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Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene

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SUMMARY

The *int-3* oncogene was identified as a frequent target in Mouse Mammary Tumor Virus (MMTV)-induced mammary carcinomas and encodes the intracellular domain of a novel mouse *Notch* gene. To investigate the role of the *int-3* proto-oncogene in mouse development and carcinogenesis, we isolated cDNA clones corresponding to the entire coding potential of the *int-3* proto-oncogene. We propose to name this gene *Notch4* and reserve the *int-3* nomenclature for references to the oncogenic form. The deduced amino acid sequence of Notch4 contains conserved motifs found in Notch proteins; however Notch4 has fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than other mouse Notch homologues. Comparison of the coding potential of the *int-3* gene to that of *Notch4* suggests that loss of the extracellular domain of Notch4 leads to constitutive activation of this murine Notch protein. In situ hybridization revealed that *Notch4* transcripts are primarily restricted to endothelial cells in embryonic and adult life. Truncated *Notch4* transcripts were detected in post-meiotic male germ cells. The distinct Notch4 protein features and its restricted expression pattern suggests a specific role for *Notch4* during development of vertebrate endothelium.

Key words: Notch, int-3, endothelial cells, mammary oncogene

INTRODUCTION

The *int-3* gene was originally identified on the basis of its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor-specific transcripts derived from the *int-3* gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 oncoprotein in mouse mammary epithelial cells (HC11) promotes anchorage-independent growth (Robbins et al., 1992). Expression of *int-3* as an MMTV-LTR-driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the *Notch/lin-12* gene family were first identified in *Drosophila* and *Caenorhabditis elegans* through genetic analysis of mutations that alter cell fate decisions (for reviews see Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). *Drosophila Notch* regulates multiple cell fate decisions that involve cell-cell interactions during fly development, for instance, control of cell fate decisions involving neural/epidermal specification in proneural clusters (Artavanis-Tsakonas and Simpson, 1991). The *C. elegans* lin-12 and glp-1 proteins are structurally related to Notch and are also involved in cell fate specifications during development in the nematode (Greenwald, 1985; Yochem and Greenwald, 1989). Genetic analysis of *Notch/lin-12* genes suggests that this family of genes controls binary cell fate decisions and inductive signaling that depend on cell-cell interactions (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Greenwald and Rubin, 1992). Alternatively, *Notch/lin-12* genes have been proposed to block cell differentiation, thus maintaining the competence of cells for subsequent cell-fate determination (Coffman et al., 1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins characterized by highly repeated, conserved domains. The amino terminus of Notch proteins encodes the extracellular domain and contains as many as 36 repeats of an EGF-like motif involved in ligand binding (Rebay et al., 1993) and three tandem copies of a Notch/lin-12 sequence motif of unknown function. The intracellular portion of Notch proteins is characterized by six tandem copies of a cdc10/ankyrin motif, thought

to be a protein-protein interaction domain (Michaely and Bennett, 1992) and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia, and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to *Drosophila Notch*, additional *Notch*-related genes have been isolated from mammals, including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991) and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et al., 1994). To date, three *Notch* homologues, *Notch1*, *Notch2* and *Notch3*, have been identified in the mouse, and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites and nervous system (Williams et al., 1995). Abundant expression of *Notch1*, *Notch2* and *Notch3* is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the *Notch1* gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death, preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

The gene products of *Drosophila Delta* (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990) and *C. elegans Lag-2* (Henderson et al., 1994; Tax et al., 1994) and *Apx-1* (Mello et al., 1994) are thought to act as ligands for Notch proteins. In the mouse, the orthologue of *Delta*, referred to a *Dll1* (*Delta-like gene 1*), is expressed during embryonic development in the paraxial mesoderm and nervous system in a pattern similar to that of mouse *Notch1* (Bettenhausen et al., 1995). A murine *Serrate*-related gene named *Jagged* has been identified and is partially co-expressed with murine *Notch* genes in the developing spinal cord (Lindsell et al., 1995).

