The Expression of the Notch Receptors,
Notch Ligands, and the Fringe Genes in Hematopoiesis

by

Nita Singh

*A thesis submitted in conformity with the requirements
for the degree of M.Sc.
Graduate Department of Immunology
University of Toronto*

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0-612-29262-2

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Nita Singh
M.Sc., 1997
Department of Immunology, University of Toronto

Abstract

Hematopoiesis is a complex process of self-renewal and differentiation in which totipotent stem cells give rise to all the mature blood cell types throughout the lifetime of an organism. How these cells undergo fate selection, however, is not fully understood.

The Notch signalling system is known to mediate cell-fate decisions of multi-potent precursors in a wide range of tissues of all organisms throughout development. Thus, we wanted to determine if Notch also plays a role in hematopoiesis. Described here is an analysis of the expression of the Notch receptors, Notch ligands, and the Fringe genes in cells of the blood system.

The expression of the four Notch receptors, Dll1 and mJagged-1 (ligands for Notch), and the Fringe genes (regulators of Notch:ligand binding) was tested by RT-PCR in adult mouse whole bone marrow. All of these genes were found to be expressed in whole bone marrow.

The expression of transcript for mNotch1, mNotch3, mNotch4, Dll1, mJagged-1, Manic Fringe, and Radical Fringe was examined on a lineage precursor blot containing amplified cDNAs from a hierarchy of hematopoietic precursors, maturing blood cell populations, and three fibroblast cell lines. Distinct transcript expression patterns were found for mJagged-1 and Manic Fringe.

To be able to perform functional experiments the cDNA for mJagged-1, not described in literature, was cloned.

Acknowledgements

I would like to thank Dr. Robert A. Phillips and Dr. Sean E. Egan for their supervision, advice, and support. Their wealth of knowledge and love of science will always be inspiring.

I thank Dr. Norman Iscove for providing the lineage blots, and helpful discussions.

I would also like to thank all the members of the Phillips/Gallie/Egan lab for all their help, advice, and ‘intriguing’ conversations. I especially thank Brenda Cohen and Dr. David Ryan for ‘showing me the ropes’, and answering my endless questions.

I thank my family for all their love and support.

Finally, I especially thank Steve Martin for alternately being my technician, graffiti artist, graphics designer, editor, supporter, and friend.

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List of Abbreviations

BFU-E - burst forming unit - erythrocyte
BHIB - brain heart infusion broth
bHLH - basic helix loop helix
BM - bone marrow
cDNA - complementary DNA
CFU-S - colony forming unit-spleen
CFU-E - colony forming unit-erythrocyte
CFU-GM - colony forming unit-granulocyte/macrophage
CFU-meg - colony forming unit-megakaryocyte
CFU-Eo - colony forming unit-eosinophil
CFU-GEMM - colony forming unit granulocyte/erythrocyte/macrophage/megakaryocyte
CNS - central nervous system
Dll1 - mouse Delta-like1
DNA - deoxyribonucleic acid
E(spl) - Enhancer of Split complex
EGF - epidermal growth factor
Epo - erythropoietin
FBS - fetal bovine serum
G-CSF - granulocyte colony stimulating factor
GM-CSF - granulocyte/macrophage colony stimulating factor
HIM - hematopoietic inductive model
IL - interleukin
kb - kilobase
KL - kit ligand
M-CSF - macrophage colony stimulating factor
mRNA - messenger RNA
MTN Blot - multiple tissue Northern Blot
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PNS - peripheral nervous system
Rh - rhodamine
RNA - ribonucleic acid
RT-PCR - reverse transcription PCR
SDS - sodium dodecyl sulphate
SF - Steel factor
Su(H) - Suppressor of Hairless
TGF β - transforming growth factor β
TNF α - tumour necrosis factor α
UTR - untranslated region

1.1 The Blood System

The circulating blood cell population consists of at least nine major cell types: erythrocytes, platelets, B lymphocytes, T lymphocytes, natural killer cells, monocytes, neutrophils, eosinophils, and basophils. These cells are critical in the maintenance of homeostasis, blood clotting, and immune function. The lifetime of blood cells ranges from a few days to several years and the turnover rate for particular cell types can be quite high. For example an adult mouse requires the production of 3×10^8 erythrocytes and 3×10^7 B lymphocytes per day. With such a high turnover rate for particular blood cells and the critical function of the blood system, it is vital that an organism be able to maintain a complete population of mature blood cells throughout its lifetime and be able to regulate the production of different blood cell types as each is required.

Hematopoiesis is the process by which the mature blood cell types are generated. Numerous studies have investigated the process of hematopoiesis in an effort to understand the origin of the different cell types, and the process of differentiation. These studies have begun to elucidate the mechanisms of the blood forming system.

1.2 In Search of the Stem Cell

Ford and his colleagues in the late 1950's performed a series of hallmark experiments in the field of hematopoiesis (Ford et al, 1956; Micklem et al, 1966). These investigators demonstrated that the blood system of an irradiated mouse could be fully reconstituted by the transplantation of syngeneic bone marrow derived donor cells. This finding suggested that bone marrow was the source of all myeloid and lymphoid cells. This discovery led to the concept of the hematopoietic stem cell. It was hypothesized that there existed a stem cell that was able to differentiate into cells of different lineages, and that this cell must be capable of extensive self-renewal.

A second influential experiment was performed by Till and McCulloch in the early 1960's (Till et al, 1961). These investigators demonstrated that the transfer of bone marrow cells into lethally irradiated mice resulted in the formation of nodules in the spleen containing a colony of hematopoietic cells between 7-14 days after transplantation. The spleen colonies were found to contain cells of the myeloid lineage. Further studies demonstrated that each colony was clonal, in that each colony arose from a single precursor cell (Becker et al, 1963). This precursor cell was termed a CFU-S (colony forming unit-spleen). The CFU-S cell was found to have self-renewal ability, since the transplantation of a spleen colony into a secondary recipient also resulted in the formation of secondary spleen colonies (Siminovitch et al, 1963).

The CFU-S assay was initially considered to be an assay for the most primitive stem cell. However, lymphocytes were not detected in the nodules. Furthermore, it was found that spleen colonies were unable to reconstitute the blood system of an irradiated mouse permanently (Lala et al, 1978; Paige et al, 1979; Kitamura et al, 1981). A few studies have shown that the

re-transplantation of spleen colonies into lethally irradiated recipients could generate B and T cells, however it was not absolutely certain whether these cells were derived from the CFU-S or another precursor (Lala et al, 1978; Lepault et al, 1993).

Additional studies proved the existence of a primitive cell that gives rise to the CFU-S, but also contributes to the lymphoid lineages (Micklem et al, 1966; Wu et al, 1967; Wu et al, 1968). Subsequently, studies by a number of groups have also shown the existence of such a primitive cell (reviewed in Lemischka, 1991; Phillips, 1991; Lemischka, 1992). These experiments used various techniques to 'track' donor cells before transplantation into lethally irradiated mice or mutant mice with hematopoietic deficiencies such that the marker could be tracked in the hematopoietic tissue of the recipient. Three techniques were used in these studies to identify cells: radiation to induce chromosome translocations in the donor cells (Wu et al, 1967; Wu et al 1968; Abramson et al, 1977); retroviruses to introduce integrants into the donor cells (Keller, 1985; Dick, 1985; Snodgrass, 1987; Lemischka, 1986); and competitive re-population in which the donor cell population consisted of a constant number of bone marrow cells mixed with a varying number of a test population of cells, where isoenzymes and hemoglobin variants were used as the markers (Harrison, 1980).

