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Isolation and Functional Analysis of a cDNA for Human *Jagged2*, a Gene Encoding a Ligand for the Notch1 Receptor

BIAO LUO, JON C. ASTER, ROBERT P. HASSERJIAN,† FRANK KUO, AND JEFFREY SKLAR*

Division of Molecular Oncology, Department of Pathology, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts 02115

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Signaling through Notch receptors has been implicated in the control of cellular differentiation in animals ranging from nematodes to humans. Starting from a human expressed sequence tag-containing sequence resembling that of *Serrate*, the gene for a ligand of *Drosophila melanogaster* Notch, we assembled a full-length cDNA, now called human *Jagged2*, from overlapping cDNA clones. The full-length cDNA encodes a polypeptide having extensive sequence homology to Serrate (40.6% identity and 58.7% similarity) and even greater homology to several putative mammalian Notch ligands that have subsequently been described. When in situ hybridization was performed, expression of the murine *Jagged2* homolog was found to be highest in fetal thymus, epidermis, foregut, dorsal root ganglia, and inner ear. In Northern blot analysis of RNA from tissues of 2-week-old mice, the 5.0-kb *Jagged2* transcript was most abundant in heart, lung, thymus, skeletal muscle, brain, and testis. Immunohistochemistry revealed coexpression of Jagged2 and Notch1 within thymus and other fetal murine tissues, consistent with interaction of the two proteins in vivo. Coculture of fibroblasts expressing human Jagged2 with murine C2C12 myoblasts inhibited myogenic differentiation, accompanied by increased Notch1 and the appearance of a novel 115-kDa Notch1 fragment. Exposure of C2C12 cells to Jagged2 led to increased amounts of *Notch* mRNA as well as mRNAs for a second Notch receptor, *Notch3*, and a second Notch ligand, *Jagged1*. Constitutively active forms of Notch1 in C2C12 cells also induced increased levels of the same set of mRNAs, suggesting positive feedback control of these genes initiated by binding of Jagged2 to Notch1. This feedback control may function in vivo to coordinate differentiation across certain groups of progenitor cells adopting identical cell fates.

The Notch gene family encodes transmembrane receptors critical for various cell fate decisions during development in both invertebrates and vertebrates (3, 57). Multiple ligands that activate Notch and related receptors have been identified, including Delta (36, 61) and Serrate (17, 59) in *Drosophila melanogaster*, Lag-2 (27, 58) and Apx-1 (44) in *Caenorhabditis elegans*, and Delta (9, 28) and Jagged1 (42, 46) in vertebrates. All ligands identified to date are transmembrane proteins with similar overall structural features, including a large extracellular domain containing a DSL (Delta-Serrate-Lag-2) motif that is specific to Notch ligands, followed by variable numbers of iterated epidermal growth factor (EGF)-like repeats, a single transmembrane segment, and a short, less well conserved cytoplasmic domain.

One important function for Notch and its ligands is to control divergent programs of differentiation among spatially proximal groups of equipotent progenitor cells (for a recent review, see reference 35). During peripheral neurogenesis in *Drosophila*, for example, Notch and Delta direct cell fate decisions within clusters of proneural cells through a process termed lateral inhibition (24–26). All cells within a proneural cluster initially express both Notch and Delta. Delta-dependent Notch signaling activates the transcription factor Suppressor of Hairless [Su(H)] (18, 55), which increases expression of basic helix-loop-helix transcription factors of the Enhancer of split (E[spl]) complex (7, 31, 39). E(spl) products in turn decrease levels of Delta by down-regulating expression of basic helix-loop-helix factors of the achaete-scute complex (30, 37, 47), proneural genes that are required for adoption of neuronal cell fate (13, 20, 51, 53) and thereby serve to complete a negative regulatory pathway operating on Delta expression (24). It is believed that random variation in Notch signaling is amplified through this negative regulatory pathway, eventuating in adjacent cells that are either Notch high/Delta low or Notch low/Delta high. Cells that are Notch low/Delta high become sensory organ precursors that ultimately give rise to neurons, whereas Notch high/Delta low cells adopt an epidermal cell fate. Similar negative regulatory pathways between Notch receptors and ligands appear to control primary neurogenesis in the frog (9–11, 40) and vulvar development in *C. elegans* (12, 63).

Recently, a cDNA encoding a mammalian Notch ligand related to *Drosophila Serrate* was isolated from a rat cDNA library (42). When expressed in feeder fibroblasts, this cDNA, termed *Jagged1*, inhibited myogenic differentiation of the murine myoblast C2C12 cell line (42), implicating mammalian Notch ligands in the negative control of certain differentiation events. Subsequently, a cDNA for a second rat homolog, termed *Jagged2*, was reported (56). In this paper, we describe the isolation of a human cDNA having sequences related to both *Drosophila Serrate* and the rat *Serrate* homologs, especially *Jagged2*. Functional analyses of the polypeptide encoded by this cDNA, which we refer to as human *Jagged2*, indicate that it too may mediate control of differentiation events in mammalian muscle. This process involves positive regulation induced by ligand binding of genes for both Notch receptors and ligands, in a manner that differs from the negative regulation of ligand genes observed during specification of neural cell fates in the developing fruit fly.

* Corresponding author. Phone: (617) 732-7446. Fax: (617) 732-7449. E-mail: jsklar@bustoff.bwh.harvard.edu.

† Present address: Department of Histopathology, Hammersmith Hospital, London, United Kingdom.

MATERIALS AND METHODS

Isolation of human Jagged1 and Jagged2 cDNAs. A database of human brain expressed sequence tags (ESTs) at the American Type Culture Collection

(ATCC) (1) was searched for sequences homologous to those of *Drosophila* Notch ligands, leading to the identification of one clone, HIBBK87, containing sequences similar to a region of *Serrate* and *Delta* encoding EGF repeats. The 5′ fragment of HIBBK87 was used to screen a randomly primed λgt11 cDNA library constructed from human umbilical vein endothelial cells (HUVEC) (21). Positive clones were purified and subcloned into the *Eco*RI site of the plasmid pBluescript II SK− (Stratagene). Sequences at the ends of the inserts were determined by the method of Sanger et al. (54) and used to rescreen the HUVEC library. Successive iterations of this procedure produced a set of overlapping partial cDNAs which were assembled into a contiguous *Jagged2* cDNA with the aid of Wisconsin Genetics Computer Group and MacVector software after sequence analysis was performed on both strands (54). During screening of the HUVEC library with a 210-bp *Pst*I fragment from one of the partial cDNAs (VEC5/SK3), multiple human *Jagged1* cDNA clones were also identified. A full-length *Jagged1* cDNA was subsequently assembled through identification of overlapping clones as described for *Jagged2*. A partial cDNA (ATCC 30425; yf98a01) for human *Delta1* was also identified by homology screening of the ATCC EST database with murine *Delta1*.

