CELL STRUCTURE AND FUNCTION 25: 21–31 (2000)

Murine *Delta* Homologue, *mDelta1*, Expressed on Feeder Cells Controls Cellular Differentiation

Keigo Mizutani,[1,2] Tadashi Matsubayashi,[1,2,] Shigeru Iwase,[1,3] Takahiro S. Doi,[1,†] Kenji Kasai,[1,††] Makoto Yazaki,[2] Yoshiro Wada,[2] Toshitada Takahashi,[1] and Yuichi Obata[1,*]**

[1]*Laboratory of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusaku, Nagoya, 464-8681,* [2]*Department of Pediatrics, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuhoku, Nagoya 467-8601, and* [3]*Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedouri, Mizuhoku, Nagoya 467-8603*

ABSTRACT. **The Delta/Serrate-Notch pathway is involved in intercellular signaling that controls cell fate during the development of invertebrates and vertebrates. Delta is a prototype of Notch ligands and has been studied extensively in Drosophila. In higher vertebrates, four *Delta*/*Serrate* homologues and four *Notch* homologues have been identified. Recent studies showed that the murine *Delta* homologue, *mDelta1*, is essential in early embryogenesis. The biological activity of mammalian *Delta* and its roles in cellular differentiation, however, have remained unclear. In this study, we first surveyed expression of *mDelta1* in the adult mouse and found it to be present in a wide range of tissues. For testing biological activity of *mDelta1*, we expressed a *mDelta1* full-length cDNA in L cells using a eukaryotic expression vector. Effects of *mDelta1* on cellular differentiation were examined in two independent systems, featuring C2C12 myogenic differentiation and multipotent murine bone marrow cell differentiation. Inhibition of the former was observed with *mDelta1* expression on L cells, associated with suppression of *myogenin*, a myogenic transcription factor. Expression of *mDelta1* in conjunction with GM-CSF promoted differentiation of bone marrow cells to myeloid dendritic cells at the expense of other lineages. Although the effects of *mDelta1* on two differentiation systems appeared opposing, as inhibition occurring in one and induction in the other, this can be understood by the unifying concept of generation of diverse cell types from equivalent progenitors. Thus, the present study provided evidence that mammalian *Delta* participates in intercellular signaling, determining the cell fate in a wide variety of tissues.**

Key words: Delta/Serrate-Notch pathway/*mDelta1*/intercellular signaling/inhibition of differentiation/inductive signal

Delta and Serrate and their receptor, Notch, are highly conserved membrane proteins in invertebrates and vertebrates, thought to play key roles in cell fate decisions during development (Artavanis-Tsakonas *et al*., 1999; Greenwald, 1998; Kimble and Simpson, 1997; Lewis, 1998; Milner and Bigas, 1999; Weinmaster, 1998). Genetic and molecular studies have shown that Delta/Serrate ligands expressed on the surfaces of cells interact with and activate Notch receptors on neighboring cells. Activated Notch then translocates into nucleus and promotes the expression of sets of genes that inhibit or induce cellular differentiation. In equipotent cells, the Delta/Serrate-Notch mediates signals to effect so-called lateral inhibition in which a limited number of cells adopt the specific cell fate whereas adjacent cells are inhibited from differentiation. Delta/Serrate-Notch interactions also occur among heterotypic or multipotent cell types with inductive signaling that permits the establishment of demarcated boundaries between cell

* To whom correspondence should be addressed. E-mail: yobata@aichi-cc.pref.aichi.jp

** Present address: Department of Pediatrics, Seirei Hamamatsu General Hospital, 2-12-12 Sumiyoshi, Hamamatsu 430-8558.

† Present address: Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA.

†† Present address: Laboratory of Developmental Neurogenetics, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892-4180, USA.

Abbreviations: DSL, Delta/Serrate/Lag-2; EGF, epidermal growth factor; *neo*, *neomycin phosphotransferase II*; CMV-*IE*, cytomegalovirus *immediate early*; PCR, polymerase chain reaction; RT, reverse transcriptase; dpc, days postcoitum; I-DMEM, Iscove's Dulbecco's modified Eagle medium; FBS, fetal bovine serum.

types. Through both lateral inhibition and inductive signaling, Delta/Serrate-Notch contributes to the generation of distinct cell lineages from initially equivalent cells. Two distinct *Notch* ligands, *Delta* and *Serrate*, have been identified in *Drosophila melanogaster* (Artavanis-Tsakonas *et al*., 1999; Greenwald, 1998; Kimble and Simpson, 1997) and several homologues are now known in other species, including *Caenorhabditis elegans* (Greenwald, 1998; Kimble and Simpson, 1997), *Xenopus laevis* (Chitnis *et al.*, 1995; Jen *et al*., 1997), the chick (Hayashi *et al*., 1996; Henrique *et al*., 1995; Myat *et al*., 1996), mouse (Bettenhausen *et al*., 1995; Dunwoodie *et al*., 1997; Jiang *et al*., 1998; Luo *et al*., 1997; Mitsiadis *et al*., 1997; Sidow *et al*., 1997), rat (Lindsell *et al*., 1995; Shawber *et al*., 1996) and man (Gray *et al*., 1999; Li *et al*., 1998; Luo *et al*., 1997). *Delta, Serrate* and their homologues share a so-called DSL (Delta/Serrate/Lag-2) domain and epidermal growth factor (EGF)-like tandem repeats in the extracellular region. Both the DSL domain and the EGF-like repeats are required for interaction with Notch receptors. Structural differences between *Delta* and *Serrate* exist in the extracellular and cytoplasmic domains. In the extracellular domain, mammalian *Delta* homologues have 8 EGF-like repeats but lack a cysteine-rich region, while *Serrate* homologues have 16 EGF-like repeats and a cysteine-rich region. It has been speculated that *Delta* and *Serrate* have overlapping but distinct functions. Spatial and temporal differences in their expression during fruit fly development have also been noted (Doherty *et al*., 1996; Muskavitch, 1994). In higher vertebrates, *Jagged, Serrate* homologues, have been extensively investigated. *Jagged1* is expressed in various human adult tissues (Li *et al*., 1998) and in rat embryos (Lindsell *et al*., 1995), and *Jagged2* is expressed in mouse embryos and adult tissues (Luo *et al*., 1997; Shawber *et al*., 1996). *hJagged1*, *hJagged2* and *rJagged1* expressed in fibroblasts were found to inhibit terminal differentiation of murine C2C12 myoblasts (Lindsell *et al*., 1995; Luo *et al*., 1997). *hJagged1* expressed on HS-27a human stromal cells inhibits G-CSF-induced differentiation of murine 32D myeloid progenitor cells manipulated to express *mNotch-1* (Li *et al*., 1998). *hJagged1* transfected in 3T3-cells also promotes proliferation of murine marrow primitive precursor cells (Varnum-Finney *et al*., 1998).

In contrast to *Jagged*, only limited information is available for the functions of mammalian *Delta1*. Bettenhausen *et al.* showed the mouse *Delta* homologue, *mDelta1* (*Dll1*), to be expressed transiently between day 7 and 12.5 of embryonic development but down-regulated by day 15.5 with expression in the adult restricted to the lung and heart (Bettenhausen *et al*., 1995). Mouse embryos deficient for *mDelta1* die around 12 days postcoitum (dpc) with severe patterning defects in the paraxial mesoderm where *mDelta1* normally is expressed (de Angelis *et al*., 1997). The expression pattern of *mDelta1* suggests that *mDelta1* is involved in the development of neural tubes and somites (Bettenhausen *et al*., 1995; de Angelis *et al*., 1997). Because of embryonic lethality of *mDelta1* deficient mice, its roles in cellular differentiation and development of adult mice remain unclear. Recently, and during the course of this study, it was found that mammalian *Delta* inhibits differentiation of C2C12 cells (Kuroda *et al*., 1999). In the present study, the expression of *mDelta1* in adult mice was therefore re-examined and found to be present in various tissues including lung, brain, kidney, heart, muscle, spleen and lymph nodes. To determine the biological effects of *mDelta1* on cell differentiation, a full-length *mDelta1* cDNA was isolated and transfected into L cells. The influence of *mDelta1* expressed on L cells was then examined in two independent systems, featuring C2C12 myogenic differentiation and multipotent murine bone marrow cell differentiation.