By using individually 'marked' cells, the self-renewal and the differentiation potential of this cell could be indirectly measured. In general, each of the mature blood cell populations was sampled as a function of time and then assayed for the presence of the marker. If the marker was present in a particular population and was present in the long-term, one could conclude that the initially marked cell was capable of extensive self-renewal and subsequent proliferation. The distribution of the marker in the different lineages would determine the differentiative potential of the initially marked cell.

Long-term reconstitution using 'marked' cells demonstrated in some studies that the reconstituted hematopoietic system was oligoclonal, sometimes monoclonal, with the long-term reconstituting cells being totipotent (Capel et al, 1989; Jordan et al, 1990). The same stem cells providing long-term reconstitution in the primary lethally irradiated mouse, could also reconstitute a second lethally irradiated mouse. Thus, these series of experiments suggested the existence of a totipotent stem cell that was capable of extensive self-renewal, and capable of stably contributing long-term to hematopoietic tissue.

1.3 Development of the Hematopoietic Lineages

Hematopoietic stem cells must possess the following characteristics: 1) they must be able to self-renew; 2) they must have extensive proliferative ability; 3) they must be able to differentiate into cells of varying lineages; and 4) they must respond to regulatory signals to maintain a constant population of mature cells (Siminovitch et al, 1963).

Figure 1 outlines the basic differentiation pathway of the pluripotent stem cell. The differentiation of a pluripotent stem cell into a mature blood cell is a multi-step process requiring regulation at each step. The first stage at which regulation is required is at the level of the stem cell. Most stem cells have been found to be quiescent (Hodgson et al, 1979; Lerner et al, 1990). In the steady state most stem cells are in G_0 of the cell cycle: only a few stem cells supply differentiated cells at a given time. Thus, a dormant stem cell has several options: 1) to remain in G_0 ; 2) to enter the cell cycle and self-renew with the resulting daughter cells returning to the dormant state; or 3) to enter the cell cycle and differentiate. Once a stem cell becomes committed

Figure 1: Hematopoietic Lineages

Fig.1 Hematopoietic Lineages. The figure shows a model for development of the hematopoietic lineages from pluripotent stem cells. Committed progenitors are termed: CFU-S (colony forming unit-spleen), -E (erythrocyte), -GM (granulocyte/macrophage), -meg (megakaryocyte), -Eo (eosinophil). Taken from Phillips, 1991.

to differentiation its self-renewal ability decreases. In fact, as a cell progresses through a differentiation pathway, its self-renewal capacity continuously decreases while its proliferative capacity increases.

It is critical that the fate of stem cells be controlled. For example, if all the stem cells differentiate, then there will be no stem cells remaining to provide mature blood cells at later times. Conversely, if all the stem cells self-renew there will be an excess of stem cells with no mature blood cells. The control of proliferation and differentiation is also important through the differentiation pathway.

The purpose of my research is to understand the molecular basis underlying the fate of hematopoietic cells. We would like to identify factors important in cell-fate decisions that determine whether a cell remains dormant, whether a cell self-renews, and finally, whether and how a cell differentiates.

1.4 Models of Hematopoiesis

There are two predominant models of hematopoiesis: the stochastic model, and the hematopoietic inductive model (Till et al, 1980). The stochastic model states that various microenvironmental signals only regulate the proliferative state of the cell. Once a cell exits G_0 , however, the decision to self-renew or differentiate is random. The hematopoietic inductive model (HIM) suggests that the microenvironment and various signals received by hematopoietic cells regulate the decision to self-renew or differentiate. Accumulated evidence suggests that some aspects of both hypotheses may be correct.

A study of CFU-Ss demonstrated that the re-transplantation of spleen colonies can generate a wide variation in the number of spleen colonies in secondary recipients (Siminovitch et al, 1963). This finding suggested that the extent of self-renewal varies between individual spleen colonies. A similar study looked at CFU-GEMM cells (colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte), a cell that overlaps with the in vivo CFU-S (Nakahata et al, 1982). Analysis of colonies generated in vitro has revealed that the decision to enter the cell cycle is not uniform. Furthermore, in vitro colonies formed from paired progenitors (two daughter cells derived from a single parent cell), were analyzed, and it was found that the type and number of lineages varied (Suda et al, 1984). These studies have revealed that the decision to self-renew or commit is at least partially stochastic.

The hematopoietic microenvironment can also regulate self-renewal and commitment decisions. Considering the high degree of regulation that is required to ensure that proliferation is controlled, that stem cells do not become extinguished, and that the systemic demands of the organism are met, it is not surprising that the microenvironment should control these decisions.

Adult hematopoiesis occurs mostly in the bone marrow of the sternum, vertebrae, iliac bones, and ribs, in the spaces between the bony trabeculae (reviewed in Lichtman, 1981). The spaces are lined with endothelial cells which form a complete inner lining, and adventitial reticular cells (the principal fibroblast-like cell in the marrow) which form an incomplete outer coat. A thin basement membrane is present between the cell layers. Endothelial cells and reticular cells together comprise the stroma of the bone marrow (though bone marrow also contains fat cells, and tissue macrophages). The reticular cells synthesize reticular fibers, which along with their cytoplasmic processes provide most of the support and intimate contact for hematopoietic cells.

Studies have shown that the stroma of the bone marrow and spleen provide different microenvironments and thus differentially specify cell fates (reviewed by Trentin, 1978). Spleen colonies and bone marrow colonies formed in heavily irradiated mice reconstituted with bone marrow were found to be distinct (Trentin, 1970; Trentin, 1971; Curry et al, 1967; Curry et al, 1967). For the first 8-10 days, most spleen colonies or bone marrow colonies consisted of only one lineage: erythroid, neutrophilic granuloid, eosinophilic granuloid, or megakaryocytic. In spleen colonies the ratio of erythroid colonies to granuloid colonies was 3:1, whereas in bone marrow colonies the ratio was 1:2 (Wolf et al, 1968). To determine if this was a result of selective homing of different committed cells to different locations, irradiated mice were injected with bone marrow, and those cells that had 'homed' to the bone marrow were re-transplanted to irradiated secondary hosts. The ratios of erythroid to granuloid colonies observed in the secondary hosts were the same as that observed in primary hosts. This suggested that the precursor cells were the same, but the microenvironment effected their outcome. In fact, erythroid colonies only appear in the red pulp of the spleen, whereas granuloid colonies appear adjacent to the splenic capsule or in the lymphoid follicles of the white pulp (Curry et al, 1967).

Experiments using hypertransfusion-induced polycythemic mice, which suppresses erythropoietin secretion and erythroid differentiation, have shown that the limitation of differentiative potential is geographic rather than temporal (Curry et al, 1967). In these mice, differentiated erythroid colonies do not appear, and the pluripotent colony forming cells which would have given erythroid colonies do not divert to a different lineage. Small undifferentiated colonies do appear that have acquired erythropoietin sensitivity, since exogenous or endogenous erythropoietin can cause these cells to undergo erythroid differentiation.

There is some controversy, however, in the role of the microenvironment in the formation of spleen colonies. Initial studies showed that spleen colonies consisted predominantly of erythroblasts for the first 8-10 days. After 10 days however, many colonies endoclonally developed a second lineage (Trentin, 1970; Trentin, 1971). Typical mixed colonies seen in the spleen consisted of a large erythroid colony, with granulopoiesis or megakaryocytopoiesis in a peripheral segment. This suggested that a pluripotent erythroid colony outgrew an erythroid environment, and interacted with a granuloid or megakaryocytic microenvironment. A subsequent study however, demonstrated that CFU-Ss giving rise to spleen colonies at day 8 were neither multi-potential nor self-maintaining, while CFU-Ss giving rise to spleen colonies at day 12 had these characteristics. This variation was found to be due to different CFU-S populations, and not due to the microenvironment (Magli et al, 1982).