Construction of *Jagged1* and *Jagged2* expression vectors. DNA of the plasmid pBluescriptSK− containing the full-length *Jagged2* cDNA was digested with *Bsu*36I and *Xho*I and ligated to a double-stranded DNA fragment created by annealing of two oligonucleotides, 5′-TCAGGCACCGC and 5′-TCGAGCGG TGCC. The cDNA insert was then excised from the plasmid with *Eco*RI and *Xho*I and ligated into pA3M (43), a derivative of pcDNA3 (Invitrogen). The product of this ligation encodes a form of Jagged2 in which the carboxy-terminal 20 amino acids of the cytoplasmic domain are replaced by three copies of a myc epitope tag. To make Jagged1-myc, pBluescriptSK− containing the full-length *Jagged1* cDNA was digested with *Sac*I and religated, resulting in deletion of sequences encoding the 3′ stop codon and untranslated region. The 3′-deletion-containing cDNA was then inserted between the *Hin*dIII and *Eco*RV sites of pA3M. This cDNA encodes a form of Jagged1 in which the carboxy-terminal 40 amino acids are replaced by three iterated copies of a myc epitope tag. To permit retroviral packaging, the Jagged1-myc and Jagged2-myc cDNAs were ligated into the retroviral shuttle vector pBABE-puro (45). The construction of pBABE plasmids encoding full-length Notch1 and two constitutively active forms of Notch1, ΔE, and ICNΔRAM23 containing amino-terminal deletions has been previously described (4, 5).

Antibodies and immunohistochemistry. To raise Jagged2-specific antibodies, a cDNA encoding the cytoplasmic domain of Jagged2 (amino acids 1088 to 1238) was amplified by PCR and ligated into the pGEX-2TK plasmid (Pharmacia). Cells of *Escherichia coli* XL1-Blue transformed with this plasmid were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Glutathione *S*-transferase-Jagged2 fusion protein was purified from extracts prepared from induced cells on glutathione-Sepharose beads (Pharmacia) as described by Kaelin et al. (33) and used to immunize New Zealand White rabbits. Anti-Jagged2 antibody was affinity purified on Jagged2-Affigel 10 (Bio-Rad) under the conditions described by Hasserjian et al. (23). Control preimmune antibody was purified on protein A-Sepharose (Pharmacia). Affinity-purified antibodies specific for Notch1 have been previously described (23). Monoclonal antibody 9E10, specific for a myc epitope, was the kind gift of Jeffrey Parvin.

Immunoperoxidase staining of paraffin sections with Notch1- and Jagged2-specific antibodies was carried out as previously described (23).

In situ hybridization. The cDNA VEC5/SK3 was used to screen an oligo(dT)-primed mouse brain cDNA library constructed in the ZAPII vector (Stratagene). One identified clone, mJagged2-1, contained a 3.7-kb cDNA insert corresponding to nucleotides 1027 to 4712 of human *Jagged2*. A 1.6-kb *Pst*I-*Xho*I fragment of mJagged2-1 was subcloned into pBluescriptSK− to create a new clone termed MJPX45. After digestion with *Pst*I, linearized MJPX45 was transcribed with T7 RNA polymerase to produce a ^{35}S-labeled antisense riboprobe. To produce a sense riboprobe, MJPX45 was digested with *Xho*I and transcribed with T3 RNA polymerase. In situ hybridization of paraffin sections prepared from formalin-fixed, whole murine embryos was performed with MJPX45 probes as described by Angerer and Angerer (2), except that hybridization was carried out at 55°C and the 50% formamide posthybridization wash was performed at 50°C.

Northern blot analysis. RNA was isolated from tissues and cultured cells by using TriZol (Life Technologies) and analyzed by the Northern blot procedure according to Fourney et al. (19). The probe used to detect *Jagged2* was a 1.6-kb *Pst*I/*Xho*I fragment from MJPX45, whereas the probe used for *Jagged1* was a 3,850-bp cDNA spanning the entire coding region of human *Jagged1*. Murine probes specific for *Notch1*, *Notch2*, and *Notch3* (38) were provided by Urban Lendahl, a probe for myosin light chain 1,3 (MLC1,3) mRNA was provided by Andrew Lassar, and a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was provided by Erle Robertson. The probes were synthesized by using the Random Primers DNA labeling system (Life Technologies). The hybridization was carried out at 42°C in a solution containing 50% formamide, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.2% tetrasodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 4% dextran sulfate, and 0.1 mg of denatured single-stranded salmon sperm DNA (Sigma) per ml. The membrane was washed at 47°C in 2× SSC–0.1% SDS for 1 h. The lengths of the RNAs corresponding to hybridizing bands were estimated by using RNA size standards (Life Technologies).

Cell lines. All cell lines were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 2 mM glutamine (D10 medium). C2C12 cells were obtained from the ATCC; NIH 3T3 cells were the gift of James Cunningham. The BOSC23 ecotropic retrovirus packaging cell line has been previously described (49).

Retroviral transduction of cell lines. To prepare ecotropic retrovirus, BOSC23 cells were transfected with pBABE DNA containing various cDNA inserts under the conditions described by Pear et al. (49). Viral supernatants were harvested 72 h posttransfection and used to infect NIH 3T3 cells or C2C12 cells in the presence of Polybrene (8 mg/ml). Transduced cells were selected 24 h postinfection with D10 containing puromycin (1 μg/ml).

C212 cell myogenesis assay. The C2C12 myogenesis assay was performed as described by Lindsell et al. (42) with minor modifications. Briefly, C2C12 cells infected with pBABE retroviruses were added to 100-mm-diameter tissue culture plates in D10 medium containing puromycin. When C2C12 cells reached ~70% confluence, 5×10^6 NIH 3T3 cells infected with pBABE retroviruses were seeded onto each dish. In experiments studying Notch1 processing and expression of endogenous *Notch1*, *Notch3*, and *Jagged1* RNA transcripts, these cells were cocultured for two additional days in D10 containing puromycin. In experiments studying myogenesis, the medium was changed to Dulbecco's modified Eagle's medium containing 10% horse serum (differentiation medium) 1 day after seeding with NIH 3T3 cells. Fresh differentiation medium was added 3 days later, and the cells were then harvested on day 6.

Immunoprecipitation and Western blot analysis. Dishes of cells were lysed for 10 min at 4°C in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% Nonidet P-40; 0.5% deoxycholate; 0.1% SDS) containing freshly added aprotinin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (0.25 mM). Cell extracts were then collected and spun in a microcentrifuge (Eppendorf) at 14,000 rpm for 10 min at 4°C. To detect recombinant Jagged2-myc in RIPA extracts, Western blots prepared as described by Aster et al. (4) with the total lysate were stained with anti-myc monoclonal antibody 9E10 by a chemiluminescent method (ECL kit; Amersham). To detect endogenous Notch1 polypeptides, immunoprecipitates were prepared from RIPA extracts by mixing with 2 μl of anti-TC Notch1 rabbit antiserum (23) for 1 h and then 20 μl of protein A-Sepharose (Pharmacia) for one additional hour at 4°C. Beads were then washed four times with 0.5 ml of ice-cold RIPA buffer and boiled in SDS-polyacrylamide gel electrophoresis loading buffer. Eluted Notch1 polypeptides were detected on Western blots stained with affinity-purified rabbit polyclonal antibody raised against the T3 domain of Notch1 (4). The anti-TC antiserum recognizes the cytoplasmic domain in Notch1 in a region that is divergent among known Notch homologs and appears to be specific for Notch1.