Materials and Methods

DNA and RNA isolation

Genomic DNA was isolated from parental and transfected L cells by a proteinase K/SDS method (Blin and Stafford, 1976). Total RNA was isolated from L cells, transfected L cells, C2C12 cells and various tissues of C3H/He mice by a guanidine thiocyanate/CsCl method (Chirgwin *et al.*, 1979).

Southern blot analysis and densitometric analysis

Aliquots of 12 μg DNA digested with restriction enzymes were separated by electrophoresis in 0.7% agarose gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). A full-length *mDelta1* cDNA was labeled with ^{32}P using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) and used as a probe for hybridization. Intensity of hybridizing bands was measured by densitometric analysis using ATTO Densitograph software (Atto Inc., Tokyo, Japan).

Northern blot analysis

Aliquots of 10 μg total RNA were separated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, and transferred to nitrocellulose membranes (Lehrach *et al*., 1977). A full-length *mDelta1* cDNA and a full-length *myogenin* cDNA (Wright *et al*., 1989; a gift of Dr. Harold Weintraub, Fred Hutchinson Cancer Center, Seattle, WA, USA) were labeled with ^{32}P and used as probes for hybridization, carried out in the presence of 50% formamide at 45°C for 16 hours. The membranes were then washed three times with 2×SSC/0.2% SDS for 15 min each followed by twice with 0.2×SSC/0.2% SDS at 45°C for 15 min each.

RT-PCR analysis for expression of mDelta1, mNotch-1, 2, 3, 4 and β-actin

The forward and reverse primers for PCR amplification were designed to produce the following fragments: *mDelta1* (Genbank Accession Number X80903; nucleotide position 596 to 1008), *mNotch-1* (Z11886; 4452 to 5218), *mNotch-2* (D32210; 4881 to 5440), *mNotch-3* (X74760; 4831 to 5240), *mNotch-4* (U43691; 4061 to 4708), and β-*actin* (X03672; 505 to 942). Five μg total RNA from various tissues was reverse transcribed and amplified through 30 cycles (20 cycles in the case of β-*actin*). Amplified products were separated by electrophoresis in 1.2% agarose gels and visualized by ethidium bromide staining.

Eukaryotic expression vector

A stable eukaryotic expression vector, pCXN (Niwa *et al*., 1991), the gift of Dr. Jun-ichi Miyazaki at Osaka University School of Medicine (Osaka, Japan), has a mutant *neomycin phosphotransferase II* (*neo*) gene driven by a HSV *tk* promoter. Due to the mutation in *neo* and a weak *tk* promoter, pCXN confers only marginal resistance to neomycin, allowing selection of transfectants with a high copy number of pCXN in the presence of a high concentration of neomycin. cDNA can be cloned at the *Eco*RI or *Xho*I sites of the rabbit β-*globin* gene 3 flanking sequence which contains a Poly-A signal. Transcription of inserted cDNA is driven by a cytomegalovirus *immediate early* (CMV-*IE*) enhancer and a chicken β-*actin* promoter.

Preparation of expression vectors containing mDelta1 constructs

Based on the published *mDelta1* sequence (Bettenhausen *et al.*, 1995; Genbank Accession Number X80903), four pairs of polymerase chain reaction (PCR) primers specific for *mDelta1* were designed to obtain full-length *mDelta1* cDNA (1-2,187). Fragment 1: 5 1-30, 3 1008-989; Fragment 2: 5 596-615, 3 1630-1605; Fragment 3: 5 1321-1346, 3 2100-2081; and Fragment 4: 5 1805-1824, 3 2187-2168. Samples of 10 μg total RNA isolated from 18.5 dpc embryonic murine brain were reverse transcribed using Superscript II reverse transcriptase (RT) (Life Technology, Rockville, MD, USA) with random primers (Boehringer Mannheim). The 1st strand cDNA was amplified through 30 cycles at 94°C for 1 min, 58°C for 2 min and 72°C for 2 min in PCR buffer (Boehringer Mannheim) with Taq DNA polymerase (Boehringer Mannheim), using a Program Temp Control System PC-800 (ASTEC Co. Ltd., Fukuoka, Japan). Four RT-PCR products were digested with appropriate restriction enzymes and ligated to produce a full-length *mDelta1* cDNA. Sequencing confirmed its identity. To express *mDelta1* in mammalian cell lines, the full-length *mDelta1* cDNA was inserted into pCXN by adding *Eco*RI-linkers or modifying *Eco*RI-termini of pCXN to blunt ends. Sense or anti-sense orientation of *mDelta1* in pCXN was determined by restriction enzyme analysis.

Transfection of mDelta1 into L cells

Murine fibroblast L cells (Wigler *et al.*, 1977) were cultured in Iscove's Dulbecco's modified Eagle medium (I-DMEM, Life Technology) containing 7% fetal bovine serum (FBS) at 37°C in 5% CO_2. DNA transfection into murine L cells was performed using a calcium phosphate co-precipitation method (Wigler *et al.*, 1977). Transfected L cells were selected in the presence of the neomycin derivative G418 (Geneticin, 1,200 μg/ml, Life Technology). A total of 26 colonies originating from *mDelta1* transfected single cells were isolated and established as cell lines, 13 with *mDelta1* ends modified by *Eco*RI-linker attachment, and the remaining 13 with blunt ends. Nine of the 13 clones in each group have the sense orientation of *mDelta1*, while the other four the anti-sense orientation.

Cell labeling with fluorescent probe

In order to distinguish myoblast C2C12 cells (Yaffe and Saxel, 1977) from parental or transfected L cells, they were labeled with the PKH2 fluorescent probe (Zynaxis Cell Science, Malvern, PA, USA), according to the manufacture's protocol. Briefly, 1×10^6 of C2C12 cells were washed in phosphate-buffered saline and resuspended in the 1 ml of supplied diluent A. Then 50 μl of 40 μM PKH-2 dye was added. After 5 min incubation at room temperature, 1 ml of I-DMEM with 7% FBS was added, and then 2 ml of FBS was slowly introduced from the underlayer to stop the labeling reaction. Labeled C2C12 cells were washed three times with I-DMEM, and used for the differentiation experiments.

Myogenic differentiation experiments

L cells transfected with sense or anti-sense *mDelta1* (5×10^6 cells in 100 mm culture dish) were lethally irradiated with 4,000 rad by Hitachi MBR-1520R (Hitachi, Tokyo, Japan). C2C12 cells (8×10^5) labeled with PKH2 dye were plated on irradiated transfectants and cultured for 1 day in I-DMEM with 7% FBS at 37°C in 5% CO_2. The culture medium was changed to FBS-free I-DMEM for the induction of myogenic differentiation (Kopan *et al*., 1994). C2C12 cells were further cultured for additional 2 days and the morphological changes were examined under fluorescence and phase-contrast microscopes (Nikon, Tokyo, Japan).