We report here the identification and expression analysis of a fourth murine *Notch* homologue, which we propose to name *Notch4*, reserving the *int-3* nomenclature for the truncated oncogene. Although the intracellular domain of the int-3 oncoprotein shares homology with the Notch/Lin-12 protein family, we now provide a comparison of the full-length Notch4 protein with that of the int-3 oncoprotein. The activated int-3 protein contains only the transmembrane and intracellular domain of the Notch4 protein. The predicted amino acid sequence of Notch4 includes the conserved features of all Notch proteins, but Notch4 has seven fewer EGF-like repeats compared to Notch1 and Notch2 and contains a significantly shorter intracellular domain. Notch4 is expressed primarily in embryonic endothelium and in adult endothelium and male germ cells.

MATERIALS AND METHODS

Isolation and sequencing of *Notch4* cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5′ primer: CGTCCTGCTGCGCTTCCTTGCA and 3′ primer: CCGGTGCCTAGTTCAGATTTCTTA) designed from the *int-3* cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned *int-3* oncogene. Two consecutive 5′ RACE reactions (5′-Amplifinder RACE kit, Clonetech) using testis and lung cDNA were done to obtain cDNA clones located 5′ of the *int-3* oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene) and the TA cloning vector (Invitrogen) and used to generate probes to screen a lung cDNA library (Clonetech). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% formamide, 3× SSC, 100 mM Tris-HCl (pH 7.4), 5× Denhardt's solution, 0.2% SDS and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1× SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5′ restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full-length *Notch4* cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems). Sequence data from both strands were obtained for the entire *Notch4* cDNA and were analyzed and assembled using computer software (MacVector, Assemblylign).

Northern blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and northern blot hybridization analysis was performed. 20 μg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes, Stratagene) by capillary blotting. ^{32}P-labeled riboprobes were transcribed (Maxiscript in vitro transcription kit, Ambion) from *Notch4* cDNA clones encoding the 5′ or 3′ UTR (untranslated region) or ORF (open reading frame). The 3′ UTR *Notch4* cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 20 mM NaH_2PO_4 (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 10 μg/ml poly(A) mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2× SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2× SSC and 1% SDS. Membranes were exposed to X-ray film (X-OMAT AR, Kodak). The integrity of the RNA, as well as comparable amounts of RNA, were tested by rehybridization with a GAPDH probe.

In situ hybridization

Staged embryos ranging from 9 days post-coitum (d.p.c.) to birth were obtained from timed breedings of CD-1 mice. The morning when the the vaginal plugs appeared was counted as 0.5 d.p.c. Lungs was obtained from adult CD-1 mice. Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak emulsion and exposed for 2 weeks at 4°C. Analyses were carried out using both light- and dark-field optics on a Leica DA microscope. To avoid potential cross-hybridization with homologous RNAs, we used an antisense ^{35}S-labeled RNA probe corresponding to the 3′ UTR of *Notch4*. Probes were used at a final concentration of 9×10^4 dpm/ml.

RESULTS

Isolation and analysis of *Notch4* cDNA clones

The *int-3* mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full-length *int-3* gene, which we will refer to as *Notch4*, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, we have cloned cDNAs containing the complete coding potential of the *Notch4* gene. Using primers derived from the published sequence of the *int-3* oncogene, RT-PCR was used to isolate a 2.4 kb *int-3* cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal *int-3* gene, cDNAs were isolated by 5′ RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of *Notch4* transcripts detected by northern blot analysis; thus, we believe the cloned cDNA represents the full-length *Notch4* gene.

Several differences (insertions, deletions and single nucleotide changes) were found between the nucleotide sequence of *Notch4* reported here and the previously published *int-3* nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or, possibly, of mutations found in the tumor-derived *int-3* transcript (Robbins et al., 1992) that are not found in the *Notch4* gene. The nucleotide sequence of mouse *Notch4* has been deposited with GenBank under the Accession number U43691.