RESULTS

Isolation of full-length human *Jagged2* cDNAs. To identify cDNAs encoding Notch ligands, a human brain EST database was screened for sequences homologous to *Drosophila Serrate* and/or *Delta*. A clone containing an EST with homology to *Serrate* and *Delta* was used to initiate screening of a HUVEC cDNA library. After four rounds of rescreening, overlapping cDNAs were assembled into a 4.7-kb cDNA with an open reading frame of 1,238 codons (Fig. 1A). Within the coding region, this cDNA is highly homologous to *Drosophila Serrate* in predicted amino acid sequence (40.6% identity and 58.7% similarity) (Fig. 1B). Subsequently, two rat homologs for *Serrate* were described, *Jagged1* and more recently *Jagged2*, both of which have significant homology to the human cDNA isolated by us. The correspondence between *Jagged2* and our cDNA (89% identity and 93% similarity in the corresponding amino acid sequences) was even higher than that between our cDNA and *Serrate*, and we consequently have referred to the human *Serrate*-related cDNA and the gene from which it is derived as human *Jagged2*. The full-length human *Jagged2* cDNA encodes a number of recognizable sequence motifs, including (beginning at the amino terminus and moving toward the carboxy terminus) a hydrophobic amino acid sequence compatible with a signal peptide, a predicted extracellular DSL domain, 16 iterated EGF-like repeats, a cysteine-rich region, a hydrophobic sequence consistent with a transmembrane domain, and a short cytoplasmic domain (132 amino acids). An unusual feature in human Jagged2 relative to Serrate is a 24-amino-acid insertion that interrupts EGF repeat 10. Similar insertions are also present in rat Jagged2 and Jagged1.

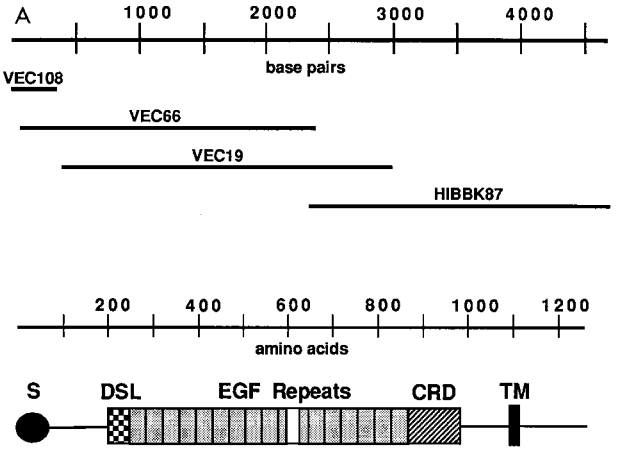
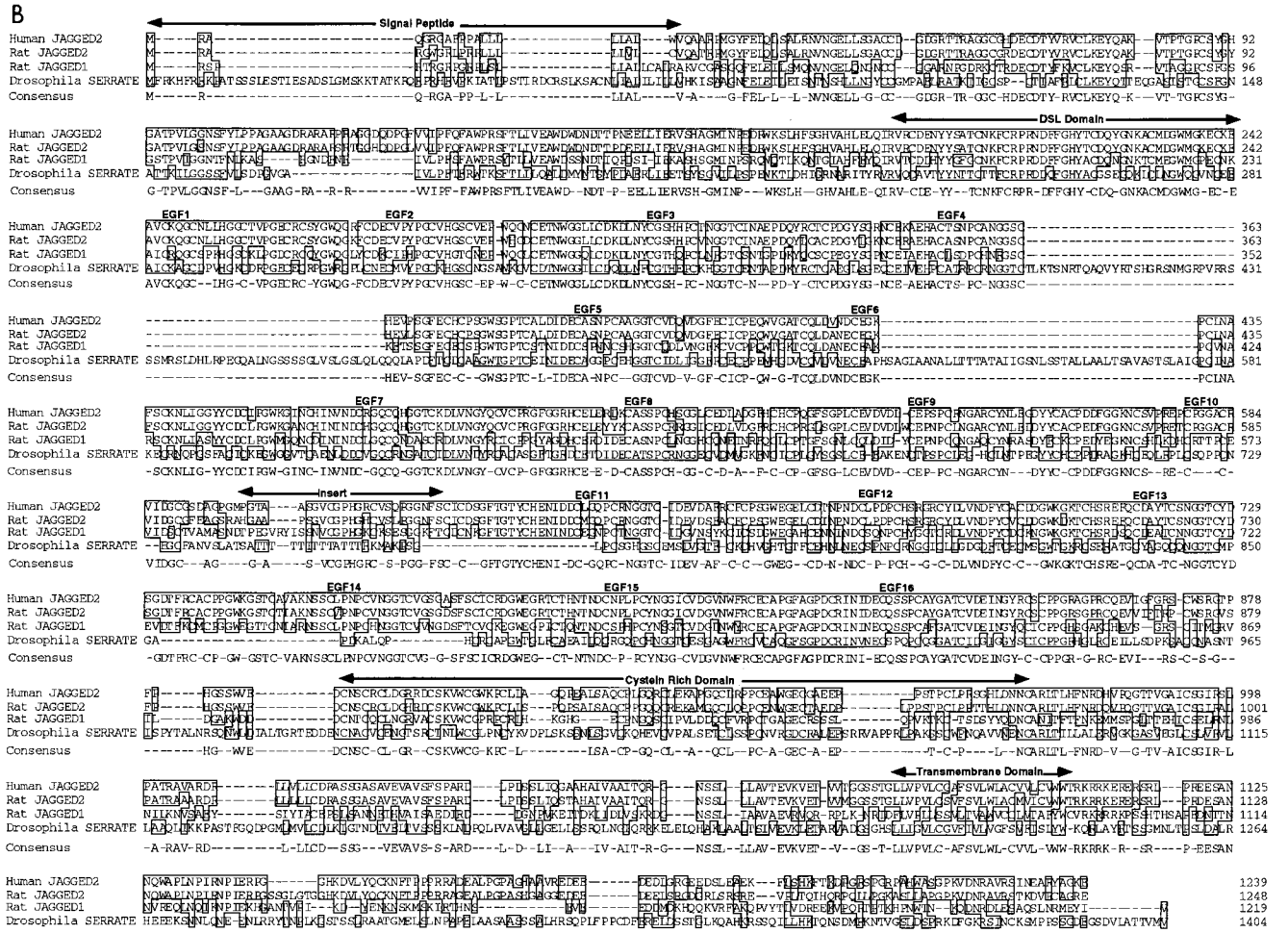
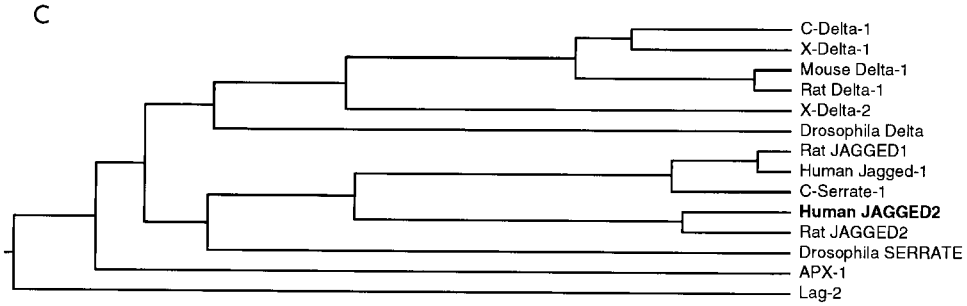