Bone marrow cultures on mDelta1 transfected L cells

1×10^5 L cells transfected with sense or anti-sense *mDelta1* were plated in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA), cultured for 3 days and then lethally irradiated with 4,000 rad by Hitachi MBR-1520R. Tg.H-2K^b-1 mice (Hamasima *et al*., 1989) at 4−6 weeks of age were used as a source of bone marrow cells in order to distinguish them from transfected L cells (H-2K^k, D^k). Induction of dendritic cells from bone marrow cells was performed as described previously (Inaba *et al*., 1992) with a few minor modifications. Briefly, Thy-1$^+$ MHC Class II$^+$ B220$^+$ cells were removed before culturing with L cells by the panning method (Wysocki and Sato, 1978). 5×10^6 cells were

cultured on the transfected L cells in 6-well plates with 5 ml of RPMI 1640 (Life Technology) containing 10% FBS, 1,000 U/ml recombinant mouse GM-CSF (a gift of Kirin Brewery Company, Tokyo, Japan) and 50 μM 2-mercaptoethanol (2-ME, Wako Pure Chemical Industries, Osaka, Japan) at 37°C in 5% CO_2. The cultures were fed every two days by adding 2.5 ml of fresh medium with FBS, 2-ME, and recombinant mouse GM-CSF. At days 2, 4 and 6, cultured bone marrow cells were analyzed for their cell surface markers by flow cytometric analysis using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Bone marrow cultures on mDelta1 transfected L cells without cell-to-cell contacts

Murine bone marrow cells were cultured in the upper compartment of cell culture inserts (0.45 μm pore size) placed on sense or anti-sense *mDelta1* transfected L cells in the lower compartment of 6-well plates (Becton Dickinson Labware). At days 2, 4 and 6, cultured bone marrow cells were analyzed by a FACScan.

Bone marrow cultures in the conditioned medium of mDelta1 transfected L cells

Supernatant of 4-day culture medium of the sense or anti-sense *mDelta1* transfected L cells was used as a conditioned medium. In the medium consisting of 50% conditioned medium, 10% FBS, 1,000 U/ml GM-CSF and 50 μM 2ME, bone marrow cells were cultured on the parental L cells as described above. At days 2, 4 and 6, cultured bone marrow cells were analyzed by a FACScan.

Antibodies

The following mAbs were used: biotin-conjugated hamster mAb against CD11c (Huleatt and Lefrançois, 1995) (PharMingen, San Diego, CA, USA), FITC-conjugated rat mAbs against CD11b (Springer *et al*., 1978) (PharMingen), rat mAbs against MHC Class II (Bhattacharya *et al*., 1981) (gift of Dr. Muneo Inaba of Kansai Medical University, Osaka, Japan), CD86 (Nakajima *et al*., 1995) (gift of Dr. Hideo Yagita of Juntendo University School of Medicine, Tokyo, Japan), SER-4 (Crocker and Gordon, 1989) (gift of Dr. Muneo Inaba), F4/80 (Austyn and Gordon, 1981) (gift of Dr. Muneo Inaba), Gr-1 (Hestdal *et al*., 1991) (CALTAG Labs., South San Francisco, CA, USA), and FITC-conjugated H-2K^b (E121.46, Seikagaku Kogyo, Tokyo, Japan). For secondary reagents, streptavidin-phycoerythrin (PharMingen) or FITC-conjugated goat anti-rat IgG (Chemicon, Temecula, CA, USA) were used.

Cell death of cultured bone marrow cells

Cultured bone marrow cells were stained with annexin V and propidium iodide (Vermes *et al*., 1995) according to the manufactures protocol (Medical & Biological Labs. Co., Nagoya, Japan) and followed by FACScan analysis.

Results

Expression of mDelta1 in the mouse

Expression of *mDelta1* in the adult mouse was examined by Northern blot analysis using a full-length *mDelta1* cDNA as the probe. Hybridizing transcripts were detected not only in the lung and heart but also in the brain, kidney, muscle, spleen and lymph nodes (Fig. 1A). The transcript was 3.8 kb in size as reported in the mouse embryo (Bettenhausen *et al*., 1995) and different from transcripts of other members of *Delta/Serrate* family of the mouse, 2.2 and 5.0 kb for *Dll3* (Dunwoodie *et al*., 1997; Kusumi *et al*., 1998), 5.0 kb for *mSerrate-2* (*mJagged2*) (Luo *et al*., 1997) and 7.2 kb for *mSerrate-1* (*mJagged1*) (Matsubayashi *et al*., unpublished data). Although the amounts of *mDelta1* transcripts in testis, bone marrow, thymus and liver were below the detection level by northern blot analysis, *mDelta1* RNA was detected by RT-PCR analysis in all the tissues tested (Fig. 1B). The previous study by Bettenhausen *et al*. showed *mDelta1* transcripts to be present in embryos from 7 to 12.5 dpc, but absent at 15.5 dpc (Bettenhausen *et al*., 1995). They also showed that in the adult mouse *mDelta1* transcripts were present only in the lung and heart, but not in other tissues. In human, *H-Delta-1* expression is limited to adult heart,

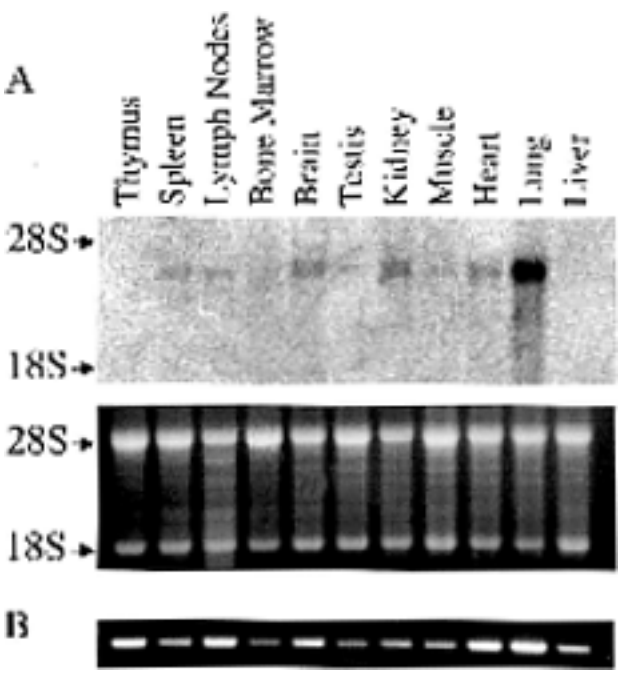

Fig. 1. Expression of *Delta1* in the mouse. RNAs prepared from various tissues of adult mice were studied for the expression of *mDelta1* by Northern blot analysis (A) and RT-PCR (B). (A) Abundant *mDelta1* transcripts are apparent in lung, kidney, brain and heart by Northern blot analysis. The bottom panel shows ethidium bromide staining of the gel. (B) RT-PCR, however, demonstrates expression of *mDelta1* in all tissues tested.

pancreas, brain and muscle (Gray *et al*., 1999). Thus, the tissue distribution of *mDelta1* in the adult mouse seems slightly different from that of *H-Delta-1*. The present study, however, showed *mDelta1* to be expressed in a wider range of tissues than originally reported, suggesting that it participates in development and differentiation at many sites.