Analysis of the deduced *Notch4* amino acid sequence

Analysis of the deduced amino acid sequence of *Notch4* reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins and 58% similar and 40% identical to *Drosophila* Notch. Lower homologies were found when compared with the *C. elegans* lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). The N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site

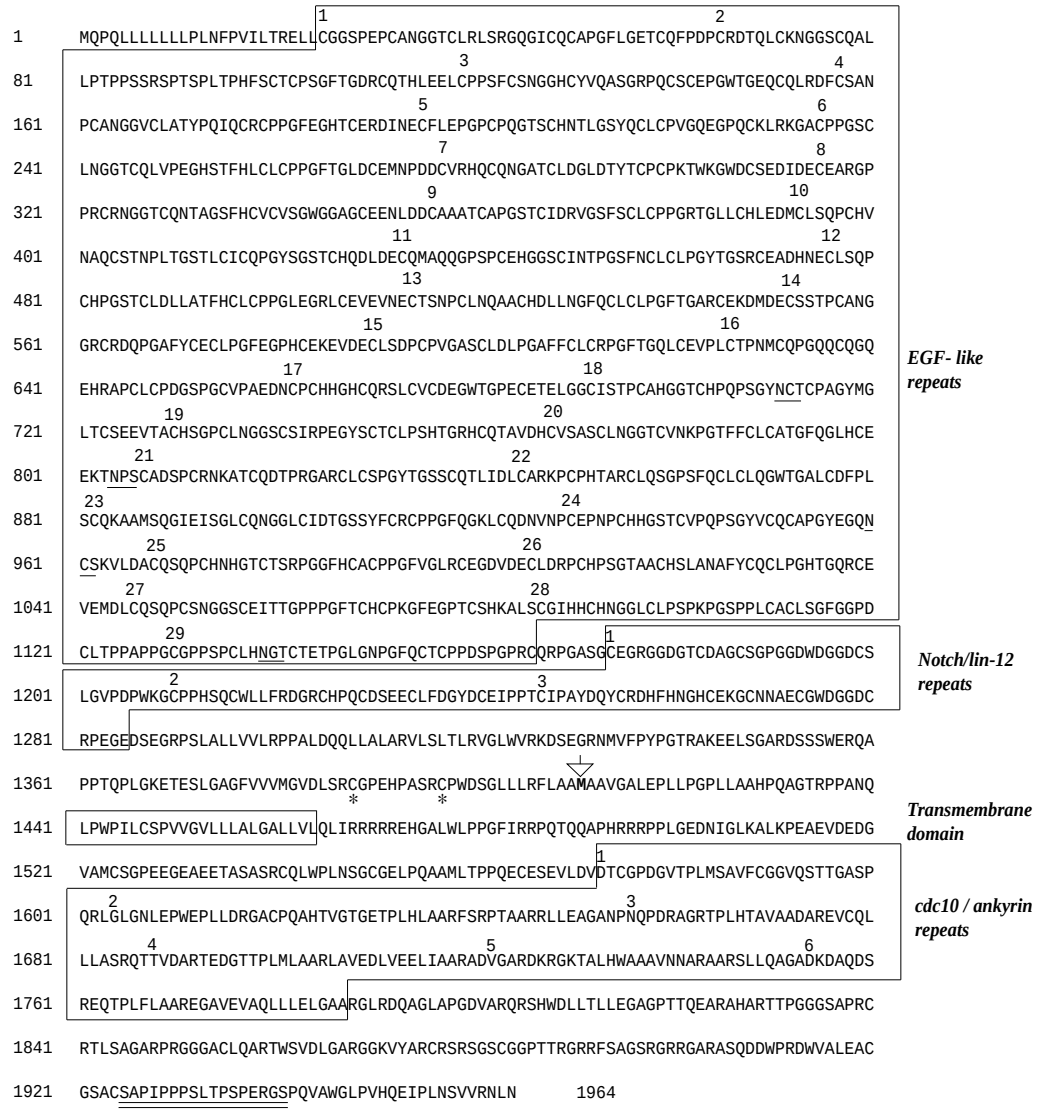

Fig. 1. Deduced amino acid sequence of Notch4 (GenBank accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, as follows: 29 epidermal growth factor(EGF)-like repeats; 3 Notch/lin12 repeats; a transmembrane domain; and 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.

was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane-spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992) and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in Notch proteins as equivalent EGF-like repeats. We analyzed the relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein. Fig. 2 schematizes the relationship of EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2, EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2 and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeat derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). For a discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2), see Lardelli et al. (1994).