A further role for the hematopoietic environment is suggested by the fact that most in vitro culture systems require an adherent layer of stromal cells. For example, the Dexter culture system allows for the long-term growth and differentiation of hematopoietic cells (Dexter et al, 1977). In this system, an adherent layer of stromal cells for the stem cells is essential. Various stromal cell lines have been established which have been found to support the differentiation of many lineages, although to differing extents (reviewed in Kincade, 1991). Biochemical analysis of a number of stromal cell lines have shown that stromal cells express a variety of cytokine receptors, adhesion molecules, and secrete a variety of cytokines. Not all stromal cells express the same set of these biochemical factors, suggesting that different stromal cells may have specialized functions.

A number of studies have focused on identifying and characterizing cytokines (reviewed by Kincade, 1991; Ogawa, 1993). Some factors are simply stimulatory or inhibitory, while others

have more complex functions. Cytokines can be divided into three categories: 1) late acting lineage-specific factors that support proliferation and maturation of committed progenitors; 2) intermediate-acting lineage non-specific factors; and 3) factors affecting cell cycle dormant stem cells.

Examples of late acting lineage-specific factors are: erythropoietin, which is important for erythropoiesis; M-CSF, which is important for macrophage/monocyte differentiation; IL-5, which is important for eosinophil differentiation; and G-CSF, which is important in the maturation of monopotent neutrophil progenitors (but may also act on earlier precursors).

Examples of intermediate acting lineage non-specific factors are GM-CSF, IL-3, and IL-4. All of these cytokines support the proliferation of multipotent progenitors, but only after they exit G₀.

Examples of factors affecting the cell cycle status include IL-6, G-CSF, IL-11, SF (Steel factor), and IL-12. The use of these cytokines in combination supports colony formation from dormant murine hematopoietic progenitors.

Examples of inhibitory cytokines include interferons, TNF α , TGF- β , and MIP1 α . In general, interferons and TNF α are lineage non-specific factors affecting a range of cells in the hematopoietic differentiation hierarchy, while TGF β and MIP1 α in general seem to effect early precursors.

Despite the discovery of various growth factors, the general consensus is that the role of growth factors is to support survival and proliferation, but not to specify cell fates. Thus, the apparent induction of proliferation and maturation by these growth factors is due to cells that are competent to receive those signals.

1.5 Clues From Parallel Systems

Cell-fate decisions of the type made during the process of hematopoiesis also occur in a wide range of tissues of all organisms throughout development. How cell-fate decisions are made in these tissues may provide clues on similar processes in hematopoiesis. One regulatory system that has been found to play a role in cell-fate determination throughout a range of tissues in a number of organisms, is the Notch signalling system. The goal of my research is to determine the expression of the Notch receptors, Notch ligands, and regulators of Notch-ligand binding in cells of the hematopoietic system, to begin to ascertain the role of the Notch receptor system in the regulation of cell-fate decisions.

1.6 The Notch Receptor System

The Notch family of proteins has been found to play a role in mediating cell-fate decisions in a wide range of tissues in a number of invertebrates and vertebrates (reviewed in Fortini et al, 1993; Artavanis-Tsakonas et al, 1995; Simpson, 1995; Kopan and Turner, 1996). The Notch receptor was first identified in *Drosophila melanogaster*, and found to play a role in determining the cell fate of equipotent neuroepidermal cells. Normally, such a population of equipotent cells becomes segregated into a population of neuroblasts and epidermoblasts. A loss-of-function mutation of the Notch gene results in potential epidermoblasts becoming neuroblasts (Poulson,

1937). A gain-of-function Notch mutation however, results in potential neuroblasts becoming epidermoblasts (Struhl et al, 1993; Lieber et al, 1993).

Subsequently, Notch was found to be expressed widely in the *Drosophila* embryo, and also in later stages in various uncommitted and proliferative cells. Notch was found not only to play a role in determining cell fates of neuroepidermal cells, but also to be important in the development of the mesoderm, adult wing, adult PNS, germline, ovarian follicle cells, larval malpighian tubules, adult sensory bristles, and eye structures (reviewed by Artavanis-Tsakonas and Simpson, 1991).

Delta and Serrate are two known ligands for the Notch receptor protein in *Drosophila* (Fleming et al, 1990; Kopczynski et al, 1988; Thomas et al, 1991; Vassin et al, 1987). Both of these ligands are transmembrane proteins. Delta and Serrate are not entirely redundant ligands. It has been found that Serrate can substitute for Delta to activate Notch during neuroblast segregation (Gu et al, 1995). During wing development however, Delta and Serrate proteins can only activate Notch in distinct compartments of the wing disc, although Notch functions in both these compartments (Doherty et al, 1996; Couso et al, 1995; Jonsson et al, 1996; Kim et al, 1995; Speicher et al, 1994).

Fringe is a secreted protein involved in the Notch pathway. A receptor for Fringe has not yet been identified. Fringe is a boundary specific signaling molecule involved in *Drosophila* wing development (Irvine, 1994). It has been demonstrated that the secreted protein Fringe controls the sensitivity of Notch for its ligand (Fleming et al, 1997; Johnston et al, 1997; Cohen et al, 1997). The mechanism by which this occurs is not understood.

The classic example of how Notch functions to specify cell fates is during neurogenesis in the ventral ectoderm of *Drosophila* (Poulson, 1937; Cabrera, 1990; Simpson et al, 1989; Skeath

et al, 1991; Cubas et al, 1991). Initially, a monolayer of cells has equal potential to become neuroblasts or epidermoblasts, all of which express Notch and Delta. A natural fluctuation in the levels of expression of Notch and/or Delta occurs, which becomes enhanced by a feedback mechanism. Cells expressing higher levels of Notch receive a stronger signal through the Notch receptor, which induces increased Notch expression and decreased Delta expression in that cell ($\text{Delta}^{\text{low}}\text{Notch}^{\text{hi}}$); resulting in some cells expressing high levels of Delta ($\text{Delta}^{\text{hi}}\text{Notch}^{\text{low}}$) to be surrounded by cells expressing high levels of Notch ($\text{Delta}^{\text{low}}\text{Notch}^{\text{hi}}$). The Delta protein expressed on the surface of a $\text{Delta}^{\text{hi}}\text{Notch}^{\text{low}}$ cell will bind to Notch on the surface of the $\text{Delta}^{\text{low}}\text{Notch}^{\text{hi}}$ cell and thus inhibit the $\text{Delta}^{\text{low}}\text{Notch}^{\text{hi}}$ cell from becoming a neuroblast, while the $\text{Delta}^{\text{hi}}\text{Notch}^{\text{low}}$ expressing cell becomes a neuroblast. The Notch expressing cell later becomes an epidermoblast.

1.7 The Mouse Notch Gene Family

Homologs of Notch, the Notch ligands, and the Fringe genes have been identified in a wide range of invertebrates and vertebrates, including *C. elegans*, *X. laevis*, zebrafish, chicken, rat, mouse and human. The conservation of Notch and Notch ligands through evolution suggests that Notch plays a role in determining cell fates in a wide range of tissues and organisms (reviewed by Egan et al, 1997).

Refer to Figure 2 for a diagram of the Notch family of proteins, and Table 1 for the expression pattern of these genes.