Establishment of mDelta1 transfected L cell lines

Based on genetic studies of the fruit fly and on structural similarities among *Drosophila Delta* and mammalian *Serrate* homologues, mammalian *Delta* has been speculated to be also involved in cell fate decisions without proof of such activity. To test whether this actually the case with *mDelta1*, a full-length cDNA (2,187 bp) was obtained by RT-PCR of RNA from the 18.5 dpc embryonic brain with four sets of *mDelta1* specific primers and by ligation of the four RT-PCR products (see *Materials and Methods*). After confirming by sequencing that the cDNA was indeed *mDelta1*, it was inserted into a eukaryotic expression vector, pCXN, in which transcription of cDNA is driven by a CMV-*IE* enhancer and a chicken β-*actin* promoter. Plasmids with *mDelta1* in the sense or anti-sense orientation in relation to the enhancer and promoter were prepared and transfected into L cells by calcium phosphate precipitation. After a few weeks of selection in the culture medium containing 1,200 μg/ml G418, over a dozen L cell lines transfected with sense or anti-sense *mDelta1* were established. Southern blot and the densitometric analysis of hybridizing fragments showed the transfectants to contain 3 to 25 times the copies of endogenous *mDelta1* DNA present in the parental L cells (data not shown). Abundant expression of sense and anti-sense *mDelta1* mRNA in transfectants was confirmed by Northern blotting (Fig. 2A). The amounts were so large that it was difficult to make precise comparisons but rough estimation demonstrated it was several hundredfold more than those in the lung, which expresses the largest amounts of *mDelta1* among normal adult tissues (Fig. 2B). L cell transfectants with high expression of sense or anti-sense *mDelta1* (* in Fig. 2A) were selected for the differentiation experiments. The size of the sense *mDelta1* transcripts was 2.8 kb and slightly smaller than that of anti-sense *mDelta1*. The reason for this size difference was unclear and remains to be determined.

Suppression of myogenic differentiation by mDelta1

The effect of *mDelta1* on cellular differentiation was examined in the C2C12 murine myogenic system (Kopan *et al*., 1994; Weintraub, 1993). C2C12 cells were cultured on L cells expressing large amounts of sense or anti-sense *mDelta1*. For ease of observation, C2C12 cells were prelabeled with a fluorescent dye. When cultured alone, C2C12 cells after serum starvation change their morphology from undifferentiated growing myoblasts (Fig. 3A) to differentiated myocytes, aligned and fused with each other to form networks of multinucleated myotubes (Fig. 3B). On sense *mDelta1* transfected L cells, C2C12 cells showed a morphology similar to the undifferentiated myoblasts even after the induction of differentiation (Fig. 3C). In contrast, on L cells transfected with anti-sense *mDelta1*, C2C12 myoblasts differentiated to myocytes (Fig. 3D).

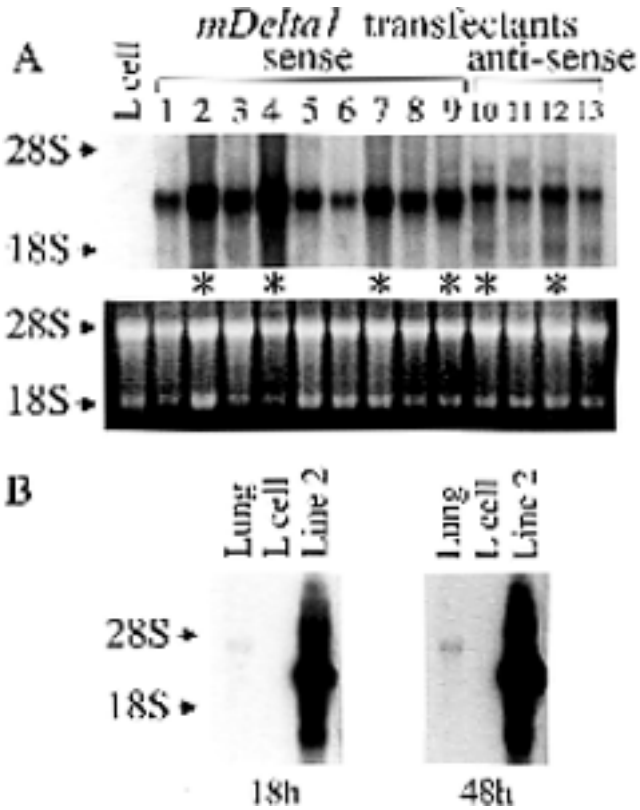

Fig. 2. Expression of *mDelta1* in transfected L cells. Northern blot analysis of *mDelta1* expression in parental and transfected L cells. (A) The ends of full-length *mDelta1* cDNA cloned into pCXN were *Eco*RI linker attached. The numbers indicate transfected cell lines. Transfectants with the sense *mDelta1* are 1-9. Transfectants with the anti-sense *mDelta1* are 10-13. Transcripts of sense *mDelta1* are about 2.8 kb and 250 bp smaller than those of anti-sense *mDelta1*. The bottom panel in (A) shows ethidium bromide staining of the gel. Asterisks indicate cell lines selected for the differentiation experiments. (B) Comparison of mDelta1 expression in transfected L cells and adult lung.

To confirm the inhibition of cell differentiation by *mDelta1*, the expression of a muscle-specific gene was examined by Northern blot analysis. It has been reported that most myoblasts in culture express *MyoD* and *Myf-5* myogenic transcription factors, but turn on the other transcription factor, *myogenin* and muscle structural genes only when induced to differentiate (Weintraub, 1993). As shown in Figure 4, C2C12 cells in the exponential growth phase did not express *myogenin* (lane 1), and after induction of differentiation by serum starvation up-regulation was observed (lane 2), correlating well with morphological