EGF-like repeats 11 and 12 of *Drosophila* Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins in vitro (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig. 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteines of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins. In addition, EGF-like repeats 22-23 of Notch4 have been conserved among murine Notch proteins (EGF-like repeats 28 and 29 of Notch1) and equivalent EGF-like repeats in *Drosophila* Notch are implicated in the regulation of Notch protein function through genetic analysis of the *Abruptex* alleles of Notch (Kelley et al., 1987).

Notch4 also contains three Notch/lin-12 repeats, which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4 are two cysteines at positions 1388 and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

The intracellular domain of Notch4 contains the six ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52% and 55% identical to the ankyrin repeat domains of Notch1, Notch2 and Notch3, respectively. In all Notch proteins the number of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1), such as that found in *Drosophila* Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant homology to other known proteins. This C terminus is also much shorter in Notch4 (177 residues), than in other Notch proteins (457 residues in Notch1, 437 in Notch2 and 329 in Notch3).

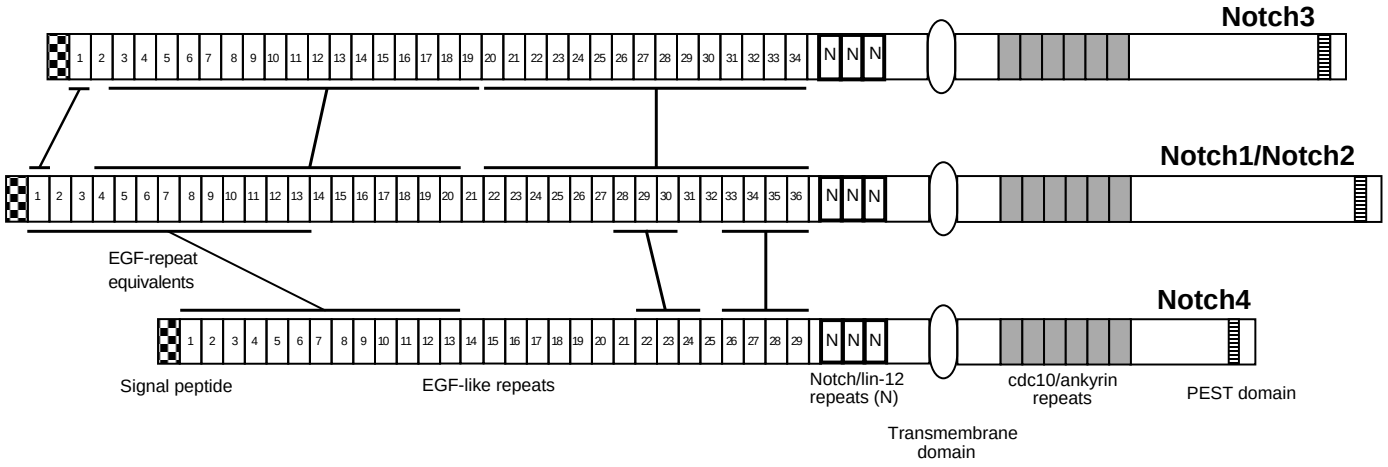

Fig. 2. Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified, connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains seven EGF-like repeats, fewer than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2, creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy terminus is shorter when compared to Notch1, 2 and 3.