In mouse, four Notch genes have been identified, mNotch1-4 (Reaume et al, 1992; Lardelli et al, 1993; Lardelli et al, 1994; Robbins et al, 1992; Uyttendaele et al, 1996). The

Figure 2: The Protein Structure of Notch, the Notch Ligands, and the Fringe Genes

Table 1: Highlights of the Expression Patterns of the Notch Receptors, Dll1, Jagged, and the Fringe Genes

<i>Gene</i>	<i>Embryonic Expression</i>	<i>Adult Expression</i>	<i>Reference</i>
mNotch1	-first detected at e8.5, peaks at e10.5 and continues throughout development - during the process of gastrulation, neuralation, and somitogenesis	-brain, heart, kidney, liver, lung, muscle, ovary, testis, thymus, and spleen, with highest levels in lung, and lowest levels in the muscle and liver.	Reaume et al, 1992
mNotch2	-first detected at e8.5, and throughout development in whole embryo	-high levels in adult brain, liver, and kidney, but low levels in adult muscle	Lardelli et al, 1993
mNotch3	-first detected at e8.5, and throughout development in whole embryo	-heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis -highest levels in heart, spleen, and lung; lowest levels in liver, and testis	Lardelli et al, 1994
mNotch4	-predominantly in endothelial cells	-highest expression in lung; intermediate expression in heart and kidney; low expression in ovary and muscle; and very low expression in brain, intestine, liver, spleen, and testis; also in mammary gland	Uyttendaele et al, 1996

Table 1: Highlights of the Expression Patterns of the Notch Receptors, Dll1, Jagged, and the Fringe Genes (cntd)

<i>Gene</i>	<i>Embryonic Expression</i>	<i>Adult Expression</i>	<i>Reference</i>
Rat Jagged-1	-nervous system, eye, ear, kidney, pancreas, limb bud, and skin	-not given	Lindsell et al, 1995
Rat Jagged-2	-coordinated expression with Notch1 in thymus; little expression in developing CNS	-not given	Shawber et al, 1996
Dll1	-between e7-e12, Dll1 expression includes the paraxial mesoderm, relating to somitogenesis, and a subset of cells in the developing nervous system	-high levels in lung, and lower levels in heart -no detectable expression was found in the spleen, muscle, kidney, testis, gut, liver, or brain	Bettenhausen et al, 1995
Lunatic Fringe	-expression through e7-e17 of whole embryo	-high levels in brain, thymus, lung, spleen; low levels in heart liver, kidney, skeletal muscle	Cohen et al, 1997; Vogt et al, 1997
Manic Fringe	-expression through e7-e17 of whole embryo	-high levels in brain, thymus, heart, lung, kidney, spleen, skeletal muscle; low levels in liver	Cohen et al, 1997; Vogt et al, 1997
Radical Fringe	-expression through e7-e17 of whole embryo	-high levels in brain, lung, kidney, skeletal muscle; low levels in thymus, heart, liver, spleen	Cohen et al, 1997; Vogt et al, 1997

extracellular domain consists of 36 EGF repeats in mNotch1/2, 34 EGF repeats in mNotch3, and 29 EGF repeats in mNotch4. An EGF repeat is approximately 40 amino acids in length with six cysteine residues forming three regularly spaced disulfide bonds. In the extracellular domain, but C-terminal to the EGF repeats is a region containing three LN repeats, named because this repeat is common to LIN-12 and GLP-1 of *C. elegans* (the *C. elegans* homologs of Notch), and Notch, as well as all other homologs of Notch. Each LN repeat is cysteine rich, consisting of approximately 40 amino acids. In the intracellular domain, close to the transmembrane domain is a Ram domain, followed by 6 cdc10/SWI6/ankyrin repeats. Each ankyrin repeat consists of approximately 35 amino acids. A version of this motif was identified in two yeast cell cycle control proteins (reviewed by Blank et al, 1992). At the C-terminus is a PEST sequence (being proline, glutamate, serine, and threonine rich) which may control protein degradation.

One mouse homolog of *Drosophila* Delta has been identified, termed Dll1 (Bettenhausen et al, 1995). The extracellular domain at the N-terminus contains a cysteine rich DSL domain. This domain is common to the Notch ligands: Delta, Serrate, and lag-2 (a *C. elegans* ligand for LIN-12 and GLP-1 Notch-like receptors). In the extracellular domain of Dll1, but C-terminal to the DSL domain, are 8 EGF repeats.

Two homologs of *Drosophila* Serrate have been identified in rat, termed Jagged-1 and Jagged-2 (Lindsell et al, 1995; Shawber et al, 1996). In both proteins, the extracellular domain contains a DSL domain followed by 16 EGF repeats. Following the EGF repeats, but still in the extracellular domain, is a cysteine rich region. Jagged-2 is of the same size and has the same domain structure as Jagged-1.

To determine which domains are required for Notch: Delta and Notch:Serrate association, a mutational analysis of *Drosophila* Notch was performed (Rebay et al, 1991). Notch constructs

with varying combinations of domains were expressed transiently in S2 cells, and co-cultured with a cell line stably expressing Delta or Serrate. It was found that EGF repeats 11 and 12 of Notch were necessary and sufficient to mediate binding to Delta or Serrate. In these experiments, it was also found that Serrate-Notch binding is weaker than Delta-Notch binding. It is not clear which domains of Delta or Serrate are specifically involved in binding Notch; however, both the DSL domain and the EGF repeats appear important.

Three mouse Fringe homologs have been identified, Lunatic Fringe, Manic Fringe, and Radical Fringe (Cohen et al, 1997; Vogt et al, 1997). The sequence of the Fringe genes has been found to be highly homologous to the Lex1 family of galactosyltransferases (Yuan et al, 1997).

1.8 Mammalian Notch Genes in Development

To examine the role of mNotch1 in development, two labs have generated mNotch1 knockout mice. Homozygous mNotch1^{-/-} mutant embryos were found to die before e11.5 (Swiatek et al, 1994). At e8.5, mutant embryos appeared to be normal. By e9.5 the mutant embryos were developmentally retarded although pattern formation was largely normal. Somitogenesis was found to be delayed and disorganized (Conlon et al, 1995). After this stage, however, there was widespread cell death. Initially, cell death was not random, but occurred particularly in the neuroepithelium and neurogenic neural crest. Cell death was not due to the lack of placentation or vascularization. In addition, erythrocytes were present in the blood vessels and heart.

There are 4 Notch genes in the mouse. In situ analysis of the expression of mNotch1, mNotch2, and mNotch3 in CNS development demonstrated that each gene had distinct but overlapping patterns of expression in the neuroepithelium (Lardelli et al, 1994). Distinct expression patterns and specific lethal phenotypes of mNotch1 mutant mice together indicate that the four mouse Notch genes are not entirely redundant.

The mNotch4 gene has only recently been identified (Robbins et al, 1992; Uyttendaele et al, 1996). A fragment of mNotch4 was first identified as the proto-oncogene int-3 (Gallahan et al, 1987). The int-3 oncogene encodes the transmembrane and intracellular domain of mNotch4, and is a frequent target for viral integration in MMTV-induced mammary carcinomas.

A functional role for rat Jagged-1 in myogenesis was examined (Lindsell et al, 1995). A fibroblast cell line, transfected with full length rat Jagged-1, and C2C12 cells, transfected with full length rat Notch1, were co-cultured under conditions that would normally cause C2C12 to differentiate into myoblasts. Rat Jagged-1 binding to Notch was able to inhibit myogenesis of C2C12 cells. Cells failed to express myogenin and MLC2, factors required for myogenesis.

To examine the role of Dll1 in development, a Dll1 knockout mouse was generated (de Angelis et al, 1997). Homozygous Dll1^{-/-} mutant embryos become severely hemorrhagic after embryonic day 10, and die around day 12 of development. Loss of Dll1 in the CNS resulted in excessive neuronal differentiation, consistent with the role of Delta in cell fate decisions in *Drosophila*. Dll1 was also found to play a role in the compartmentalization of somites.