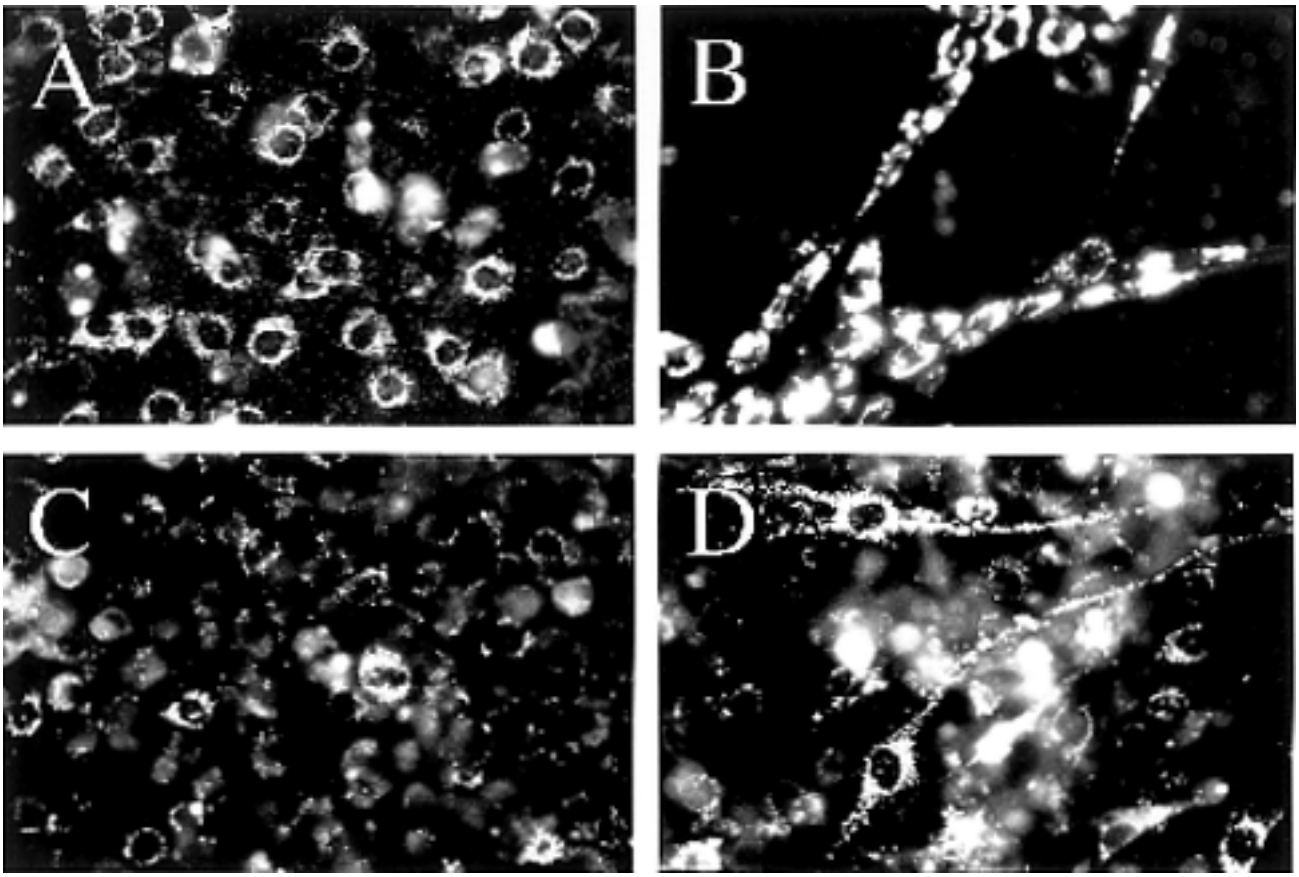

Fig. 3. Morphological changes of C2C12 cells cultured on *mDelta1* transfected L cells. C2C12 cells cultured alone are shown in (A) and (B). C2C12 cells cultured on the L cells transfected with the sense *mDelta1* are shown in (C), while those on L cells transfected with the anti-sense *mDelta1* are shown in (D). (A) C2C12 cells prior to induction of differentiation. (B)–(D), C2C12 cells after induction of differentiation by serum starvation. C2C12 cells cultured on sense *mDelta1* transfectants showed morphology similar to the undifferentiated myoblasts even after the induction of differentiation. C2C12 cells were pre-labeled with the PKH2 fluorescent dye. The photographs were taken using a fluorescence microscope at 400×.

change. C2C12 cells on anti-sense *mDelta1* transfectants also expressed *myogenin* after being induced to differentiate (Fig. 4, lanes 7, 8), but this was not the case in sense *mDelta1* transfected L cells (Fig. 4, lanes 3-6). L cells transfected with *mDelta1* themselves did not express *myogenin* (Fig. 4, lanes 9, 10). Thus, the results indicated that *mDelta1* expressed in feeder fibroblasts inhibits myogenic differentiation of C2C12 cells. These results of suppression of C2C12 differentiation by *mDelta1* were in complete agreement with recently published findings (Kuroda *et al.*, 1999).

Accelerated differentiation of myeloid dendritic cells mediated by mDelta1

To test the influence of *mDelta1* on differentiation of multipotent precursor cells, murine bone marrow cells were co-cultured with *mDelta1* transfected L cells in the presence of GM-CSF. Without feeder fibroblasts, GM-CSF promotes differentiation of bone marrow progenitors along the myeloid lineage to mature dendritic cells (Inaba *et al.*, 1992). Myeloid lineage dendritic cells derived from bone marrow express specific cell surface markers of integrin family CD11c ($\alpha_X\beta_2$) and CD11b ($\alpha_M\beta_2$). As dendritic cells mature, they upregulate expression of both MHC Class II and CD86 (B7-2) adhesion molecules (Inaba *et al.*, 1992; Inaba *et al.*, 1994). After 6 days under our culture conditions, approximately 20% were $CD11c^+CD11b^+$ myeloid lineage cells and half expressed MHC class II and CD86. The majority were positive for CD11b but negative for CD11c, MHC class II or CD86, and a proportion expressed F4/80 or SER-4, suggesting they belong to another hematopoietic lineage, for example, macrophages (data not shown). As shown in Figure 5, bone marrow cells co-cultured with *mDelta1* transfected L cells in the presence of GM-CSF readily differentiated to myeloid lineage dendritic cells. On day 2, $CD11c^+CD11b^+$ cells already comprised one third of the population and increased to become predominant on day 6. Furthermore, most $CD11c^+CD11b^+$ cells expressed both MHC Class II and

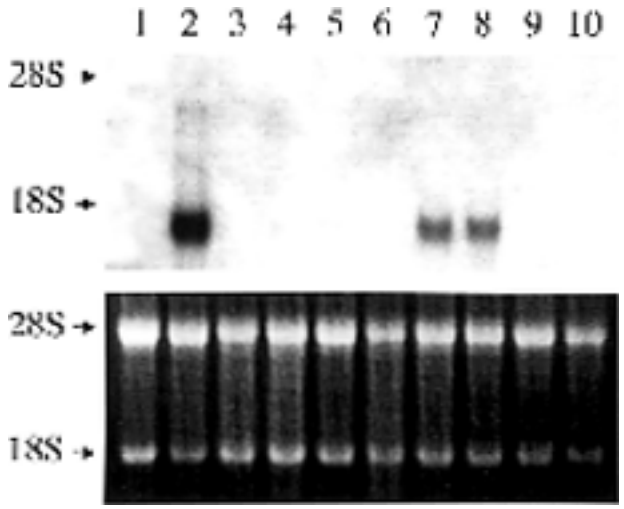

Fig. 4. Expression of *myogenin* in C2C12 cells cultured on *mDelta1* transfected L cells. Lane 1: Undifferentiated C2C12 cells. Lane 2: Differentiated C2C12 cells after serum starvation. Lanes 3-6: C2C12 cells on sense *mDelta1* transfected L cell lines (Nos. 2, 4, 7 and 9 in Fig. 2A) after induction of differentiation. Lanes 7 and 8: C2C12 cells on anti-sense *mDelta1* transfected L cell lines (Nos. 10 and 12 in Fig. 2A) after induction of differentiation. Lane 9: L cells transfected with sense *mDelta1* (No. 4 in Fig. 2A). Lane 10: L cells transfected with anti-sense *mDelta1* (No. 12 in Fig. 2A). C2C12 cells cultured on sense *mDelta1* transfectants did not express *myogenin* even after being induced to differentiate. The bottom panel shows ethidium bromide staining of the gel.

CD86, indicating that they differentiated to mature dendritic cells. Cells belonging to other lineages represented only a minor population. In contrast, with anti-sense *mDelta1* transfected L cells, much fewer bone marrow cells differentiated to $CD11c^+CD11b^+$ myeloid dendritic cells. A population of $SER\text{-}4^+$ or $F4/80^+$ cells was prominent in bone marrow cells cultured for 6 days on the anti-sense *mDelta1* transfected L cells while such cells were absent with sense *mDelta1* transfected L cells (data not shown). Bone marrow cells on anti-sense *mDelta1* transfected L cells differentiated slightly more efficient than those without any feeder cells, probably due to their production of cytokines/growth factors. Thus, *mDelta1* in conjunction with GM-CSF activity promotes differentiation of myeloid dendritic cells at the expense of other lineages.