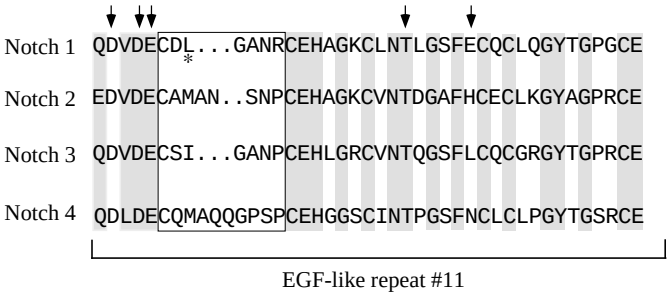

Fig. 3. Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium-binding sites are marked with arrows. A region within EGF-like repeat #11 of the Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in Xenopus Notch that obliterates binding to Delta is marked with an asterisk (*).

Analysis of *Notch4* transcripts in adult tissues

Several adult tissues were examined for the presence of *Notch4* transcripts by northern blot analysis. To minimize cross-hybridization with other mouse *Notch* transcripts, we used a riboprobe derived from the 3′ UTR of *Notch4*. In most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned *Notch4* cDNA. The 6.7 kb transcript is most highly expressed in lung, at lower levels in heart and kidney and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues, including brain, intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, *Notch4* expression varies widely in adult tissues. Other than in testis, we did not detect transcript size variation in different tissues.

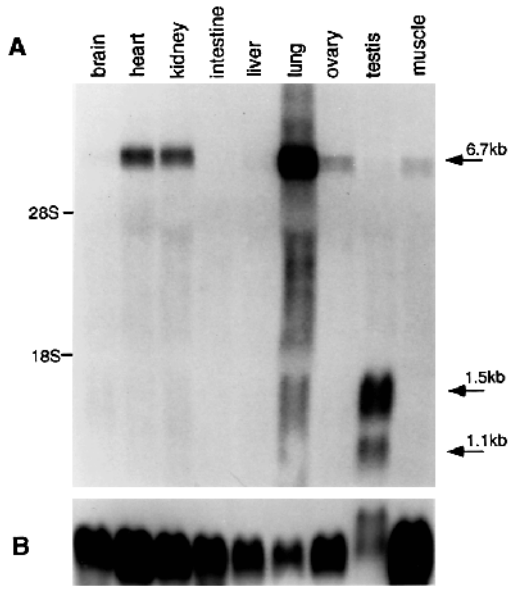

Fig. 4. Expression analysis of *Notch4* in adult mouse tissues. (A) Northern blot using a riboprobe transcribed from the 3′ UTR of *Notch4* (probe D in Fig. 5). (B) The same blot reprobed with a GAPDH probe. The transcript sizes of 6.7 kb, 1.5 kb and 1.1 kb are indicated and were estimated with reference to 28 S and 18 S rRNA migration.

Analysis of testis-specific truncated *Notch4* transcripts

To determine the cell lineage specificity of *Notch4* expression in the murine testis, RNA was analyzed in the germ cell-deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^v) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli and peritubular myoid cells (Mintz and Russell, 1957). Heterozygous litter mates ($W/+$) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^v) and testes with normal germ cells [$W/+$ and adult (+/+)] was done using a riboprobe derived from the 3′ UTR (probe D in Fig. 5C). Transcripts of 1.5 kb and 1.1 kb were detected in RNA from the testes of adult wild type and $W/+$ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