1.9 The Notch Signal Transduction Pathway

The signal transduction pathway activated by ligand binding to Notch is not fully understood; however, there have been a number of studies looking at this pathway in *Drosophila* (reviewed in Artavanis-Tsakonas et al, 1995; Kopan and Turner, 1996). A nuclear protein, Suppressor of Hairless (Su(H)), was found to bind Notch (Fortini et al, 1994). Su(H) can be found in the cytoplasm or in the nucleus, and contains a putative nuclear localization signal. Su(H) in the cytoplasm interacts strongly with the Ram domain of Notch and with low affinity to the ankyrin repeats of the intracellular domain of Notch (Honjo, 1996; Roehl et al, 1996).

In *Drosophila* there are a number of loci encoding putative nuclear proteins that are thought to act downstream of Notch activation. One locus that becomes transcriptionally activated in subsets of CNS neuronal precursor cells by Notch activation is the Enhancer of Split complex (E(spl)) (Bailey et al, 1995; Jennings et al, 1995; Jennings et al, 1994; Lecourtois et al, 1995). The E(spl) locus encodes 7 small basic-helix-loop-helix (bHLH) transcriptional repressor proteins and Groucho (Delidakis et al, 1991; Delidakis et al, 1992; Knust et al, 1992). E(Spl)bHLH/E(spl)Groucho complexes act as transcriptional repressors (Tietze et al, 1992). A putative binding site for Su(H) has been found in regulatory sequences of the E(spl) genes, and the transcription of this locus has been found to require the Su(H) protein (Tun et al, 1994; Henkel et al, 1994; Grossman et al, 1994; Zimmer-Strobl et al, 1994).

A specific example of the Notch signal transduction pathway can be given for the segregation of neuroblasts and epidermoblasts from equipotent cells during CNS development (Cabrera, 1990; Simpson et al, 1989; Skeath et al, 1991; Cubas et al, 1991). Delta expressing cells in this tissue come to be surrounded by Notch expressing cells as discussed in section 1.6. Delta

on one cell binds Notch on another cell and activates Notch in that cell. The E(spl) locus becomes transcriptionally activated in cells receiving a Notch signal, possibly by the translocation of Su(H) from the cytoplasm into the nucleus. Induction of the E(spl) bHLH repressor proteins causes the down-regulation of neuroblast-specific genes which drives cells towards the epidermoblast lineage.

Su(H) is closely related to the mammalian DNA binding protein variously known as RBP-J κ , KBF2, or CBF1. RBP-J κ is involved in the Epstein-Barr virus induced immortalization of B cells (Zimmer-Strobl, 1994; Henkel, 1994; Grossman, 1994). Mammalian homologs of the E(spl) locus have also been identified, and are termed HES proteins (Sasai et al, 1992). The HES-1 locus has been found to contain target DNA binding sequences for RBP-J κ . An activated form of Notch (described below), was found to strongly stimulate the HES-1 promoter (Jarriault et al, 1995). This activation depended on the binding of RBP-J κ to the activated form of Notch.

Dominant transforming mutants of human Notch1, feline Notch2, and mouse Notch4 have been isolated (Ellisen et al, 1991; Rohn et al, 1996; Robbins et al, 1992). These dominant active mutants consist of only the cytoplasmic domain, or the cytoplasmic domain with the transmembrane domain. Experiments in *Drosophila* and *C. elegans* have shown that the expression of the cytoplasmic domain of Notch induces cell fates that are normally dependent on Notch activation by ligand (Fortini et al, 1993; Greenwald, 1994; Lieber et al, 1993; Struhl et al, 1993). Interestingly, the cytoplasmic domain of Notch has been found in the cytoplasm and nucleus of cells. Also, the intracellular domain of Notch contains two putative nuclear localization signals on either side of the ankyrin repeats (Stifani et al, 1992). Thus, a cleaved form of wild type Notch may be involved in the signalling pathway.

To examine this possibility, the proteolytic processing of mNotch1 was studied in 3T3 fibroblasts (Kopan et al, 1996). A mNotch1 construct consisting of the transmembrane and intracellular domains was found to become proteolytically cleaved, releasing the activated intracellular domain which translocated to the nucleus and inhibited activity of muscle-specific promoters and myogenesis. It has also been found that LN repeats of Notch1 negatively regulated cleavage of the intracellular domain. It has been hypothesized that the LN repeats may prevent the dimerization of Notch in the absence of ligand; where dimerization by ligand is thought to be required for Notch activation.

1.10 Notch in Hematopoiesis

Despite evidence for the role of Notch in cell-fate decisions in many systems, few studies have examined its role in cell fate determination in hematopoiesis.

mNotch1 appeared to play a role in the differentiation of CD4⁺ and CD8⁺ lineages in developing T cells of the mouse (Robey et al, 1996). The intracellular domain of mNotch1 was expressed exclusively in thymocytes of transgenic mice, and development was monitored. An activated form of Notch was found to bias differentiation to favour CD8⁺ T cells over CD4⁺ T cells. The activated form of mNotch1 allowed the development of thymocytes to CD8⁺ T cells, even in the absence of MHC Class I. However, when both MHC Class I and II were missing, very few mature T cells developed.

During thymocyte development in mice expression of mNotch1 is precisely regulated (Hasserjian et al, 1996). mNotch1 expression was first detected throughout the fetal murine

thymus at e13.5. At e18.5, the thymic cortex stained strongly, with less staining of the medulla. At the same time point in the liver, there was staining for mNotch1 in mononuclear cells of unknown lineage; however, mNotch1 was undetectable in maturing erythroid and megakaryocytic cells. At 4 weeks and 7 weeks, there was strong staining in the superficial thymic cortex, with little or no staining of the deep cortex or medulla.

Using three colour flow cytometry and immunohistochemical analysis of flow -sorted cells, highest Notch1 expression was found in immature cortical thymocytes that were $CD24^{high}CD4^{-}CD8^{-}$, lowest expression was found in more mature cortical thymocytes that were $CD24^{low}CD4^{+}CD8^{+}$, and intermediate expression was found in mature $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ cells.

Truncated versions of the Notch1 gene in humans, TAN-1, have been found in T cell leukemias with translocations involving chromosomes 7 and 9 (Ellisen et al, 1991). In the translocation, the transmembrane and intracellular domain of TAN-1 on chromosome 9 becomes fused to the TCR β locus on chromosome 7. This translocation produces an activated form of TAN-1, presumably resulting in T cell leukemias.

A related study has found that activated Notch alleles result in the exclusive development of T cell neoplasms (Pear et al, 1996). Various retroviral activated TAN-1 (Notch1) constructs were used to infect bone marrow cells in vitro, which were then used to reconstitute 10 lethally irradiated syngeneic mice. The observed frequency of tumorigenicity was 30-50%. An analysis of the tumours showed that they were mostly immature T cells. The cell populations ranged from $Thy1.2^{+}CD4^{-}CD8^{-}$ to $Thy1.2^{+}CD4^{+}CD8^{+}$.

In another study, RT-PCR analysis demonstrated Notch1 (termed TAN-1 in humans) expression in human $CD34^{+}$ hematopoietic precursors (Milner et al, 1994). $CD34^{+}$ cells are a

population of cells enriched for hematopoietic precursors and stem cells. Quantitative RT-PCR for Notch1 (TAN-1) was performed in unseparated mononuclear cells, CD34⁻ cells, CD34⁺ cells, CD34⁺lin⁺ cells, and CD34⁺lin⁻ cells (an immature subset of cells that lacks the expression of antigens associated with commitment to various lineages). Notch1 expression was found in all samples, but at higher levels in CD34⁺ populations. Both CD34⁺lin^{+/+} populations expressed TAN-1, which is expected since both of these populations contain multipotent progenitors in the process of making cell-fate decisions.