To test whether the action of *mDelta1* on dendritic cell differentiation requires the cell-to-cell contacts, a microporous membrane was inserted between bone marrow cells and L cell transfectants. Without direct contact, the rate of dendritic cell differentiation was not different whether bone marrow cells were cultured with sense *mDelta1* transfectants or with anti-sense *mDelta1* transfectants (data not shown). The results indicated that accelerated differentiation of myeloid dendritic cells by sense *mDelta1* transfected L cells was mediated by direct cell-to-cell contact, but not by a soluble protein(s) produced by the sense *mDelta1* transfected L cells. To further confirm this, the conditioned medium of sense or of anti-sense *mDelta1* transfected L cells was added to the culture of bone marrow cells with parental L cells. No difference in the dendritic cell differentiation was observed between the two conditioned media (data not shown), indicating that no differentiation promoting factors were produced specifically by *mDelta1* transfectants. Thus, these results showed that direct interaction of *mDelta1* expressing L cells with the receptor expressing bone marrow cells was necessary to promote dendritic cell differentiation.

To examine the possibility that *mDelta1* influences cell survival, i.e., preferentially protects myeloid/dendritic cells from cell death or promotes cell death of other lineages, cultured bone marrow cells were examined by staining with annexin V-FITC and propidium iodide. No differences in total viable cell numbers or in the mode of cell death (apoptosis/necrosis) were observed between bone marrow cells cultured on sense *mDelta1* transfected L cells and those on anti-sense *mDelta1* transfected L cells (data not shown). Thus, the preferential expansion of myeloid dendritic cells is the result of direct action of *mDelta1* on the differentiation rather than an indirect influence on cell death.

Expression of Notch receptors in C2C12 and bone marrow cells

In the present study, *mDelta1* inhibited C2C12 myogenic differentiation or induced myeloid dendritic cell differentiation without engineered over-expression of Notch receptors, unlike in the previous report (Lindsell *et al.*, 1995). Thus, natural receptors for mDelta1 must exist on the cell surfaces of C2C12 and bone marrow cells. Members of the Notch family are most likely candidates, although there is a possibility that other receptor molecules may mediate signals from mDelta1. Assessment of expression of *Notch* family members by RT-PCR demonstrated that C2C12 cells expressed *mNotch-1*, *mNotch-2*, *mNotch-3*, but not *mNotch-4* and that bone marrow cells expressed all four members (data not shown). We are now producing antibodies specific to mNotch-1 to allow determination of whether it is responsible for affected differentiation of C2C12 and of bone marrow cells by interacting with mDelta1.

Discussion

Our results clearly demonstrated that *mDelta1* can influence the differentiation of target cells by inhibiting or promoting differentiation depending on the system. Almost all previous experiments using cultured cells have resulted in inhibition of differentiation by Delta/Serrate-Notch signaling, as we demonstrated here for the C2C12 myogenic system. During development, Delta/Serrate-Notch mediates not only lateral inhibition but also inductive signals, both of which are important to generate distinct sets of tissues and

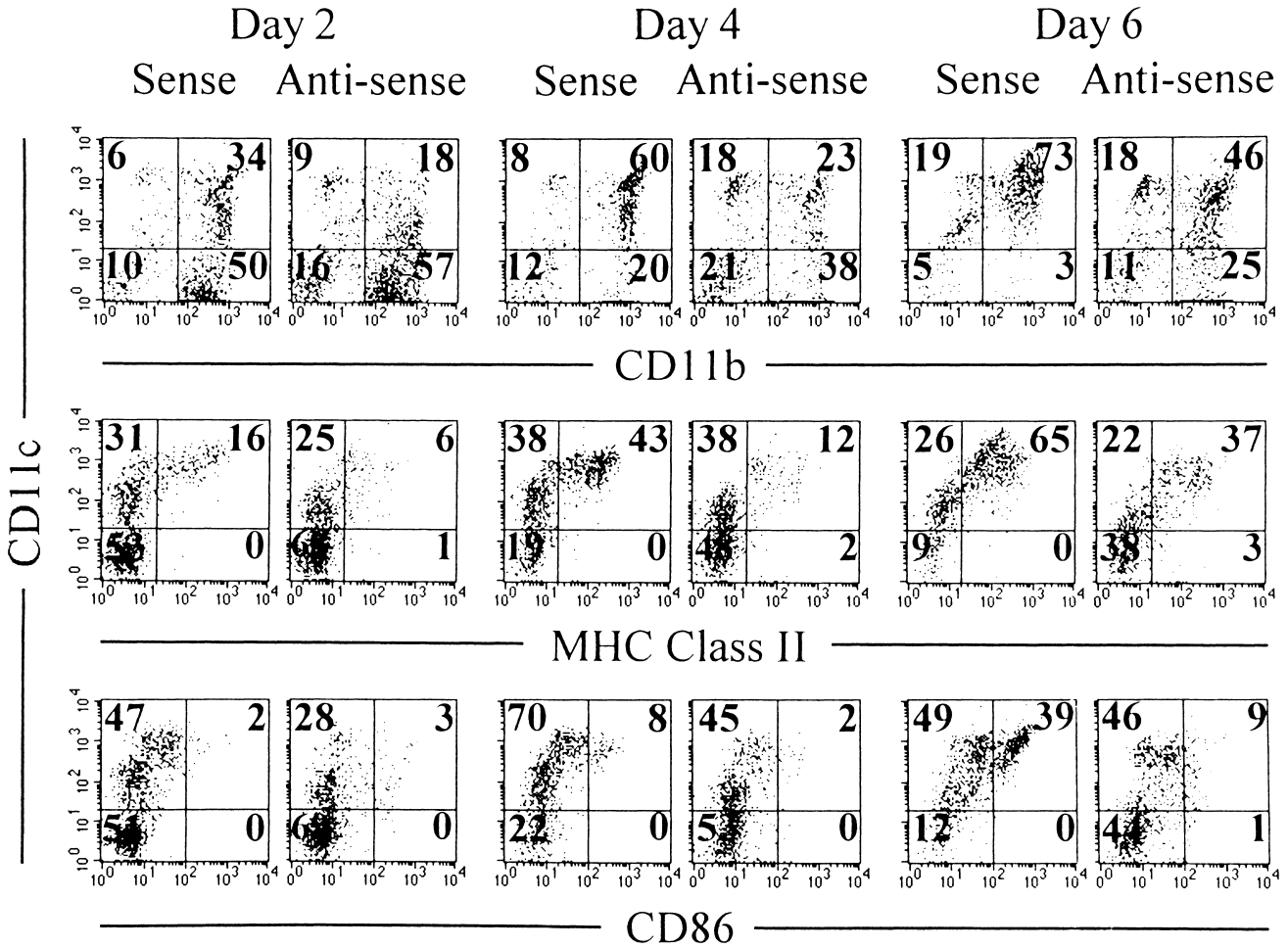

Fig. 5. Flow cytometric analysis of the bone marrow cells cultured on *mDelta1* transfected L cells in the presence of GM-CSF. FACS profile of the bone marrow cells cultured on the L cells transfected with sense or anti-sense *mDelta1* stained with anti-CD11c, anti-CD11b, anti-MHC Class II, and anti-CD86 antibodies. The analysis was performed at days 2, 4 and 6 of culture.

cell types from initially equivalent cells. The inductive signals of the Delta/Serrate-Notch pathway play a role in formation of demarcated tissue boundaries as in vertebrate limb development (Gaunt, 1997) and dorsal-ventral formation during fly wing margin specification (de Celis *et al.*, 1996). Another example of inductive signals *in vivo* is the increase in CD8 T cells and corresponding decrease in CD4 T cells in the T cell lineage of transgenic mice with activated *mNotch1* (Robey *et al.*, 1996). However, no *in vitro* culture systems for inductive signals of Delta/Serrate-Notch have been reported. The most likely explanation of the preferential differentiation of myeloid dendritic cells by *mDelta1* demonstrated in this study is that while *mDelta1* inhibits cellular differentiation of multipotent bone marrow cells, the targets remain sensitive to GM-CSF and differentiate to myeloid dendritic cells. This can be considered as an example of inductive signaling. Delta/Serrate-Notch effects are undoubtedly exerted in cooperation with other molecules. Molecules involved in lateral inhibition have been actively investigated (Artavanis-Tsakonas *et al.*, 1999; Greenwald, 1998; Kimble and Simpson, 1997; Kuroda *et al.*, 1999; Weinmaster, 1998), while those in inductive signals have remained unresolved. Molecular dissection of effects of *mDelta1* on myeloid dendritic cell differentiation from multipotent bone marrow cells in the presence of GM-CSF should yield valuable information not only on inductive signals of the Delta/Serrate-Notch pathway but also on myeloid differentiation.