FIG. 1. Structural analysis of human *Jagged2* cDNA. (A) Partial *Jagged2* cDNAs used to assemble the complete coding sequence and diagram of motifs in the deduced amino acid sequence. The four overlapping cDNAs, VEC108, VEC66, VEC19, and HIBBK87, used to construct the composite *Jagged2* cDNA are shown in the upper portion of the panel. A diagram of the primary protein structure of human Jagged2 is shown at the bottom with motifs labeled as follows: S, signal peptide; EGF repeats, EGF-like repeats; CRD, cysteine-rich domain; TM, transmembrane domain. (B) Alignment of the amino acid sequence of human Jagged2, rat Jagged2, rat Jagged1, and *Drosophila* Serrate. The amino acid sequences deduced from the cDNAs for these proteins were aligned by using the MegAlign program of the DNAStar software package, and a consensus sequence was derived. Conserved residues are boxed. The EGF repeats of human Jagged2 are labeled over the first cysteine of each repeat. The regions for the signal peptide, the DSL domain, the insert in EGF repeat 10, the cysteine-rich domain, and the transmembrane domain of human Jagged2 are indicated. (C) Phylogenetic relationship of Notch ligands. The amino acid sequences of human Jagged2, human Jagged1, and other previously described Notch ligands were used to generate a phylogenetic tree with the MegAlign program.





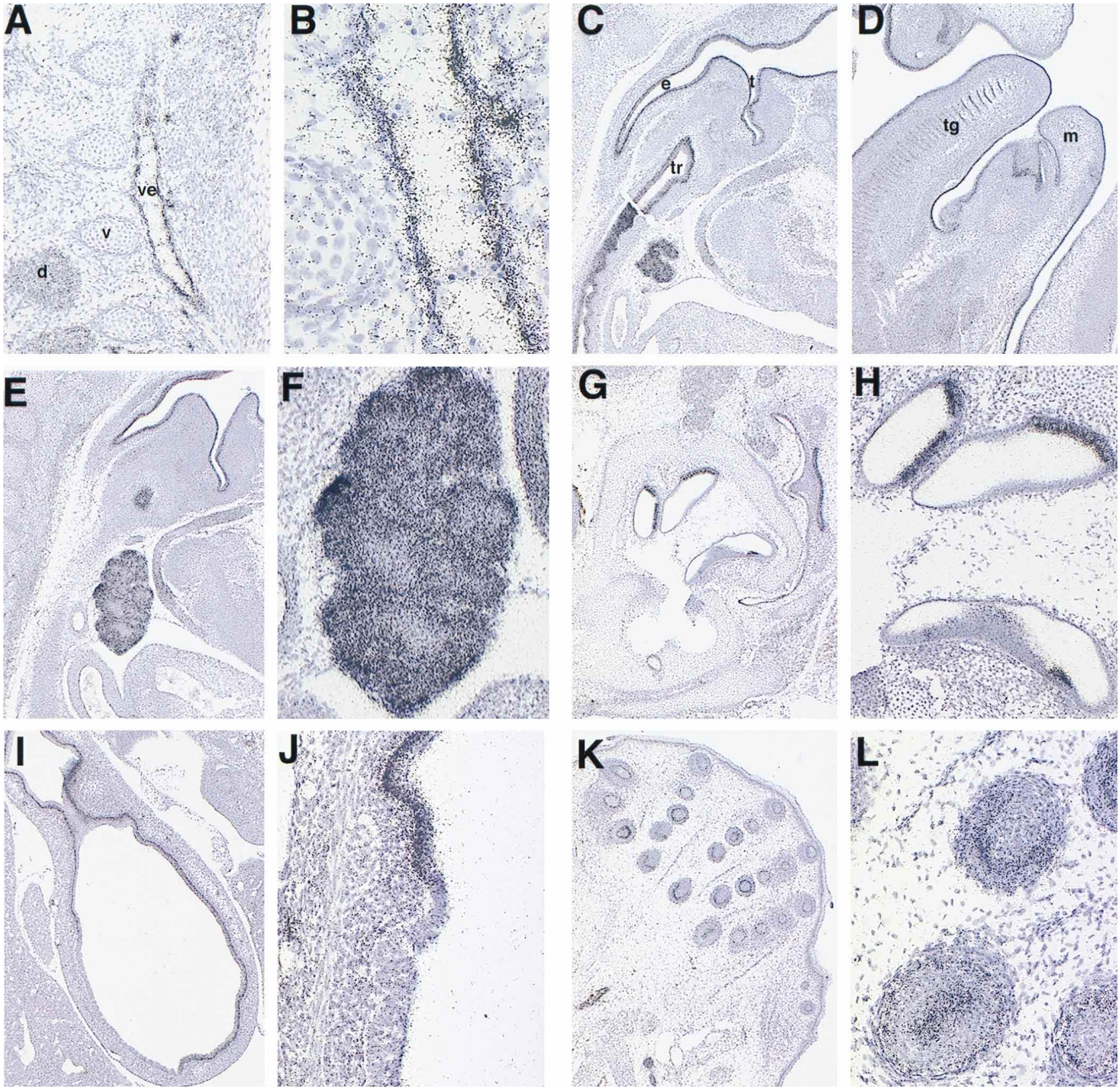


FIG. 2. *Jagged2* expression in murine embryos. Jagged2 expression in murine embryos was analyzed by in situ hybridization with a ^{35}S-labeled antisense *Jagged2* riboprobe. After development of the photographic emulsion, embryos were counterstained with hematoxylin. (A through D) Structures showing significant hybridization include dorsal root ganglia (d), vertebral cartilages (v), blood vessel (ve), trachea (tr), esophagus (e), thyroglossal duct (t), tongue (tg), and mandible (m). (E and F) Intense hybridization to the thymus, particularly within the cortex. (G and H) Sections through the inner ear, indicating hybridization to the sensory hair cells of the semicircular canals (upper two structures) and cochlea (lower structure). (I) Section through the esophagus and proximal stomach, showing hybridization to the basal layer of the squamous mucosa but not to columnar mucosa. The transition from squamous to columnar epithelium is shown at higher power in panel J. (K and L) Sections through multiple whisker follicles in the snout. The ages of the embryos were 13 dpc (A and B), 14 dpc (C through F), and 15.5 dpc (G through L).

During the screening of the HUVEC library, clones distinct from those of human *Jagged2* were also identified. These cDNAs were assembled into a composite cDNA having sequences highly homologous to rat *Jagged1*, which we have therefore referred to as human *Jagged1* (data not shown).

The sequence of human Jagged2 was compared to those of other known Notch ligands using MegAlign software from the DNAStar package, which permits construction of phylogenetic trees (Fig. 1C). This analysis confirmed the interpretation that both human *Jagged1* and *Jagged2* appear to have evolved from an ancestral *Serrate*-like gene.