A functional study looked at mNotch1 in granulocytic differentiation (Milner et al, 1996). 32D cells, a myeloid progenitor cell line, proliferate as undifferentiated blasts in IL-3, but differentiate into mature neutrophilic granulocytes when stimulated with G-CSF. The intracellular domain of mNotch1 was transfected into 32D cells, and their ability to differentiate was studied. In medium containing G-CSF, constitutively mNotch1 expressing 32D cells did not differentiate, but rather continued to expand as undifferentiated cells. Interestingly, the intracellular domain of mNotch2 had no effect on granulocytic differentiation.

1.11 The Expression of the Notch receptors, Notch Ligands, and the Fringe genes in Hematopoiesis

The pleiotropic expression of the Notch family of proteins and its known role in cell-fate decisions suggest that the Notch family of proteins may be important regulators of the blood forming system.

My project involved performing an analysis of the expression of the Notch receptors, Notch ligands, and the Fringe genes in cells of the blood system. The first part of my project involved performing RT-PCR analysis of whole bone marrow to determine which of these genes were being expressed.

The second part of my project involved identifying which cells of the bone marrow express which candidate genes. Currently, it is extremely difficult to sort bone marrow cells on the basis of various markers to identify and obtain a pure population of cells at stages in the hematopoietic hierarchy, particularly for early precursors. Since Notch signalling is important in cell-fate decisions, it is important to determine the expression of the candidate genes in each cell population along the differentiation pathway. This goal was achieved by probing a lineage precursor blot (described in Brady et al, 1995; and in Section 2.7). The lineage precursor blot consists of amplified cDNAs from a hierarchy of hematopoietic precursor cells whose potential is known, from maturing hematopoietic cells, and three fibroblast cell lines. From the precursor lineage blot, one is able to determine which genes are expressed in the different hematopoietic populations. The amplified cDNAs were constructed such that they consist of the most 3' untranslated region (3' UTR) of the mRNAs. Thus, to screen the lineage blot it was first necessary to generate 3' UTR probes for mNotch1, mNotch2, mNotch3, mNotch4,Dll1, mJagged-1, Lunatic Fringe, Manic Fringe, and Radical Fringe.

To perform functional assays on the role of Notch in hematopoiesis, it is necessary to clone the cDNAs for the various genes of interest. Thus, the third part of my project involved cloning the cDNA for mouse Jagged-1.

2.1 Isolation of Bone Marrow Cells

Bone marrow cells were obtained from adult BALB/c mice. Femur and tibia of mice were removed, and bone marrow was flushed out with PBS. Cells were spun down twice at 1200 rpm at 5°C for 10 minutes to remove any debris, and re-suspended in PBS. Viable cells (dead cells and debris stain blue) were counted using 0.4% Trypan Blue stain (Gibco BRL).

2.2 RNA Isolation

Total RNA was extracted from the various cells/tissues assayed using the TRIzol reagent following manufacturer's instructions (Gibco BRL). After counting, the cells were re-pelleted and lysed in the TRIzol reagent using 1 mL of reagent per $5-10 \times 10^6$ cells. For tissue, 1 mL of TRIzol reagent was used to homogenize 50-100 mg of tissue. After lysis, chloroform was added followed by centrifugation, to separate the solution into an aqueous phase and organic phase. RNA remains in the aqueous phase. The RNA was recovered by precipitating the aqueous phase with isopropanol, followed by washing with 75% ethanol. The final RNA pellet was dissolved in 20-100 μ L DEPC- H_2O .

The RNA yield was determined by measuring the A_{260} of the samples, using the conversion that an OD_{260} of 1 corresponds to an RNA concentration of 40 $\mu\text{g/mL}$.

2.3 RT-PCR

The total RNA of all the cells/tissues assayed were reverse transcribed using the following method. 1 μg of total RNA was brought up to a volume of 9 μL in DEPC- H_2O . The total RNA was heated at 90°C for 5 min, then chilled on ice. The denatured RNA was added to other reaction components in a final volume of 20 μL which contained: 200 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Gibco BRL), 1X First Strand buffer (Gibco BRL), 1 mM dNTPs (Pharmacia Biotech), 10 mM DTT, 20 U RNAGuard (Pharmacia Biotech), and 250 ng random hexamer primers. The reaction was incubated at room temperature for 10 minutes, and then at 37°C for one hour.

The various PCR reactions consisted of a 100 μL reaction containing: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM MgCl_2 , 0.2 mM dNTPs (Pharmacia Biotech), 0.01% gelatin, 0.2-0.5 μg of each upstream and downstream primer, 20 μL of the RT reaction described above, and 2.5 U of Taq DNA polymerase.

The Notch primers, Dll1 primers, mJagged-1 primers, and Fringe primers used for RT-PCR are diagrammatically illustrated in Figures 3A-C.

Figure 3A: Sequence of PCR Primers Used for mNotch1, mNotch2, mNotch3, and mNotch4 RT-PCR

* **Note:** Nucleotide numbers (nts) are given with the 'A' of the start codon as nucleotide 1, except for mNotch2

⁺ **Note:** Sequence as written is sense. Downstream primers used for RT-PCR were anti-sense

Figure 3B: Sequence of PCR Primers Used for Dll-1 and mJagged-1 RT-PCR

*Note: Nucleotide numbers (nts) are given with the 'A' of the start codon as nucleotide 1.

*Note: Sequence as written is sense. Downstream primers used for RT-PCR were anti-sense.

*Note: All nucleotide numbering given is for rat Jagged-1.

Size of cDNA from start codon *

Lunatic Fringe

Manic Fringe

Radical Fringe

Figure 3C: Sequence of PCR Primers Used for Lunatic Fringe, Manic Fringe, and Radical Fringe RT-PCR

* Note: Nucleotide numbers (nts) are given as 'A' of the start codon as nucleotide 1.

+ Note: Sequence is given as sense. Downstream primers used for RT-PCR were anti-sense.

For mNotch1, the upstream primer used was 5'-CAATCAGGGCACCTGTGAGCCCACAT-3', and the downstream primer used was 5'-TAGAGCGCTTGATTGGGTGCTTGCGC-3', to give a 709 base pair product.

For mNotch2, the upstream primer used was 5'-CATTGACGAGTGCACTGA-3', and the downstream primer used was 5'-GAGTGCTGGCACAAGTGT-3' to give a 407 base pair product.

For mNotch3, the upstream primer used was 5'-GTGGATGTCAACGTCCGA-3', and the downstream primer used was 5'-TGCCAGGATCAGTGCAGT-3', to give a 403 base pair product.

For mNotch4, the upstream primer used was 5'-CAGAACGTGGATCCCCTCAAGTTGC-3', and the downstream primer used was 5'-AGGCAGAGAGAGGGCAAGGAGTCAT-3', to give a 497 base pair product.

For Dll1, the upstream primer used was 5'-CATGGCACCTGCCAGCAACCCTGGCAGTGTAAGTGC-3', and the downstream primer used was 5'-GAGAGAAAGCTTAGGGGCTAGGAGCACACTCATCTACTT-3', to give a 229 base pair product.

For mJagged-1, the degenerate upstream primer used was 5'-GATGAC T/G T/C CTT C/T GG A/G/C/T CA C/T T T/A C/T G/A C A/G/C/T TG-3', and the degenerate downstream primer used was 5'-CCACAGTA G/A TT A/G/C/T AG G/A TC T/C T T/G G/A TC G/A CA-3', to give a 293 base pair product. The specific upstream primer used was 5'-GACCAGAACGGCAACAAAACCTTGCATGGAA-3', and the specific downstream primer

used was 5'- TTGGTCTCACAGAGGCACTGCCAGGGTTCA-3', to give a 224 base pair product.

