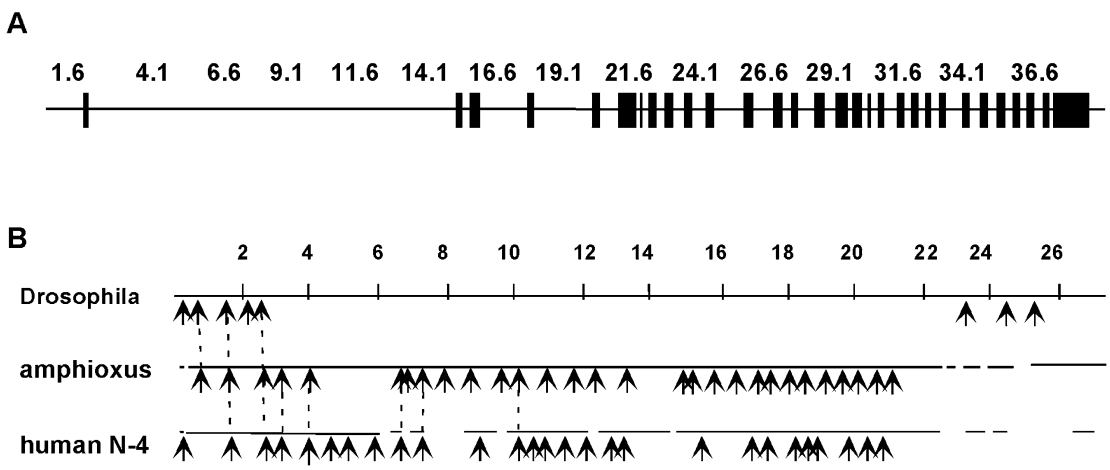

FIG. 4. Intron/exon organization of *Notch* genes. (A) Intron and exon positions in *AmphiNotch*. Exons are indicated by black bars. Numbers indicate positions within the *AmphiNotch* locus in kb. (B) Comparison of intron positions (arrows) among the *Drosophila* Notch, AmphiNotch, and human Notch4 cDNAs. Numbers are amino acid position $\times 10^{-2}$. Dotted lines indicate identical intron positions. Gaps in sequences of AmphiNotch and human Notch4 are introduced for alignment with the longer *Drosophila* cDNA.

DISCUSSION

Molecular Evolution of Notch Genes

In our phylogenetic tree, the single *AmphiNotch* gene branches at the base of the four vertebrate *Notch* genes. Together with the presence of single *Notch* genes in sea urchins and ascidian tunicates, this result suggests that *Notch* duplicated within the vertebrate lineage. However, *Notch4,* which is known only in mammals (Li *et al.,* 1998), branches before the divergences of the other *Notch* genes. Given the low bootstrap value (50) and long branch length, the position of vertebrate *Notch4* may simply reflect the extreme divergence of this gene. Indeed, homologs of three of the vertebrate *Notch* genes (*Notch1, 2, 3*) have been found both in mammals and in birds, while those of *Notch1* and *3* occur in fish and amphibians as well (Maine *et al.,* 1995; Williams *et al.,* 1995; Larsson *et al.,* 1994; Westin and Lardelli, 1997), suggesting that they must be basal to the mammalian *Notch4.* Notch genes have not yet been described in agnathans. However, if the current paradigm of one round of whole genome duplication at the base of the vertebrates and a second round after the split of gnathostomes and agnathans (Holland *et al.,* 1994) holds true, two *Notch* genes, a *Notch1* and a *Notch2/3* gene, would be expected in lampreys and hagfish.