Expression of *Jagged2* during murine development. The expression of *Jagged2* during murine embryonic development was studied by in situ hybridization with ^{35}S-antisense riboprobes. At 13 days postcoitum (dpc), expression of *Jagged2* transcripts was found in paravertebral vessels (Fig. 2A and B) and dorsal root ganglia (Fig. 2A). At 14 dpc, expression of *Jagged2* in oropharyngeal epithelium (Fig. 2C through 2E), developing

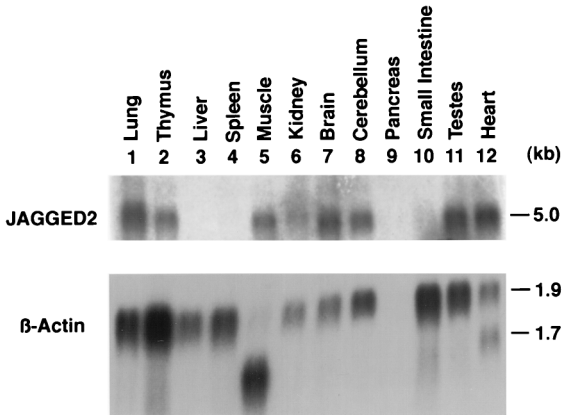


FIG. 3. *Jagged2* expression in postnatal murine tissues. Total RNA isolated from various tissues of a 2-week-old mouse was analyzed by Northern blotting. The blot was initially hybridized with a probe for murine *Jagged2* and then stripped and rehybridized with a probe for β-actin. The resulting autoradiograms are shown.

thymus (Fig. 2C, E, and F), and muscles in the tongue (Fig. 2D) was found. By 15.5 dpc, expression was noted in many tissues, including the germinative epithelium of whiskers (Fig. 2K and L), the basal layer of the developing skin (Fig. 2K), and the hair cells of both the semicircular canals and the cochlea within the inner ear (Fig. 2G and H). Oropharyngeal epithelium and esophagus also showed high levels of expression at 15.5 dpc. A striking feature of expression detected in foregut structures was the sharp drop-off in expression in epithelium at the transition from squamous to columnar in the proximal part of the stomach (Fig. 2I and J). A similar sharp demarcation between levels of *Jagged2* expression was observed at the squamocolumnar junction in developing hindgut (data not shown). Control studies with ^{35}S-sense probes from MJPX45 showed little background and no specific hybridization (data not shown).

Murine *Jagged2* expression in various tissues was also investigated by Northern blot analysis performed on RNA isolated from tissues of 2-week-old mice (Fig. 3). This revealed relatively high levels of a single ~5-kb transcript in heart and lung; moderately high levels in thymus, muscle, brain, cerebellum, and testis; and low levels in kidney and small intestine. In contrast, in tissues from animals at this age, murine *Jagged1* was found to be highly expressed in lung and kidney, while murine *Delta1* was found to be preferentially expressed in lung, spleen, and small intestine (data not shown).

Correlation of *Notch1* and *Jagged2* by immunohistochemistry. Affinity-purified antibodies directed against the cytoplasmic domain of *Jagged2* were used to study the expression of *Jagged2* and *Notch1* in murine embryos. Tissues showing high levels of *Jagged2* mRNA by in situ analysis with antisense riboprobes also showed relatively high levels of immunoreactivity with anti-Jagged2 antibody. Furthermore, the pattern of immunoreactivity with anti-Jagged2 antibody partially overlapped that observed with anti-Notch1 antibody (Fig. 4). For example, Jagged2 and Notch1 immunoreactivity coincided in dorsal root ganglia (Fig. 4A and C), thymus (Fig. 4B), the apical aspect of the germinative layer of hair follicles (Fig. 4E), the apical aspect of basal keratinocytes (Fig. 4F), and the osteoblasts of developing bone (Fig. 4H), whereas strong Notch1 immunoreactivity in the forebrain was accompanied by weak Jagged2 immunoreactivity (Fig. 4D). In developing skeletal muscle, strong Notch1 immunoreactivity was found in developing myotubes together with lower-intensity reactivity for Jagged2, which appeared to be more easily detected in scattered perimysial cells (Fig. 4G).

Inhibition of differentiation in C2C12 cells by Jagged2. Expression of relatively high levels of Jagged2 mRNA and protein in developing skeletal muscle suggested a role for Jagged2 in the control of myogenesis. Myogenesis has previously been studied in vitro by using the C2C12 cell line, which undergoes terminal differentiation in mitogen-poor medium, as manifested by the fusion of cells to form multinucleated myotubes and expression of high levels of muscle-specific mRNAs, such as that for MLC1,3. These events were previously shown to be inhibited by cocultivation with layers of feeder cells expressing rat *Jagged1* (42).

To investigate the effect of human *Jagged2* on myogenesis, C2C12 control cells or C2C12 cells infected with pBABE-Notch1 retrovirus were cocultivated with NIH 3T3 control cells or NIH 3T3 cells infected with pBABE-Jagged2 retrovirus. When cocultivated with control NIH 3T3 cells, C2C12 control cells and C2C12-Notch1 cells differentiated normally, fused into numerous multinucleated myotubes (data not shown), and expressed high levels of MLC1,3 mRNA (Fig. 5). In contrast, both C2C12 control cells and C2C12-Notch1 cells cocultured with NIH 3T3-Jagged2 cells showed diminished myotube formation (data not shown) and markedly decreased expression of MLC1,3 mRNA relative to cocultures with control NIH 3T3 cells. Identical results were observed with C2C12/NIH 3T3-Jagged1 cocultures (Fig. 5), indicating that human Jagged2 and Jagged1 are similar with respect to their capacity to inhibit C2C12 cell myogenesis.

Increased levels of full-sized Notch1 (p350) and the appearance of a novel Notch1 polypeptide of 115 kDa induced by Jagged2. Inhibition of C2C12 differentiation by human Jagged2 independent of Notch1 overexpression suggested that C2C12 cells expressed one or more endogenous Notch receptors. When analyzed by Western blot analysis (Fig. 6), immunoprecipitates prepared with an antibody specific for the cytoplasmic domain of Notch1 showed that control C2C12 cells contained appreciable amounts of p120, a presumably inactive, truncated form of Notch1 created by ligand-independent posttranslational proteolytic cleavage at a site 70 amino acids external to the transmembrane domain (3a, 4, 34), and undetectable amounts of p350, which corresponds to the full-size Notch1 receptor (4). NIH 3T3 and NIH 3T3-Jagged2 cultures contained no detectable p120 or p350, while extracts prepared from C2C12/NIH 3T3 cell cocultures contained mainly p120 in amounts similar to those observed in C2C12 cells cultured alone. In contrast, extracts prepared from C2C12/NIH 3T3-Jagged2 cocultures showed increased levels of p350 and the presence of a new cross-reactive polypeptide of approximately 115 kDa (p115).

To determine the basis for increased p350 and the appearance of p115 in C2C12/NIH 3T3-Jagged2 cell cocultures, Northern blot analysis was performed (Fig. 7). C2C12/NIH 3T3-Jagged2 cocultures showed up-regulation of a 9.3-kb *Notch1* transcript, whereas no change in this transcript was observed in C2C12/NIH 3T3 cocultures or in NIH 3T3-Jagged2 cells cultured separately (a second hybridizing transcript of 8.1 kb derived from cross-hybridization of the probe with *Notch3* mRNA, as discussed below, was also up-regulated in C2C12/NIH 3T3-Jagged2 cell cocultures). These results suggest that the Jagged2-dependent increase in p350 is due, at least in part, to increased levels of the 9.3-kb *Notch1* transcript. In addition, detection of only one *Notch1* transcript in C2C12/NIH 3T3-Jagged2 cocultures suggests that p115 is created by posttranslational proteolytic processing.