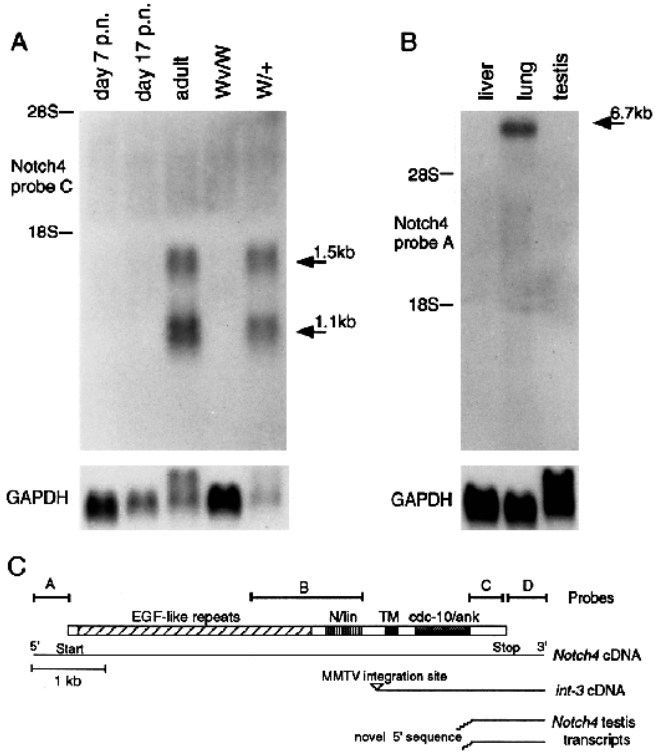

Fig. 5. Expression analysis of *Notch4* testis transcripts. (A) *Notch4* testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell-deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, W^v/W and $W/+$ mice, as indicated. (B) Northern blot analysis of several adult tissues with probe A, derived from the 5′ UTR of Notch4 and a GAPDH probe. (C) Schematic representation of truncated *Notch4* transcripts as compared to the full-length coding potential. Relative positions of probes used in the northern blot analysis are shown. Conserved elements of Notch family proteins are indicated. The MMTV integration site reported by Robbins et al. (1992) is indicated by an arrow. Novel 5′ sequences of testes cDNAs are indicated.

Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia, while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes (Nebel et al., 1961). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by northern blot hybridization to determine stage-specific expression of *Notch4* transcripts during male germ cell development. Both *Notch4* transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis (Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb *Notch4* transcripts is restricted to post-meiotic germ cells.

To determine the nature of the short *Notch4* transcripts in adult mouse testis, northern blot analysis was done using riboprobes derived from different regions of the *Notch4* coding sequence, as well as from 5′ and 3′ UTR (Fig. 5B). A riboprobe derived from the 5′ UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B), whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3′ UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Fig. 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3′ untranslated region of Notch4. Two independent clones of distinct size contained novel 5′ sequences unrelated to that found in the full-length *Notch4* cDNA (schematized in Fig. 5C, *Notch4* testis transcripts). Based upon the northern blot analysis described above and the sequence of the cloned testis cDNAs, we believe that *Notch4* transcripts are either derived from an alternate intronic promoter that is active in post-meiotic germ cells or that they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5′ untranslated region upstream of what we have currently identified. The predicted amino acid sequence of the testis *Notch4* transcripts with the novel 5′ sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode protein products. The testis transcripts may thus represent aberrant transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of *Notch4* during development and in adult lung

A 6.7 kb *Notch4* transcript was detected by northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and Sarkar et al., 1994) and adult lung (Fig. 4). To determine the spatial and temporal pattern of *Notch4* transcript accumulation during development, we examined mouse embryo tissue sections from 9.0 d.p.c. to birth using in situ hybridization. During embryonic development, as well as in postnatal tissues, *Notch4* is highly expressed in endothelial cells. Intense labeling for *Notch4* is observed in embryonic blood vessels at 9.0 d.p.c. (Fig. 6A,B). As shown in Fig. 6C,D, strong labeling is observed over the dorsal aorta, the aortic tract and the pulmonary artery in a 13.5 d.p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, we note that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6D,E) and no labeling is detected in the red blood cells