In the present study, the degree of *mDelta1* expression in transfected L cell clones was, however, extremely high, far above physiological levels. Derivation of mice that lack *mDelta1* only in targeted tissues and generation of an antibody that inhibits activity of mDelta1 should facilitate studies on defining the physiological functions of mDelta1 in development and cellular differentiation. Similar biological activity has been described for *rJagged1* (Lindsell *et al.*, 1995), *hJagged1* and *hJagged2* (Luo *et al.*,

1997). Despite of the presence of gene products with apparently overlapping functions, *mDelta1* deficient mice demonstrate embryonic lethality indicating that *mDelta1* is essential for the development of vital organs or tissues (de Angelis *et al*., 1997). It has been suggested that differential temporal-spatial expression of each Delta/Serrate ligand determines their specific roles in development (Doherty *et al*., 1996; Kimble and Simpson, 1997; Muskavitch, 1994). Furthermore, overlapping but distinct function of each member of *Notch* family has been suggested by the embryonic lethality of *mNotch-1* deficient mice (Swiatek *et al*., 1994). Thus, differential expression of Notch receptors and variation in their affinity for Delta/Serrate ligands could also contribute to specificity of action.

We are now in the process of testing whether *mDelta1* has effects on other differentiation systems. It is of particular interest to test whether hematopoietic stem cells can be maintained in a multipotential state by culturing with *mDelta1* transfected L cells. It has been demonstrated that *hJagged1* influences development of hematopoietic precursor cells, and the possibility of *ex vivo* expansion or maintenance of stem cells was suggested (Jones *et al*., 1998; Varnum-Finney *et al*., 1998).

Abnormalities in the Delta/Serrate-Notch pathway have been reported to be involved in pathogenesis of several diseases. Developmental abnormalities caused by mutations in the Delta/Serrate-Notch pathway in humans and mice have been described. Mutations in *hJgged1* cause an autosomal dominant developmental disorder, known as the Alagille syndrome, one of the most common genetic causes of chronic liver diseases in childhood (Li *et al*., 1997; Oda *et al*., 1997). Mutations in *hNotch-3* have similarly been causally implicated in a hereditary adult-onset condition causing stroke and dementia known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Joutel *et al*., 1996). A mutation in *Dll3*, the other divergent *Delta* homologue in the mouse, is responsible for a mouse mutant *Pudgy* with patterning defects during embryogenesis and severe vertebral and rib deformities in the adult mice (Kusumi *et al*., 1998). A spontaneous mutation (*syndactylism*) and targeted disruption of *mSerrate-2* (*mJagged2*) result in abnormal development of limb and T-cells (Jiang *et al*., 1998; Sidow *et al*., 1997). In addition to developmental disorders, it is conceivable that abnormal activation of Delta/Serrate or Notch, under certain circumstances, leads to cell proliferation rather than terminal differentiation, ultimately resulting in neoplasia. The first mammalian gene found for this pathway, a human *Notch* homologue *TAN-1* (*hNotch-1*), was in fact identified during a study of a t(7;9)(q34;q34.3) reciprocal translocation in acute T lymphoblastic leukemia, resulting in production of an activated form of truncated Notch (Ellisen *et al*., 1991). Up-regulation of *hNotch-1*, *hNotch-2*, *hJagged1* and *H-Delta-1* expression has been reported in cervical neoplasms (Gray *et al*., 1999; Zagouras *et al*., 1995). In addition, the *int-3* identified as an oncogene activated by insertion of a mouse mammary tumor virus has been found to be a member of the *Notch* family and designated as *mNotch-4* (Jhappan *et al*., 1992). *H-Delta-1* and *mDelta1* have been mapped to human chromosome 6q27 (Gray *et al*., 1999) and the mouse chromosome 17 *t*-complex (Bettenhausen *et al*., 1995), respectively. It is clearly of interest to survey whether causal genes for any inherited disorders or cancer of man and the mouse are located at these loci.

Acknowledgements. We thank Dr. Kazunari Yokoyama at the Institute of Physical and Chemical Research (RIKEN, Tsukuba, Japan) for cDNA probes for myogenic differentiation. We also thank Dr. Malcolm A. Moore for his assistance in English editing. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas for Cancer Research from the Ministry of Education, Science, and Sports and Culture, Japan and by a Bristol-Myers Squibb Biomedical Grant.

References

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. 1999. Notch signaling: cell fate control and signal integration in development. *Science*, **284**: 770–776.

Austyn, J.M. and Gordon, S. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.*, **11**: 805–815.

Bettenhausen, B., de Angelis, M.H., Simon, D., Gunet, J-L., and Gossler, A. 1995. Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely related to *Drosophila Delta*. *Development*, **121**: 2407–2418.

Bhattacharya, A., Dorf, M.E., and Springer, T.A. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.*, **127**:2488–2495.

Blin, N. and Stafford, D.W. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.*, **3**: 2303–2308.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, **18**: 5294–5299.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. 1995. Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature*, **375**: 761–766.

Crocker, P.R. and Gordon, S. 1989. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J. Exp. Med.*, **169**: 1333–1346.

de Angelis, M.H., McIntyre II, J., and Gossler, A. 1997. Maintenance of somite borders in mice requires the *Delta* homologue *Dll1*. *Nature*, **386**: 717–721.

de Celis, J.F., Garcia-Bellido, A., and Bray, S.J. 1996. Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. *Development*, **122**: 359–369.

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N. 1996. Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. *Genes & Dev.*, **10**: 421–434.

Dunwoodie, S.L., Henrique, D., Harrison, S.M., and Beddington, R.S.P. 1997. Mouse *Dll3*: a novel divergent *Delta* gene which may complement

the function of other *Delta* homologues during early pattern formation in the mouse embryo. *Development*, **124**: 3065–3076.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., and Sklar, J. 1991. *TAN-1*, the human homolog of the Drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell*, **66**: 649–661.

Gaunt, S.J. 1997. Chick limbs, fly wings and homology at the fringe. *Nature*, **386**: 324–325.

Gray, G.E., Mann, R.S., Mitsiadis, E., Henrique, D., Carcangiu, M-L., Banks, A., Leiman, J., Ward, D., Ish-Horowitz, D., and Artavanis-Tsakonas, S. Human ligands of the Notch receptor. 1999. *Am. J. Pathol.*, **154**: 785–794.

Greenwald, I. 1998. LIN-12/Notch signaling: lessons from worms and flies. *Genes & Dev.*, **12**: 1751–1762.

Hamasima, N., Takahashi, T., Taguchi, O., Nishizuka, Y., Stockert, E., Old, L.J., and Obata, Y. 1989. Expression of TL, H-2, and chimeric H-2/TL genes in transgenic mice: Abnormal thymic differentiation and T-cell lymphomas in a TL transgenic strain. *Proc. Natl. Acad. Sci. USA*, **86**: 7995–7999.