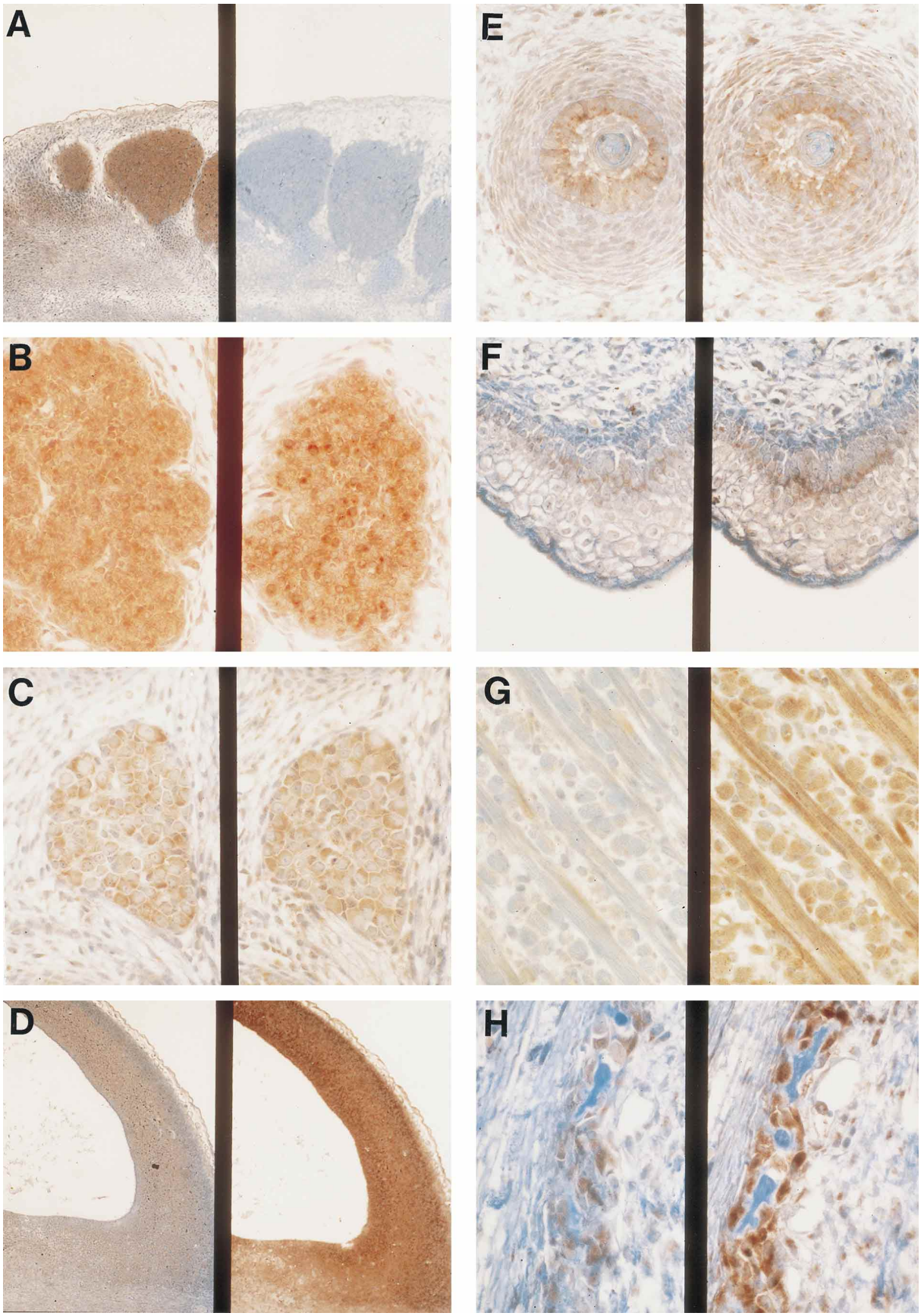

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FIG. 4. Immunohistochemical detection of Notch1 and Jagged2 in murine embryos. Paraffin sections of mouse embryos were stained with preimmune immunoglobulin, anti-Jagged2 antibody, or anti-Notch1 antibody by an immunoperoxidase method that results in a brown color and then counterstained with methyl green. Staining with anti-Jagged2 antibody, shown in the left-hand panel of each pair of photomicrographs, was compared to staining with either preimmune serum (A) or anti-Notch1 antibody (B through H) in adjacent sections of tissue. (A) Dorsal root ganglia, 14 days dpc; (B) thymus, 14 dpc; (C) dorsal root ganglion, 18 dpc; (D) forebrain, 14 dpc; (E) hair follicle, 15 dpc; (F) epidermis, 15 dpc; (G) skeletal muscle, 15 dpc; (H) skull, 18 dpc.

Altered expression of *Notch3* and *Jagged1* but not of *Notch2* by Jagged2. Ligand-dependent up-regulation of Notch expression is one component of an intercellular feedback loop that involves levels of Notch receptor and ligand and appears to control neurogenesis in *Drosophila* (24–26) and *Xenopus* (9, 10). Up-regulation of Notch1 expression in C2C12/NIH 3T3-Jagged2 cocultures appeared consistent with at least one part of a similar feedback signaling system in C2C12 cells. To explore regulatory effects on other genes in the Notch pathway, we studied the consequences of Jagged2 binding on expression of *Notch2*, *Notch3*, *Jagged1*, and *Delta1* (Fig. 7). In addition to increased expression of *Notch1*, expression of *Notch3* was also up-regulated in C2C12/NIH 3T3-Jagged2 cocultures, while *Notch2* expression was not significantly altered. Unexpectedly, expression of *Jagged1* was also up-regulated in C2C12/NIH 3T3-JAGGED2 cocultures. In contrast, *Delta1* expression was not detected in C2C12 cells cultured alone or together with NIH 3T3-Jagged2 cells (data not shown). These data suggest the existence of positive-feedback regulation of gene expression initiated by ligand binding and involving the gene for one ligand, Jagged1, and two receptors, Notch1 and Notch3.

Effects comparable to those of Jagged2 produced by constitutively active forms of Notch1 in C2C12 cells. While the above data seemed to point to a positive-feedback signaling mediated directly through interaction of Jagged2 in NIH 3T3 cells and Notch1 on C2C12 cells, it remained possible that the observed effects in C2C12/NIH 3T3-Jagged2 cocultures could arise by Jagged2 acting in some way on NIH 3T3 cells or by Jagged2 acting on C2C12 cells through receptors other than Notch1. However, overexpression of Jagged2 in C2C12 cells cultured alone also up-regulated expression of *Notch1*, *Notch3*, and *Jagged1* relative to C2C12 control cells (Fig. 8), suggesting that the only contribution of the feeder cells was expression of Jagged2. Support for the direct involvement of Notch1 in mediating the effects of Jagged2 was obtained using C2C12 cells expressing two forms of constitutively active Notch1 carrying deletions for various parts of the extracellular domain of the full-length protein. One of these polypeptides, ΔE, is a membrane-bound form of Notch1 retaining the signal peptide and 61 amino acids of the extracellular domain; the second, ICNΔRAM23, accumulates in the nucleus and has the amino terminus of the protein deleted up to amino acid residue 1858, which lies 101 amino acids internal to the transmembrane domain. Both of these polypeptides activate transcription from promoter elements having iterated binding sites for RBP-Jκ (5), a mammalian homolog of Su(H) that functions as a transcription factor downstream of activated Notch1 (29) in the