Comparisons of Notch Genes and Proteins

Variability among EGF repeats may be responsible for the tissue-specific expression of different *Notch* homologs. EGF repeats can differ in their affinity for Ca^{2+} and in the presence or absence of O-linked fucose and/or glucose. In addition, the number of EGF repeats varies among *Notch* genes both within an organism and among different organisms. Calcium-binding sites on EGF repeats have been recognized as including a consensus sequence, Cys_3-x-ASP/Asn-x-x-x-Tyr/Phe-x-Cys_4, necessary for β-hydroxylation of Asp/Asn residues, plus the sequence As/Asn/Glu-Ile/Val-Asp/Asn/Glu-Glu/Asp/Glyn-Cys_1 preceding the first Cys (Rand *et al.,* 1997). As Fig. 3B shows, these sites are highly conserved between *Drosophila* Notch, AmphiNotch, and mouse Notch1 and are absent from the 4 most N-terminal and 4 most C-terminal EGF repeats. EGF repeats 11 and 12 are necessary for Ca^{2+}-dependent ligand-mediated cell aggregation and bind Ca^{2+} directly (Rand *et al.,* 1997). The arrangement of Ca^{2+}-binding and non-Ca^{2+}-binding EGF repeats in *Notch* proteins together with differences in Ca^{2+} affinity may modulate ligand binding. In addition, differences in relative position of Ca^{2+}-binding EGF repeats among the four mammalian Notch homologs may contribute to the differences in their ligand specificity (Rand *et al.,* 1997).

In contrast, O-linked glycosylation of EGF repeats does not appear to affect the affinity of ligand binding. However, it can modulate the functions of EGF-containing proteins induced by ligand binding (Rabbani *et al.,* 1992; Moloney *et al.,* 2000). As Fig. 3A shows, these sites are moderately conserved between *Drosophila* Notch, AmphiNotch, and mouse Notch1; however, they are not as conserved as the Ca^{2+}-binding sites. Interestingly, two EGF repeats in all three Notch homologs, Nos. 22 and 31, lack sites both for Ca^{2+} binding and for glycosylation.

All *Notch* genes described to date have three Notch/lin-12 repeats. In contrast, the number of EGF repeats is variable, with a maximum number of 36 in insect *Notch* (Wharton *et al.,* 1985), vertebrate *Notch1* and *2* (Coffman *et al.,* 1990; Weinmaster *et al.,* 1991, 1992), and *AmphiNotch.* However, there are fewer EGF repeats in *Notch* genes of *Caenorhabditis* (Yochem and Greenwald, 1989), lower deuterostomes (sea urchin and ascidian) (Sherwood and McClay, 1997; Hori *et al.,* 1997), and vertebrate *Notch3* and *Notch4* (Lardelli *et al.,* 1994; Uyttendaele *et al.,* 1996). Amino acid alignments (data not shown) show that the positions of the missing EGF modules vary from organism to organism. For example, sea urchin EGF repeat 15 and ascidian EGF repeats 2 and 5 are missing. Mouse Notch3 lacks repeat 21 and parts of 2 and 3, while vertebrate Notch4 is missing Nos. 15, 17, 19, 21–23, 25, and 31. The *Caenorhabditis Notch* homologs, glp-1 and lin-12, are missing a total of 26 and 23 EGF repeats, respectively (Yochem and Greenwald, 1989). Since neither the number nor the position of the absent EGF repeats (with the exception of repeat 15 in sea urchin Notch and vertebrate Notch4) correlates with the phylogenetic position of the organisms, it seems likely that both the ancestral bilaterian and the ancestral deuterostome *Notch* genes had 36 EGF repeats and that losses of EGF repeats have occurred independently.

Different ligand specificities have been ascribed to different EGF modules. For example, modules 11 and 12 are involved in binding of Delta, and modules 19–36 bind Wingless (Wesley, 1999). Thus, the absence of specific EGF repeats could affect tissue-specific expression. Notch genes with 36 repeats (e.g., amphioxus Notch, *Drosophila* Notch, and vertebrate Notch1 and 2) are typically expressed widely in early embryos. In contrast, *Notch* genes of lower deuterostomes, *Caenorhabditis,* and *Notch3* and *4* of vertebrates, which all have fewer EGF repeats, tend to be expressed in fewer tissues. For example, zygotic expression of the ascidian *Notch* is largely restricted to ectodermal lineages during gastrulation and later to the neuroectoderm, particularly in the dorsal anterior portion (Hori *et al.,* 1997). Whether the restricted expression of ascidian *Notch* is related to the absence of specific EGF repeats remains to be determined. Expression of sea urchin *Notch* is initially restricted to the animal half of the early blastula and then becomes localized to a ring of cells around the vegetal plate which corresponds to the boundary between the presumptive secondary mesoderm and the endoderm (Sherwood and McClay, 1997, 1999; Sweet *et al.,* 1999); neural expression has not been described. Mammalian Notch4, which has only EGF modules, is apparently expressed only in developing macrophages, endothelial cells, mammary gland tissue, and the pancreas (Uyttendaele *et al.,* 1996, 1998; Lammert *et al.,* 2000; Singh *et al.,* 2000). Furthermore, mutations in human