Hayashi, H., Mochii, M., Kodama, R., Hamada, Y., Mizuno, N., Eguchi, G., and Tachi, C. 1996. Isolation of a novel chick homolog of *Serrate* and its coexpression with *C-Notch-1* in chick development. *Int. J. Dev. Biol.*, **40**: 1089–1096.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. 1995. Expression of a *Delta* homologue in prospective neurons in the chick. *Nature*, **375**: 787–790.

Hestdal, K., Ruscetti, F.W., Ihle, J.N., Jacobsen, S.E.W., Dubois, C.M., Kopp, W.C., Longo, D.L., and Keller, J.R. 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J. Immunol.*, **147**: 22–28.

Huleatt, J.W. and Lefranois, L. 1995. Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and $CD8^+$ T cells *in vivo*. *J. Immunol.*, **154**: 5684–5693.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.*, **176**: 1693–702.

Inaba, K., Witmer-Pack, M., Inaba, M., Hathcock, K.S., Sakuta, H., Azuma, M., Yagita, H., Okumura, K., Linsley, P.S., Ikehara, S., Muramatsu, S., Hodes, R.J., and Steinman, R.M. 1994. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation *in vitro*. *J. Exp. Med.*, **180**: 1849–1860.

Jen, W-C., Wettstein, D., Turner, D., Chitnis, A., and Kintner, C. 1997. The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in *Xenopus* embryos. *Development*, **124**: 1169–1178.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G., and Callahan, R. 1992. Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes & Dev.*, **6**: 345–355.

Jiang, R., Lan, Y., Chapman, H.D., Shawber, C., Norton, C.R., Serreze, D.V., Weinmaster, G., and Gridley, T. 1998. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes & Dev.*, **12**: 1046–1057.

Jones, P., May, G., Healy, L., Brown, J., Hoyne, G., Delassus, S., and Enver, T. 1998. Stromal expression of Jagged 1 promotes colony formation by fetal hematopoietic progenitor cells. *Blood*, **92**: 1505–1511.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cécillion, M., Maréchal, E., Maciazek, J., Vayssière, C., Cruaud, C., Cabanis, E-A., Ruchoux, M.M., Weissenbach, J., Bach, J.F., Bousser, M.G., and Tournier-Lasserve, E. 1996. *Notch3* mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature*, **383**: 707–710.

Kimble, J. and Simpson, P. 1997. The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol*., **13**: 333–361.

Kopan, R., Nye, J.S., and Weintraub, H. 1994. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development*, **120**: 2385–2396.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. 1999. Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J. Biol. Chem.*, **274**: 7238–7244.

Kusumi, K., Sun, E.S., Kerrebrock, A.W., Bronson, R.T., Chi, D-C., Bulotsky, M.S., Spencer, J.B., Birren, B.W., Frankel, W.N., and Lander, E.S. 1998. The mouse pudgy mutation disrupts *Delta* homologue *Dll3* and initiation of early somite boundaries. *Nat. Genet.*, **19**: 274–278.

Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry*, **16**: 4743–4751.

Lewis, J. 1998. Notch signalling and the control of cell fate choices in vertebrates. *Semin. Cell Dev. Biol*., **9**: 583–589.

Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., Costa, T., Pierpont, M.E.M., Rand, E.B., Piccoli, D.A., Hood, L., and Spinner, N.B. 1997. Alagille syndrome is caused by mutations in human *Jagged1*, which encodes a ligand for Notch1. *Nat. Genet.*, **16**: 243–251.

Li, L., Milner, L.A., Deng, Y., Iwata, M., Banta, A., Graf, L., Marcovina, S. Friedman, C., Trask, B.J., Hood, L., and Torok-Storb, B. 1998. The human homolog of rat *Jagged1* expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity*, **8**: 43–55.

Lindsell, C.E., Shawber, C.J., Boulter, J., and Weinmaster, G. 1995. Jagged: a mammalian ligand that activates Notch1. *Cell*, **80**: 909–917.

Luo, B., Aster, J.C., Hasserjian, R.P., Kuo, F., and Sklar, J. 1997. Isolation and functional analysis of a cDNA for human *Jagged2*, a gene encoding a ligand for the Notch1 receptor. *Mol. Cell. Biol.*, **17**: 6057–6067.

Milner, L.A. and Bigas, A. 1999. Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood*, **93**: 2431–2448.

Mitsiadis, T.A., Henrique, D., Thesleff, I., and Lendahl, U. 1997. Mouse *Serrate-1* (*Jagged-1*): expression in the developing tooth is regulated by epithelial-mesenchymal interactions and fibroblast growth factor-4. *Development*, **124**: 1473–1483.

Muskavitch, M.A.T. 1994. Delta-Notch signaling and *Drosophila* cell fate choice. *Dev. Biol.*, **166**: 415–430.

Myat, A., Henrique, D., Ish-Horowicz, D., and Lewis, J. 1996. A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. *Dev. Biol.*, **174**: 233–247.

Nakajima, A., Azuma, M., Kodera, S., Nuriya, S., Terashi, A., Abe, M., Hirose, S., Shirai, T., Yagita, H., and Okumura, K. 1995. Preferential dependence of autoantibody producion in murine lupus on CD86 costimulatory molecule. *Eur. J. Immunol*., **25**: 3060–3069.

Niwa, H., Yamamura, K., and Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**: 193–200.

Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S., and Chandrasekharappa, S.C. 1997. Mutations in the human *Jagged1* gene are responsible for Alagille syndrome. *Nat. Genet.*, **16**: 235–242.

Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. 1996. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell*, **87**: 483–492.

Shawber, C., Boulter, J., Lindsell, C.E., and Weinmaster, G. 1996. *Jagged2*: a Serrate-like gene expressed during rat embryogenesis. *Dev. Biol.*, **180**: 370–376.

Sidow, A., Bulotsky, M.S., Kerrebrock, A.W., Bronson, R.T., Daly, M.J., Reeve, M.P., Hawkins, T.L., Birren, B.W., Jaenisch, R., and Lander, E.S. 1997. *Serrate2* is disrupted in the mouse limb-development mutant *syndactylism*. *Nature*, **389**: 722–725.

Springer, T., Galfrè, G., Secher, D.S., and Milstein, C. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.*, **8**: 539–551.

Swiatek, P.J., Lindsell, C.E., del Amo, F.F., Weinmaster, G., and Gridley, T. 1994. *Notch1* is essential for postimplantation development in mice. *Genes & Dev.*, **8**: 707–719.

Varnum-Finney, B., Purton, L.E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K.A., Le Roux, I., Mann, R., Gray, G., Artavanis-Tsakonas, S., and Bernstein, I.D. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood*, **91**: 4084–4091.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, **184**: 39–51.

Weinmaster, G. 1998. Notch signaling: direct or what? *Curr. Opin. Genet. Dev.*, **8**: 436–442.

Weintraub, H. 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell*, **75**: 1241–1244.

Wigler, M., Silverstein, S., Lee, L-S., Pellicer, A., Cheng, Y., and Axel, R. 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell*, **11**: 223–232.

Wright, W.E., Sassoon, D.A., and Lin, V.K. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell*, **56**: 607–617.

Wysocki, L.J. and Sato, V.L. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA*, **75**: 2844–2848.

Yaffe, D., and Saxel, O. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*, **270**: 725–727.

Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L., and Artavanis-Tsakonas, S. 1995. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc. Natl. Acad. Sci. USA*, **92**: 6414–6418.

(*Received for publication, October 1, 1999*

and in revised form, November 26, 1999)