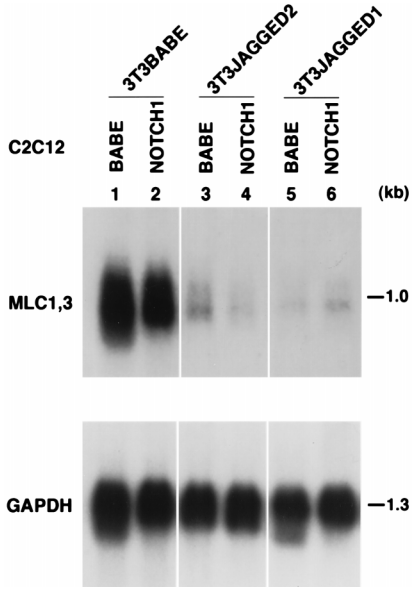

FIG. 5. Inhibited expression of the MLC1,3 gene in C2C12 cells by human Jagged2. C2C12 cells stably infected with the retrovirus pBABE (C2C12BABE) or pBABE carrying a human *Notch1* cDNA (C2C12 Notch1) were cocultured in differentiation medium with NIH 3T3 cells stably infected with pBABE (3T3BABE) or with pBABE carrying cDNAs for either *Jagged2* (3T3Jagged2) or *Jagged1* (3T3Jagged1) engineered to direct synthesis of myc epitope-tagged polypeptides. Total RNA was isolated from cultured cells and analyzed by Northern blot hybridization. A single blot was hybridized with a murine MLC1,3 probe and then stripped and rehybridized with a probe for GAPDH RNA. The resulting autoradiograms are shown.

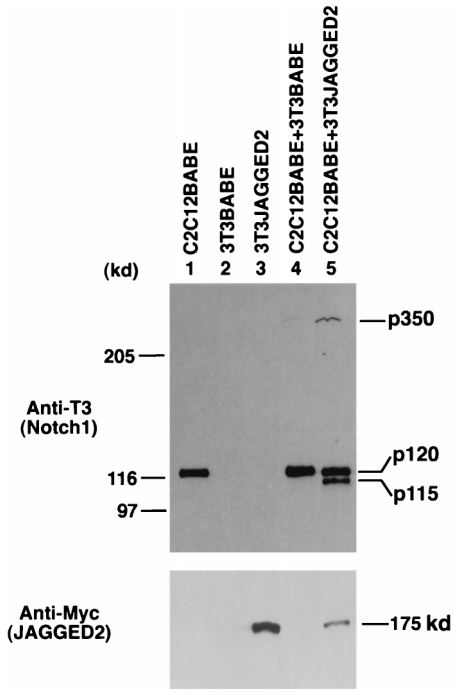

FIG. 6. Ligand-dependent processing of Notch1 in C2C12 cells induced by Jagged2. Notch1 polypeptides were immunoprecipitated with anti-TC (rabbit affinity-purified anti-Notch1 antibody) from whole-cell extracts prepared from C2C12BABE, 3T3BABE, and 3T3Jagged2 cells cultured separately or cocultured for 2 days. Western blots were then prepared from immunoprecipitated polypeptides (upper panel) or whole-cell extracts (lower panel, 25 μg of protein/lane) after electrophoresis in 6% polyacrylamide gels. The blot displaying immunoprecipitated polypeptides was stained with anti-T3, a second rabbit affinity-purified antibody against Notch1, and the blot displaying polypeptides found in whole-cell extracts was stained with anti-myc monoclonal antibody 9E10. Molecular masses of standard polypeptides and Jagged2-myc are given on the left.

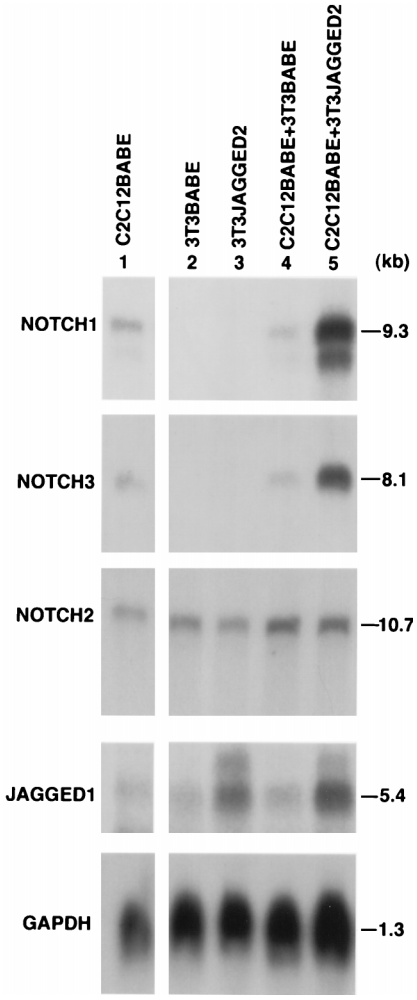

FIG. 7. Increased expression of *Notch1*, *Notch3*, and *Jagged1* in C2C12 cells induced by feeder cells carrying *Jagged2* cDNA. Total RNA isolated from C2C12BABE, 3T3BABE and 3T3Jagged2 cells cultured separately or cocultured for 2 days was analyzed by a Northern blot hybridization. The blot was initially hybridized with a probe for *Notch1* and then repeatedly stripped and rehybridized with probes for *Notch2* and *Notch3*, *Jagged1*, and GAPDH RNA. The resulting autoradiograms are shown.

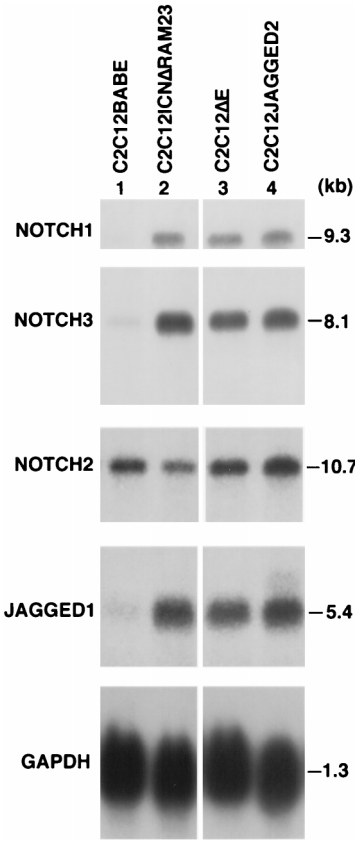

FIG. 8. Increased levels of *Notch1*, *Notch3*, and *Jagged1* mRNA induced by Jagged2 or constitutively active forms of Notch1 in C2C12 cells. Total RNA was prepared from C2C12 cells stably infected by the retrovirus pBABE (C2C12BABE), pBABE retroviruses carrying cDNAs encoding constitutively active forms of Notch1 (C2C12ICNΔRAM23 and C2C12ΔE), or pBABE retrovirus carrying a cDNA encoding a myc epitope-tagged form of Jagged2 (C2C12Jagged2). RNA was analyzed by Northern blot hybridization initially by using a probe for *Notch1* RNA and then by repeatedly stripping and rehybridizing with probes for *Notch2* and *Notch3*, *Jagged1*, and GAPDH RNA. The resulting autoradiograms are shown.

signal transduction pathway. When overexpressed in C2C12 cells, both ΔE and ICNΔRAM23 increased expression of *Notch1*, *Notch3*, and *Jagged1* but not *Notch2*, mimicking the effects of Jagged2 (Fig. 8). Therefore, activation of Notch1 by Jagged2 may be sufficient to trigger positive gene regulation in C2C12 cells.