For Lunatic Fringe, the upstream primer used was 5'-GTGCATAGCCTCTCCGAGTACTTCA-3', and the downstream primer used was 5'-CTCCTCCGGTGGCAAACCAAAAGTG-3', to give a 560 base pair product.

For Manic Fringe, the upstream primer used was 5'-CGTGATGTCTATGTGGGCAAG-3', and the downstream primer used was 5'-TCAGGGCGCTGCCAGCAG-3', to give a 481 base pair product.

For Radical Fringe, the upstream primer used was 5'-GGCAGACGTTTCATTTTCACCGATGG-3', and the downstream primer used was 5'-GAAGTCCTTCCACGATGTATCCCAC-3', to give a 480 base pair product.

For β -actin, the upstream primer used was 5'-TGGAATCCTGTGGCATCCATGAAAC-3', and the downstream primer used was 5'-TAAAACGCAGCTCAGTAACAGTCCG-3', to give a 350 base pair product.

The PCR amplification was carried out as follows: initial denaturation at 94°C for 5 minutes.; addition of Taq polymerase at 72°C; followed by cycles of denaturation at 94°C for 1 minute; primer annealing at 58°C for 1 minute for mNotch1, 56°C for 1 minute for mNotch2/3, 58°C for 1.5 minutes for mNotch4, 56°C for 1.5 minute for Dll1, 62°C for 1.5 minutes for mJagged-1 (specific primers), 58°C for 1.5 minutes for Lunatic Fringe, and 60°C for 1.5 minutes for Manic/Radical Fringe; primer extension at 72°C for 1 minute; all followed by a 10 minute extension at 72°C. 30 cycles were used for the Notch primers, and 35 cycles were used for the Dll-1, specific Jagged primers, and the Fringe primers. For the degenerate mJagged-1 primers,

the amplification was carried out as follows: initial denaturation at 94°C for 5 minute, addition of Taq polymerase at 72°C; followed by 2 cycles at 94°C for 50 seconds, 50°C for 2 minutes, and 72°C for 2 minutes; followed by 35 cycles of 94°C for 50 seconds, 56°C for 2 minutes, and 72°C for 1.5 minutes; all followed by a 10 minute extension at 72°C.

The PCR products were visualized by gel electrophoresis of 30 µL of the PCR reaction on an agarose gel polymerized with ethidium bromide. 1 µg of molecular weight standards (1 kb DNA ladder, Gibco BRL) were also run to determine the size of the PCR products.

2.4 Cloning and Sequencing the PCR Product Derived From Degenerate Jagged primers

Because the sequence of mouse Jagged-1 was unknown, it was necessary to design and use degenerate primers for PCR. The degenerate primers were designed based on the known sequence for the rat Jagged gene. Upon obtaining a PCR product of expected size from adult mouse bone marrow and mouse e15 trunk total RNA, cloned PCR products were sequenced to confirm that the mouse Jagged-1 gene had been amplified.

The PCR product was excised from the gel and isolated using the Qiaex II gel extraction kit following manufacturer's instructions (Qiagen). The PCR product was ligated into the PCR II vector using the TA Cloning kit, following manufacturer's instructions (Invitrogen).

Transformation of One Shot INVαF' competent cells was carried out by heat shock. The resulting mixture was plated on LB-agar containing 2.5 mg ampicillin, 100 µL of 100 mM IPTG, and 20 µL of 4% X-Gal in DMF. The plates were incubated at 37° overnight. 20 white colonies were

picked from both RNA sources, and grown in 5 mL liquid culture of BHI + 50 µg/mL of ampicillin at 37° with shaking at 225 rpm overnight.

Plasmids were isolated using the QIAprep Spin Plasmid kit using 1.5 mL of the 5 mL cultures, following manufacturer's instructions (Qiagen). The PCR II vector contains EcoR1 sites on either side of the cloning site, allowing for the release of the insert. To check for inserts, the plasmids were digested in a 20 µL reaction containing 5 µL of the spin plasmid prep, 1X React 3 buffer, and 10 U EcoR1 (Gibco BRL), at 37°C for 2 hours.

Those plasmids containing an insert were sequenced using the Sequenase Version 2.0 sequencing kit, following manufacturer's instructions (Amersham Life Science). The sequencing primer used was the T7 primer. Since the PCR product can insert into the cloning site in either orientation, sequencing from one end of a number of clones would give sequence information on both ends of the insert.

The sequence of the 293 base pair insert was determined and analyzed using the MacDNAsis sequence analysis program. The DNA sequence was compared to sequences in Genbank using the BLASTn program. The input sequence had very high identity to the rat Jagged-1 sequence (one substitution at the amino acid level), confirming that the cloned cDNA fragment was from the mouse ortholog of rat Jagged-1. Based on this sequence, specific mouse Jagged-1 primers were designed for further analysis.

2.5 3' Untranslated Region Probes

The lineage precursor blot is a slot blot of amplified cDNAs made from precursors whose cell fate is known, from maturing cells, and three fibroblast cell lines (Brady et al, 1995; described in more detail below). The library was constructed such that it consists of the most 3' untranslated region (3' UTR) of the genes expressed in a particular precursor. Thus, to probe the lineage precursor blot for the expression of the Notch receptors, Notch ligands, and the Fringe genes it was first necessary to obtain the appropriate probes. The probes were obtained through a combination of library screening, RT-PCR and 3' RACE techniques. See Table 2 for a description of the 3' UTR probes.

The mNotch1 3' UTR probe was obtained by a library screening technique. A mNotch1 fragment from nucleotides 7557 - 7945 was cloned by Dr. David Ryan in Dr. Phillip's lab. This clone is in the pBK-CMV vector from Stratagene (e14.5 mouse embryo cDNA library; Stratagene). The stop codon of mNotch1 is at 7594 bp, and the poly-A tail begins at 7976 bp. An EcoR1 site and BamH1 site on either side of the insert in the polylinker was used to release the insert, to give a 388 bp probe.

3' RACE was used to try to obtain the mNotch2 3' UTR (3' RACE described below). The published mNotch2 sequence is only given to nucleotide 3609 (Lardelli et al, 1993). Thus, primers for 3'RACE were designed based on homology between the rat and human Notch2 orthologues. One gene specific sense primer used was 5'-TACCTGACACCATCCCCAGAGTCTC-3', corresponding to nucleotides 7240-7264 (rat sequence). A second gene specific sense primer used was 5'-

Table 2: 3' Untranslated Region Probes

Candidate Gene	Known 3' UTR Information From Literature *	Source of 3' UTRs⁺	3' UTR Probe Used on Lineage Blot
mNotch1	nt 7597-7976 (seq. available to polyA)	library screen: nt 7557-7945	nt 7557-7945
mNotch2	only 3609 nt of coding seq. available	not obtained	---
mNotch3	nt 6958-7884 (seq. not available to polyA)	3' RACE: nt 6937-polyA (1.0 kb product)	nt 7309-7879
mNotch4	nt 5896-6331 (seq. not available to polyA)	RT-PCR: nt 5810-6306	nt 5810-6306
Dll1	nt 2170-2844 (seq. available to polyA)	library screen: nt 582-polyA	nt 2008-2636
mJagged-1	unknown sequence; stop codon for rat Jagged-1 at nt 3663	3' RACE: nt 3604-polyA (1.7 kb product)	641 bp; 23 nt away from polyA
Lunatic Fringe	unknown sequence; stop codon at nt 1135	not obtained	---
Manic Fringe	unknown sequence; stop codon at nt 964	3' RACE: nt 821-polyA (0.8 kb product)	nt 821 - polyA
Radical Fringe	unknown sequence; stop codon at nt 1003	3' RACE: nt 901-polyA (1.0 kb product)	nt 901 - polyA

* Note: Nucleotide numbers (nts) are given with the 'A' of the start codon as being nucleotide 1. Listed is known information from literature on the 3' untranslated regions of the genes.