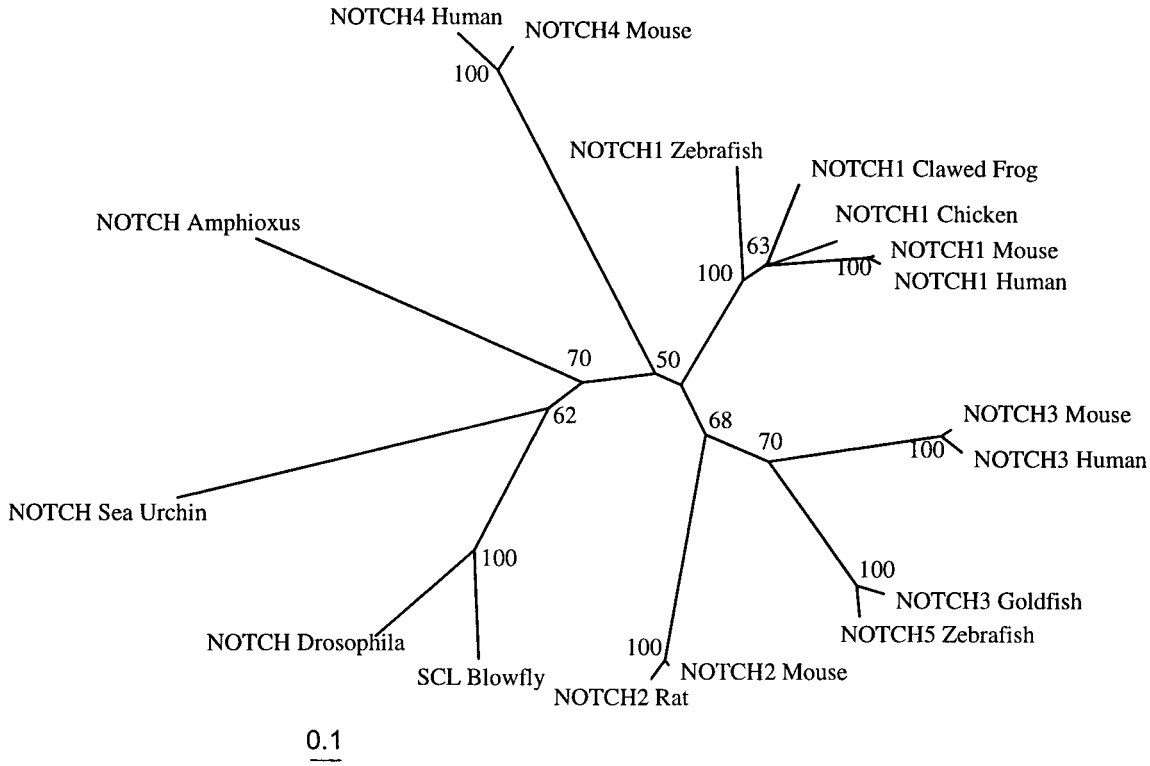

FIG. 5. Phylogeny of Notch proteins based on the Lin/Notch repeat regions, neighbor-joining method. Bootstrap values >50 are given. Scale line for branch lengths is the number of changes between character-states.

Notch3, which cause a defect in vascular smooth musculature that affects the function of several organs, tend to be clustered in the 5 most N-terminal EGF repeats (Joutel and Tournier-Lasserve, 1998). In addition, the EGF repeat that is missing in sea urchin *Notch* is the same repeat mutated in the *split* mutation of *Drosophila Notch,* which has an eye-specific phenotype (Hartley *et al.,* 1987).