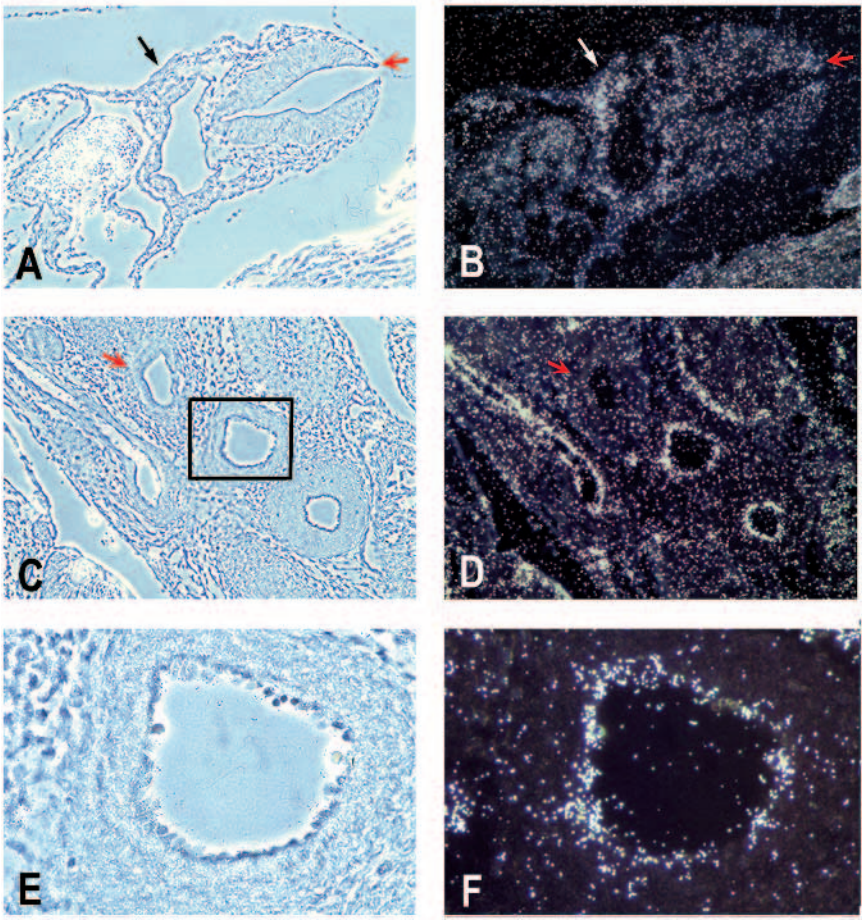

Fig. 6. *Notch4* is expressed in embryonic endothelial cells. (A,B) Phase contrast and dark-field photomicrograph of a horizontal section of a 9 d.p.c. embryo hybridized with a cRNA probe corresponding to *Notch4*. Strong labeling is detectable over the anterior cardinal vein (white/black arrows). Diffuse labeling is also present throughout the developing nervous system and at higher levels over the tip of the neural folds (red arrows). (C-F) Phase and darkfields images of a horizontal section of a 13.5 d.p.c. embryo hybridized for Notch4, showing the venous and arterial system anterior to the lung, including dorsal aorta arch, aortic and pulmonary tract. E and F are higher magnifications of the area framed in C. Embryonic vessels are labeled and, as shown in E and F, labeling is restricted to the endothelial cells lining the vessels. Arrows denote the gut, which does not have a detectable signal in the epithelium.

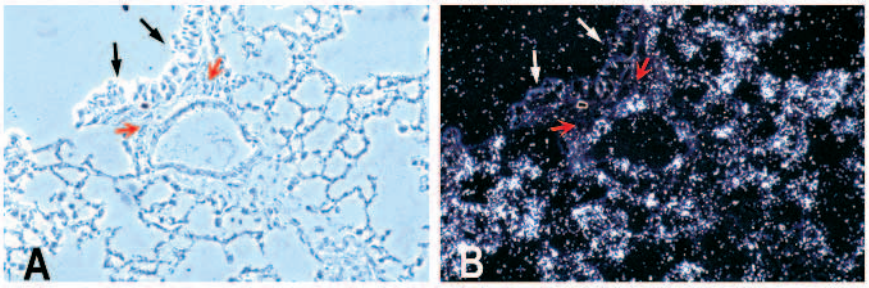

Fig. 7. *Notch4* is expressed in adult lung endothelial cells. (A,B) Phase contrast and dark-field photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to *Notch4*. Punctate staining is observed over the alveolar walls, which are predominantly composed of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).

in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 d.p.c. embryos. As shown in Fig. 6A,B, a light diffuse labeling is detected in the developing nervous system and a more distinct signal is observed at the tip of the neural folds. *Notch4* transcripts in the nervous system are still detectable at 11.5 d.p.c., but by 13.5 d.p.c. no labeling for *Notch4* is detectable in the nervous system (data not shown).