DISCUSSION

The results of our studies indicate that mammalian *Jagged2* loci likely encode functional ligands for Notch. Evidence supporting this conclusion includes the close homology of Jagged2 to *Drosophila* Serrate and rat Jagged1, both previously characterized Notch ligands. Furthermore, many tissues that express Jagged2 also express Notch1 (this study and reference 56), consistent with Notch1 being a cognate receptor for Jagged2. Perhaps most importantly, the functional effects of exposure of cultured C2C12 cells to Jagged2 can be simulated by expression of constitutively active Notch1, suggesting that Jagged2 engages the Notch1 pathway of signal transduction.

Additional circumstantial evidence for the interaction of Jagged2 and Notch1 is the appearance of the novel Notch1 polypeptide, p115, in C2C12 cells cultured together with feeder cells expressing Jagged2. Since ligand-treated cells and control cells both express a single 9.3-kb Notch1 mRNA, it seems likely that p115 is created through proteolytic processing of full-sized Notch1 (p350). The most straightforward interpretation of these observations is that proteolytic cleavage is a direct consequence of binding of Jagged2 to Notch1. However, even given the similar patterns of Jagged2 and Notch1 expression in tissues and the ability of constitutively active Notch1 to mimic effects of Jagged2, it is not absolutely certain that Jagged2 acts through Notch1 rather than through either of the other members of the Notch family of receptors or, less likely, through some pathway of signal transduction not involving any Notch protein. Accordingly, cleavage of Notch1 from p350 to p115 could theoretically occur by an indirect mechanism set in motion by Jagged2 binding to some receptor other than Notch1 and mediated by one or more intermediary factors.

The position of the cleavage site giving rise to p115 is of some interest. One proposed mechanism for Notch receptor signaling supposes that ligand binding induces an intracellular cleavage that permits nuclear translocation of the cytoplasmic

domain (29, 34), which then directly interacts with RBP-Jκ bound to DNA. However, p115 is only slightly smaller than p120, a ubiquitous form of truncated Notch1 created by ligand-independent proteolytic cleavage at a site 70 amino acids external to the transmembrane domain (3a, 4, 34). Since the molecular mass of the 70 amino acids of the extracellular domain retained in p120 is 7.44 kDa, the molecular mass of p115 suggests that it is produced by an alternative or second cleavage event just external to the transmembrane domain. Our antibodies against Notch1 do not detect endogenous C2C12 Notch1 polypeptides in situ, but Western blot analysis of cell fractions suggests that p115 remains membrane associated (42a), implying that p115 arises through cleavage in the extracellular domain.

That cleavage of Notch1 to yield p115 may play a role in activation of Notch1 by Jagged2 is still possible. Forms of Notch1 that are missing most of the extracellular domain of the protein yet remain membrane associated and extranuclear are both potent oncoproteins (48) and activators of RBP-Jκ (5). This is further supported by the equivalent ability of membrane-bound ΔE and soluble, nuclear ICNΔRAM23 (5) to produce gain-of-function phenotypes in C2C12 cells. In this regard, it may be noteworthy that the *Drosophila* mutation *kuz*, which produces Notch-like phenotypes during neurogenesis, was recently shown to result from alterations in an extracellular metalloprotease (52).

The definitive determination of *Jagged2* function in development and normal cell physiology is complicated by the existence in higher vertebrates of multiple homologous genes for both Jagged (two known genes) and Notch (four known genes) (41, 60, 64, 65). Homologous genes for these two types of proteins display overlapping but nonidentical patterns of expression among different tissues. For example, several tissues expressing high levels of Jagged2 also express abundant Jagged1 (skin epithelia, olfactory epithelia, tooth buds, and dorsal root ganglion [56]). However, Jagged2 is preferentially expressed over Jagged1 in the thymus (56). Based on observations showing that Notch1 is highly expressed in thymocytes (16, 23), that expression of constitutively active forms of Notch1 in T-cell progenitors leads to T-cell malignancy (4, 48), and that overexpression of activated Notch1 in the thymuses of transgenic mice perturbs CD4/CD8 and αβ/γδ cell fate specification (50, 62), it has been hypothesized that the Notch signaling pathway directs T-cell differentiation. Jagged2 may therefore be an important physiologic regulator of Notch1 signaling in T-cell progenitors. At the same time, it may be that Jagged2 interacts with different Notch receptors in other tissues. Further work will be needed to determine if preferred cognate ligand-receptor pairs act in separate tissues and whether different receptors generate similar or distinct downstream responses in various cell types.

Most work on functional consequences of Notch signaling has focused on the ability of this receptor to direct divergent pathways of cellular differentiation among pluripotent stem cells, a process termed lateral inhibition. These developmental fate decisions appear to be accompanied by negative regulation of genes for the ligands among groups of cells that initially coexpress Notch and Delta. In contrast, Jagged1, which belongs to the Serrate-like family of ligands, as well as Notch1 and Notch3, are apparently positively regulated by Notch activation in C2C12 cells. We hypothesize that this positive-feedback loop operates in vivo to control Notch signaling across groups of cells undergoing similar differentiation, a process that might be termed lateral reinforcement. In this situation, uniform high levels of both receptor and ligand would ensure homogeneous differentiation of cells within a tissue along a single lineage. For example, skin epithelium expresses abundant Jagged1, Jagged2, and Notch1 (this study and reference 56) and gives rise to terminally differentiated cells of a single type (keratinized squamous epithelium). Similarly, positive-feedback regulation in C2C12 cells could reflect a role in coordinating the differentiation of groups of committed myoblasts in vivo, a possibility consistent with the observation that conditional loss-of-function *Notch* mutations in *Drosophila* disrupt the terminal differentiation of committed myoblasts for which cell fate has already been specified (8).

The potential ability of activated Notch to participate in both positive- and negative-feedback loops could be explained by ligand-specific differences in the signal transduced by Notch or by cell-type-specific differences in other signaling pathways that interact with and modify the activity of Notch or downstream factors (6). Negative feedback signaling in *Drosophila* requires Notch-mediated activation of the transcription factor Su(H) (18, 55). Since Serrate can substitute for Delta in developing fly tissues where lateral inhibition operates (22), it seems likely that mammalian Serrate-like ligands will also be shown to activate Su(H)-like transcription factors, such as RBP-Jκ, in certain cell types. However, ectopically expressed Serrate and Delta produce different phenotypes in the developing fly wing (15, 32), indicating that these two ligands may produce distinct signals in some tissues. Furthermore, some cell fate decisions involving Notch in the fly appear to occur through Su(H)-independent signaling mechanisms (7, 14). Additional studies will be required to determine if positive-feedback regulation of genes in the Notch pathway and inhibition of myogenesis in C2C12 cells proceeds through RBP-Jκ or other downstream factors and how widespread this regulatory mechanism is among other types of tissues.

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