Evolutionary Conservation of the Notch Pathway in Patterning the Mesendoderm

Notch is expressed in the mesendoderm in a wide variety of protostome and deuterostome embryos. In *Drosophila, Notch* functions in both mesoderm and endoderm, in patterning of the heart and somatic musculature, and in the midgut (Corbin *et al.,* 1991; Hartenstein *et al.,* 1992; Schnabel, 1994; Park *et al.,* 1998; Rusconi and Corbin, 1998), while in *Caenorhabditis,* a role in morphogenesis of the intestine has been described (Hermann *et al.,* 2000). Vertebrate *Notch* genes are expressed like *AmphiNotch* in the posterior mesoderm and forming somites as well as in the gut. They are also expressed in several gut derivatives such as the pancreas and lung (Weinmaster *et al.,* 1992; Conlon *et al.,* 1995; Lammert *et al.,* 2000). Thus, the lack of mesendodermal expression in ascidian tunicates (Hori *et al.,* 1997) may represent a loss, which might have evolved in connection with early determination of cell fate and reduction of the embryonic gut to an endodermal strand.

In most deuterostomes, *Notch* genes are expressed very early in the posterior mesendoderm. They are coexpressed with genes of the *Wnt* signaling pathway and transcription factors such as *brachyury.* This coincidence suggests the interaction of these genes in patterning along the anterior/posterior axis. Indeed, there is considerable experimental evidence for interaction of the *wingless* and *Notch* pathways and *brachyury* at several levels in a number of tissues in embryos of several species (reviewed in Panin and Irvine, 1998; Dierick and Bejosovec, 1999). In both *Drosophila* and vertebrates, *Notch* and *wingless/Wnt* can play opposing roles in developing tissues (Brennan *et al.,* 1999; Uyttendaele *et al.,* 1998). In *Drosophila,* Wingless is a ligand of Notch, binding to the EGF repeats (Wesley, 1999; Wesley and Saez, 2000). Wingless may also affect Notch signaling through interaction of Dishevelled, a downstream component of the *Wnt*-signaling pathway, with the intracellular domain of Notch (Axelrod *et al.,* 1996). Conversely, Notch signaling can regulate *wingless* expression (Rulifson and Blair, 1995; reviewed in Panin and Irvine, 1998).

There is evidence that *brachyury* is a target of both the *Notch* and the *wingless* pathways. In ascidian tunicates, Suppressor of Hairless [Su(H)]/RBP-J_κ binds to the *brachyury* promoter and activates *brachyury* expression in