Since adult lung exhibited the highest levels of *Notch4* transcripts, in situ hybridization was performed on lung sections to determine whether *Notch4* expression remains endothelial cell-specific in adult life. Intense punctate staining was observed over the alveolar wall, indicative of capillary-specific expression (Fig. 7). The central component of the alveolar wall is the capillary flanked by pneumocyte type I epithelial cells, which line the alveolar lumen (Ross and Reith, 1985). Capillaries are highly localized in the alveolar wall and would give the punctate localized signal observed, as opposed to a more uniform pattern for epithelial cells lining the alveolar cavity. There is clearly no hybridization signal over other cellular components of the lung, that is, pseudostratified squamous epithelium, smooth muscle and connective tissue cells. The endothelial-specific expression probably underlies the abundance of *Notch4* transcripts found by northern blot analysis of highly vascularized adult tissues (lung, heart and kidney in Fig. 4).

DISCUSSION

We report here the identification of a novel mouse gene whose protein product exhibits structural homology with the vertebrate Notch protein family. We have named this gene *Notch4*, as it is the fourth murine *Notch* gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, but little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11, which has been proposed to be crucial for ligand binding. Structural variation in this repeat and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; *C. elegans* glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins, despite structural differences between them.

Notch4 is distinct from other Notch family proteins, based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of *Notch4*. This endothelial-specific expression of *Notch4* remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The *Notch4* expression pattern is in sharp contrast to the expression patterns of *Notch1*, *2* and *3*. These *Notch* genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). *Notch1* is the only other *Notch* gene reported to be expressed in endothelial cells (Reaume et al., 1992; Bettenhausen et al., 1995; Lindsell et al., 1995). Expression of *Notch1* and *4* in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development. Endothelial cell-specific expression has recently been reported for a putative Notch ligand, the chick *Serrate* homologue (Myat et al., 1996).

Since Notch proteins have been implicated in binary cell fate specification, regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebrafish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells, suggesting a common embryonic precursor which has been referred to as the ‘hemangioblast’. The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. *Cloche*, *bloodless* and *spadetail* are mutants isolated in zebrafish that display phenotypes defective in either hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the *Flk-1* and the *Flt-1* genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995; Fong et al., 1995). Null mutants for the *Flk-1* gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the *Flt-1* gene display only hematopoietic cell development defects (Fong et al.,

1995). Mutational analysis of the *Notch4* gene in whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division, or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. In the normal mouse mammary gland, endogenous int-3 protein has been detected at low levels in mammary stroma and epithelium (Smith et al., 1995). Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in *int-3* transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins function are becoming understood through genetic studies in *Drosophila*. Deltex and Suppresser of Hairless [Su(H)] have been demonstrated to bind to the cdc10 repeats of the intracellular domain of *Drosophila* Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus. where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch proteins containing only the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993), suggesting a nuclear function for this domain. We have found that the int-3 oncoprotein, modified to encode a flu epitope-tag at the C terminus, is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). The activated int-3 protein lacks a signal peptide but contains a membrane-spanning domain and thus is not likely to enter the secretory pathway. This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

We show that the *int-3* gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described by Robbins et al. (1992), the oncogenic affects are probably the result of both overexpression or ectopic expression of *Notch4* mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural alterations reported to activate the effector function of *Drosophila* Notch and *C. elegans* lin-12 proteins (Greenwald, 1994) or oncogenic activation of TAN-1. Thus, loss of the extracellular domain is likely to lead to loss of the regulatory controls provided by the ligand-binding domain believed to reside in the EGF-like repeats of Notch4.

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