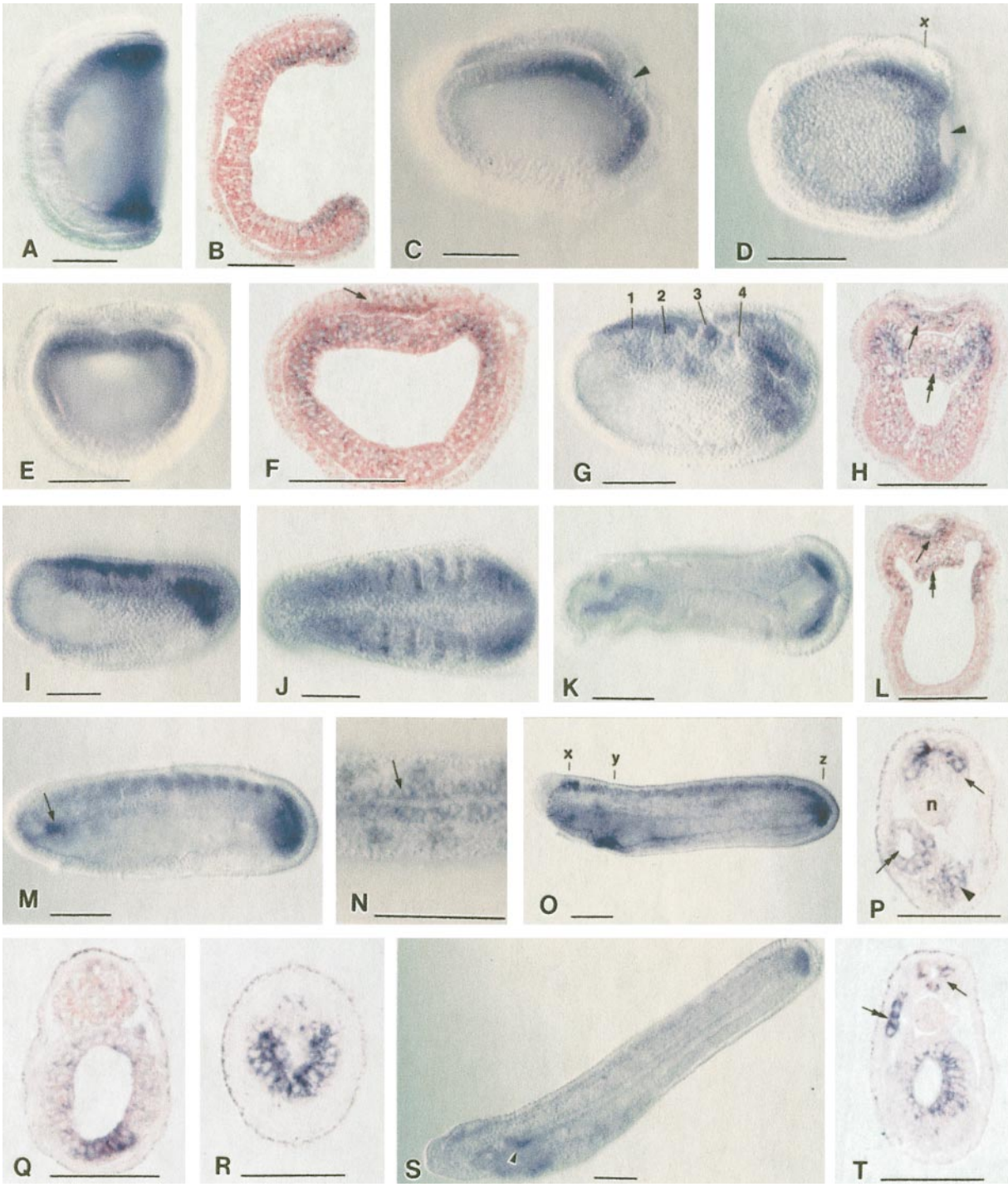

FIG. 6. *In situ* hybridization of *AmphiNotch* showing expression in amphioxus embryos shown as whole mounts (scale, 50 μm) with anterior toward the left (A, C–E, G, I–K, M–O, S) or cross sections (scale, 25 μm) (B, F, H, L, P–R, T). In all side-view whole mounts and sections dorsal is up. (A) Side view of cup-shaped gastrula (6 h) with blastopore opening toward the right. *AmphiNotch* is expressed in the presumptive mesendoderm around the blastopore and dorsally in presumptive notochord and somites. (B) Sagittal section of embryo in A showing expression in mesendoderm. (C) Side view of late gastrula (8 h). Blastopore (arrowhead) is at top right. Expression of *AmphiNotch* is strongest in the dorsolateral mesoderm and just within the blastoporal lip. (D) Dorsal view of the previous embryo showing mesodermal expression just within the blastopore (arrowhead) and extending dorsolaterally. (E) Posterior view of embryo in D in optical section through level X; expression is strong dorsally and dorsolaterally in presomitic and prechordal mesoderm. (F) Cross section through level X in D;

the notochord (Corbo *et al.*, 1997, 1998). Su(H) is a downstream component of the Notch signaling pathway; upon binding to the ankyrin repeat region of Notch, Su(H), either alone or together with the Notch intracellular domain, is translocated to the nucleus where it acts as a transcription factor (reviewed in Wu and Rao, 1999). Not surprisingly, expression of a constitutively activated Notch receptor alters tail morphology (Corbo *et al.*, 1998). In addition, in *Xenopus* the *brachyury* promoter also binds the downstream component of the Wnt signaling pathway, LEF-1/β-catenin (Arnold *et al.*, 2000). Thus, interactions between *Notch* and *wingless* pathways and *brachyury* are evidently complex.

In amphioxus, *Notch*, *wingless/Wnt*, and *brachyury* are all expressed around the blastopore in the early gastrula. The first of these posterior markers to be expressed is *brachyury* (*AmBra1* and *AmBra2*), which turns on in a ring around the equator of the late blastula/very early gastrula—the future blastoporal lip (P. W. H. Holland *et al.*, 1995; Terazawa and Satoh, 1995; Zhang *et al.*, 1997). Next, *Wnt1* turns on in the blastoporal lip (Holland *et al.*, 2000a), followed by *Wnt8* (Schubert *et al.*, 2000a; M. Schubert pers. commun.), then by *Notch*, *Wnt4*, and *Wnt7b* (Schubert *et al.*, 2000b), and finally in the late gastrula by *Wnt11* (Schubert *et al.*, 2000c). Expression of other amphioxus *Wnt* genes has not been determined. In the late gastrula and neurula, expression of *Wnt1* remains restricted to the region of the blastopore. However, as expression of *Notch* and *brachyury* expands into the somites, notochord, and neural plate, expression of *Wnts 4*, *8*, and *7b* also expands into some of these domains. For example, *Wnts 4*, *8*, and *11* and *brachyury* are coexpressed with *Notch* in the presomitic mesoderm with expression continuing into the somites (Schubert *et al.*, 2000a,c; P. W. H. Holland *et al.*, 1995). The spatiotemporal expression of these genes in amphioxus suggests that in amphioxus the *Notch* and *Wnt/wingless* pathways and *brachyury* may cooperate in patterning the mesendoderm.

Although sea urchin embryos form neither somites, a notochord, nor a nerve cord, there is also a posterior/vegetal *Notch* and *Wnt* signaling center in the early embryo. In the late blastula, the vegetal pole flattens to form the vegetal plate, which will give rise to the mesoderm and invaginate to form the embryonic gut. At this stage, Notch protein becomes localized to the apical surfaces of cells at the edges of the vegetal plate. Subsequently, by midgastrula, Notch is localized on the apical surfaces of cells around the blastopore and in the invaginating endoderm, predominantly along the dorsal side (Sherwood and McClay, 1997, 1999; Sweet *et al.*, 1999). This pattern is reminiscent of *Notch* expression in early amphioxus embryos. Similarly, genes of the Wnt-signaling pathway (*Wnt8* and β-catenin) are localized to the vegetal region of the sea urchin embryo (reviewed in Angerer and Angerer, 2000). Experimental evidence shows that *Wnt* signaling is involved in patterning along the anterior/posterior (= animal/vegetal) axis and that the Wnt and Notch pathways interact. Treatment of embryos with LiCl, which upregulates the *wingless* signaling pathway by inhibiting the negative regulator GSK3βI, alters the pattern of *Notch* expression and vegetalizes embryos (Sherwood and McClay, 1997). Moreover, effects of manipulating GSK3β levels are in agreement with the presence of a posterior Wnt-signaling center involved in patterning along the anterior/posterior axis (Emily-Fenouil *et al.*, 1998). In sea urchins, *Brachyury* is also expressed in the vegetal plate and later in the secondary mesenchyme

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expression is conspicuous through the cells of the dorsolateral mesoderm and also beginning in the basal cytoplasm of cells of the neural plate (arrow). (G) Side view early neurula (10 h); expression is detectable in the posterior mesoderm and in the dorsal part of somites 1–3, but not in somite 4. (H) Cross section of the embryo in G through the level of somite 2; the strongest expression is in dorsal cells of the forming somites; weaker expression is visible in cells of the forming notochord (tandem arrow) and in cells of the neural plate (single arrow), which is overgrown by epidermis. (I) Side view of whole mount of hatched neurula (13 h) showing expression in the posterior mesoderm, in all but the most posterior (youngest) somite, in the neural plate and notochord. (J) Dorsal view of a whole mount of the 6-somite neurula in I with the dorsal portion of the somites in focus. (K) The same embryo as in J viewed in optical section through the gut showing strong expression in the posterior mesoderm and weak expression in anterior endoderm. (L) Cross section through the embryo in J and K at the level of somite 5. *AmphiNotch* is expressed in the walls of the forming somites, in the neural plate (single arrow), and in the forming notochord (tandem arrow). (M) Side view of a late neurula (18 h) showing strong expression in the posterior mesoderm, somites, and anterior pharyngeal endoderm (arrow). (N) Enlargement of the preceding embryo in dorsal view with the neural canal in focus. Expression is detectable in the somites and in many cells of the dorsal nerve cord (arrow). (O) Side view of 22-h embryo; the most conspicuous expression is in dorsal cells of the cerebral vesicle (level x), in some ventral pharyngeal cells (level y), and in the posterior mesoderm (level z). There is less conspicuous expression in the remainder of the nerve cord and in the somites. (P) Cross section through level x of the embryo in O showing strong expression in dorsal and lateral cells of the cerebral vesicle (single arrow), in the wall of the left anterior gut diverticulum (tandem arrow), and in the anterior extremity of the pharynx (arrowhead). The notochord (n) no longer contains detectable transcripts of *AmphiNotch*. (Q) Cross section through y in the embryo in O showing strong expression in the pharyngeal endoderm especially on the ventral side. (R) Cross section through level z of the embryo O showing conspicuous expression in the posterior mesoderm. (S) Side view of a 28-h embryo with strong expression in the posterior mesoderm, in some cells of the dorsal nerve cord, in the pharynx, and in some mesothelial cells (arrowhead) that may be forming part of Hatschek's nephridium. (T) Cross section through the embryo in S at the level indicated by the arrowhead; expression is in the dorsal nerve cord (single arrow) and in mesothelial cells (tandem arrow) that are apparently part of Hatscheck's nephridium. Expression is undetectable in the notochord.

(Harada *et al.*, 1995), while in starfish it is expressed around the blastopore (Shoguchi *et al.*, 1999).

Both vertebrate and amphioxus *Notch* genes are expressed in the posterior mesendoderm, in the forming somites, and later in the tailbud (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997; Beck and Slack, 1999). *Brachyury* and several *Wnt* genes are expressed in patterns overlapping with that of *Notch* (Gont *et al.*, 1993; Beck and Slack, 1999; Tada and Smith, 2000). Although in the *Xenopus* blastula the *Wnt*-signaling pathway first establishes dorsoventral polarity, there is a second late phase of *Wnt* signaling, in which β-catenin is translocated to nuclei of cells around the lateral and ventral margins of the blastopore (Schneider *et al.*, 1996). This phase is involved in posteriorization of the neuroectoderm, formation of paraxial mesoderm, and tailbud extension. There is experimental evidence for the interaction of the *Notch* and *Wnt* pathways and *Brachyury* both in elongation of the tailbud and in patterning of the somites. In *Xenopus*, expression of *Notch* together with *Xwnt3a* provokes elongation and formation of neural tubes in animal caps and has been implicated in outgrowth of the tailbud (Beck and Slack, 1999), as has *Brachyury* (Gont *et al.*, 1993). It has been suggested that the mechanism for tail extension involving *Notch* and *Wnt3a* may be common among vertebrates. Wnt3a and Wnt11 are targets of Brachyury during gastrulation and in paraxial mesoderm, respectively (Tada and Smith, 2000; Yamaguchi *et al.*, 1999). Conversely, *Brachyury* can also be a target of the Wnt-signaling pathway, at least in embryonic stem cell cultures (Arnold *et al.*, 2000). Our results suggest that *Notch*, *Wnts*, and *Brachyury* may also cooperate in patterning the amphioxus mesoderm and in elongation of the tailbud. Although the Notch-signaling pathway has not been shown to be a direct target of Brachyury, in amphioxus, expression of *Brachyury* in the future blastoporal lip, before both *Wnt1* and *Notch* are turned on in the same cells, suggests that *Brachyury* may act upstream of *Notch* either directly or via signaling through the *wingless* pathway. These possibilities could be tested experimentally and by *in vitro* analyses of the *Notch* promoter.

In vertebrates, expression of *Notch* in the presomitic mesoderm and in early somites is required for normal segmentation, acting upstream of cyclically expressed genes such as *her1*and *HES1* (Jouve *et al.*, 2000; Aulehla and Johnson, 1999; Takke and Campos-Ortega, 1999). The Wnt signaling pathway is also involved in somitogenesis, although a direct link between Notch and Wnt signaling in somitogenesis has not been shown. In amphioxus, the somites extend the full-length of the body. The anteriormost somites are formed by enterocoely and the more posterior ones by schizocoely, more like the somites of higher vertebrates. Although there are some differences in gene expression in the two types of somites (e.g., *engrailed* is expressed during segmentation in the anteriormost somites only), *Notch* and *Wnt* genes are expressed in both the anterior and the posterior somites, indicating that later development involves common genetic pathways. Although homologs of vertebrate genes with cyclic expression in the somites (e.g., *her1, HES1*) have not been cloned from amphioxus, it is likely that they will similarly be expressed in amphioxus as in vertebrates.

Roles of AmphiNotch in Neurogenesis

In both early and late amphioxus development, the expression of *AmphiNotch* in the neural plate and nerve cord closely parallels that in vertebrate embryos. In amphioxus, ectodermal expression begins in the posterior neural plate, extends to the entire neural plate, and later becomes restricted to anterior regions of the neural tube, chiefly in dorsal cells in the cerebral vesicle. Similarly, in the mouse and *Xenopus*, *Notch* homologs are expressed in the neural plate and neural tube, especially in dorsal regions of the hindbrain, brachial spinal cord, and infundibular recess of the diencephalon (Coffman *et al.*, 1990, 1993; Bierkamp and Campos-Ortega, 1993). In the zebrafish, *Notch* homologs are expressed in the neural plate and later on in much of the brain (Westin and Lardelli, 1997). Thus, in both amphioxus an the vertebrates, *Notch* genes are initially broadly expressed in the neural plate and later become restricted to anterior regions of the nerve cord.

Activation of *Notch1* in the zebrafish in turn activates the bHLH gene *her4*, suppresses *neurogenin* expression, and reduces the number of primary neurons (Takke *et al.*, 1999). In amphioxus, *neurogenin* is broadly expressed in the posterior part of the dorsal ectoderm of the early gastrula, but by the late gastrula turns off in the posterior region of the neural plate in a pattern complementary to that of *AmphiNotch*. As the neural tube forms, *AmphiNotch* becomes weakly but broadly expressed in the neural plate, unlike neurogenin, which becomes restricted to two columns of cells on either side of the floor plate. Subsequently, both *Notch* and *neurogenin* (Holland *et al.*, 2000b) become restricted to subsets of cells in the nerve cord, particularly in dorsal regions of the cerebral vesicle, the homolog of the diencephalon. These domains are not entirely congruent, although the possibility cannot be excluded that they may include some of the same cells. These expression patterns suggest that Notch may have similar roles in neurogenesis in amphioxus and in vertebrates.

Notch Expression in the Developing Kidney

The homology of vertebrate kidneys and amphioxus nephridia has long been controversial. The controversy has centered on whether the amphioxus larval kidney is ectodermal (and thus homologous to protostome nephridia) or mesodermal (and thus homologous to the vertebrate kidney). More recent morphological studies indicate a mesodermal origin for the amphioxus larval kidney (Ruppert, 1996; Stach and Eisler, 1998). In amphioxus, Notch is expressed in the primordium of the larval kidney. Similarly, mouse *Notch* homologs are also expressed in the early kidney (Franco del Amo *et al.*, 1992; Williams *et al.*, 1995).

The finding that both express *Notch* homologs as well as homologs of *Pax2/5/8* in early development (Kozmik *et al.*, 1999) supports ideas of the common ancestry of the vertebrate pronephros and amphioxus kidney.

In summary, the presence in amphioxus of a single *Notch* gene with a full complement of 36 EGF repeats expressed in multiple tissues in embryogenesis in patterns similar to those of all the vertebrate *Notch* genes put together underscores the utility of amphioxus as a stand-in for the ancestral vertebrate. From the accumulating evidence, it is becoming increasingly apparent that the amphioxus and vertebrate body plans are established by very similar mechanisms. Given the simple genome of amphioxus and the diagrammatic clarity of embryogenesis, amphioxus embryos promise to be a simplified model for helping to elucidate the evolution of developmental mechanisms.

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