⁺ Note: Method used to generate 3' UTR cloned probes

CTTTCCCAGCCTCTGTGGGCAAGTA-3', encompassing nucleotides 7124-7148 (rat sequence). In the rat sequence, the stop codon is at nucleotide 7414. Despite numerous attempts at 3'RACE using the two different primers, and varying the PCR conditions, the correct product could not be obtained.

The mNotch3 3'UTR was obtained by 3' RACE PCR using a 3' RACE kit consisting of an e15 whole mouse embryo cDNA library (Marathon-Ready cDNA, Clontech). The gene-specific sense primer used was 5'- AAGAGGCAGGTGATGGCCTAAGTTC - 3', which begins at nucleotide 6937. The stop codon is at 6955 bp. The 3' RACE PCR reaction consisted of a 50 μ L reaction containing 1X KlenTaq PCR reaction buffer, 0.2 mM dNTPs, 1X Advantage KlenTaq Polymerase mix, 10 μ M AP1 primer, 10 μ M gene-specific primer, and 5 μ L of Marathon-Ready cDNA. The 3' RACE PCR amplification was carried out as follows: 94°C for 1 min; followed by 5 cycles of 94°C for 30 seconds, and 72°C for 4 minutes; followed by 5 cycles of 94°C for 30 seconds, and 70°C for 4 minutes; followed by 25 cycles of 94°C for 30 seconds, and 68°C for 4 minutes.

30 μ L of the 3'RACE PCR reaction was electrophoresed and visualized on a 1.5% agarose gel. A 1.0 kb product was obtained. The product was excised from the gel and isolated using the Qiaex II gel extraction kit, following manufacturer's instructions (Qiagen). The PCR product was ligated into the PCR 2.1 vector using the TA Cloning kit, following manufacturer's instructions (Invitrogen). DH5 α heat shock competent cells were transformed with 2.5 μ L of the ligation mixture. The resulting mixture was plated on LB plates containing ampicillin, IPTG, and X-Gal as described above and incubated at 37° overnight. 10 white colonies were picked and grown in 5 mL liquid culture of BHI + 50 μ g/mL of ampicillin at 37° with shaking at 225 rpm

overnight. Plasmids were isolated and were checked for inserts as described above. 3 clones were sequenced using the T7 and M13 (reverse) primers. Sequence of approximately 150 bp from each end of the clones confirmed that they were Notch3 and that each contained a polyA-tail. A DraI site at nt 7307 and an EcoRI site at nt 7879 were used to release a 573 bp product to be used as the probe.

The mNotch4 3' UTR probe was obtained by RT-PCR. The RT reaction was carried out on e15 whole mouse embryo, as described above. The upstream primer used was 5'-CAGAACGTGGATCCCCCTCAAGTTGC-3', which starts at nucleotide 5810, and the downstream primer used was 5'-AGGCAGAGAGAGGGCAAGGAGTCAT-3', which starts at nucleotide 6306, resulting in a 497 bp product. The stop codon is at 5893 bp. The PCR reaction components were as described above. The PCR amplification was carried out as follows: 94°C for 5 minutes; addition of Taq at 72°C for 5 minutes; followed by 35 cycles of 94°C for 1 minute, 60°C for 1.5 minute, and 72°C for 1 minute; followed by 72°C for 10 minutes. The PCR product was isolated and cloned into the PCR 2.1 vector as described above. Sequencing confirmed the identity of this clone. EcoRI sites in the vector were used to release the insert to be used as the mNotch4 probe.

The Dll1 3'UTR was obtained by a library screening technique. A fragment of Dll1 from nucleotide 582 - polyA tail was cloned by Dr. David Ryan in Dr. Phillip's lab. This clone is in the pBK-CMV vector from Stratagene (e14.5 mouse embryo cDNA library; Stratagene). The stop codon is at nucleotide 2167 bp, and the poly-A tail begins at nucleotide 2843. A SacI site at nt 2008 and a HindIII site at nt 2636 were used to release a 629 bp product to be used as the Dll1 3'UTR probe.

The mJagged-1 3' UTR probe was obtained by 3' RACE PCR as described above. The gene specific sense primer used was 5'-CAGGACAACAGAGACTTGGAAAGTG-3'. Since the sequence for mouse Jagged-1 was not known, this primer was designed based on sequence alignment between rat Jagged-1, human Jagged-1, and chicken Jagged-1 near the stop codon. The 3' RACE PCR amplification was carried out as follows: 94°C for 1 minute; followed by 5 cycles of 94°C for 30 seconds, and 72°C for 4 minutes; followed by 5 cycles of 94°C for 30 seconds, and 70°C for 4 min; followed by 25 cycles of 94°C for 30 seconds, and 68°C for 4 min. A 1.7 kb product was obtained, which was cloned and sequenced. Sequencing confirmed that this was mouse Jagged-1 and that the clone contained a polyA-tail. An SspI site and an AatII site, which were 23 and 644 nucleotides away from the polyA tail respectively, were used to obtain a 641 bp fragment of the 3'UTR.

3' RACE was used to try to obtain the Lunatic Fringe 3'UTR. One gene specific primer used was 5'-AGGGACCATTCTCTGTGGAAGCTGA-3'. A second gene specific primer used was 5'-TTGAGAACAAGCGGAACGCAGTGCA-3'. Despite numerous attempts at 3' RACE using two different primers, and varying the PCR conditions, the correct product could not be obtained. A Lunatic Fringe cDNA clone obtained by Dr. Sean Egan's lab contained part of the 3'UTR sequence. This sequence was used to screen the EST database. An EST clone from LifeTech. mouse adult brain matched this sequence (IMAGE Consortium Clone ID 370297). Despite analyzing 42 clones by sequencing and PCR, the correct 3' UTR clone could not be identified in the mixture of clones supplied by the IMAGE Consortium.

The Manic Fringe 3'UTR probe was obtained by 3' RACE PCR as described above. The gene specific sense primer used was 5'-TCAGCTACGGTGTCTTTGAGGGGAA-3', starting at

nucleotide 821. The stop codon is at nucleotide 964. The 3' RACE PCR amplification was carried out as follows: 94°C for 1 minute; followed by 5 cycles of 94°C for 30 seconds, and 72°C for 4 minutes; followed by 5 cycles of 94°C for 30 seconds, and 70°C for 4 minutes; followed by 25 cycles of 94°C for 30 seconds, and 68°C for 4 minutes. A 800 bp product was obtained, which was cloned and sequenced. Sequencing confirmed that this was Manic Fringe, and that the clone contained a polyA-tail. EcoR1 sites in the vector were used to release the insert to be used as a probe.

The Radical Fringe 3'UTR probe was obtained by 3' RACE PCR as described above. The gene specific sense primer used was 5'-AACATACAGCAGGACCCTACACGGT-3', starting at nucleotide 901. The stop codon is at nucleotide 1003. The 3' RACE PCR amplification was carried out as follows: 94°C for 1 minute; followed by 5 cycles of 94°C for 30 seconds, and 72°C for 4 minutes; followed by 5 cycles of 94°C for 30 seconds, and 70°C for 4 minutes; followed by 25 cycles of 94°C for 30 seconds, and 68°C for 4 minutes. A 1.0 kb product was obtained, which was cloned and sequenced. Sequencing confirmed that this was Radical Fringe, and that the clone contained a polyA-tail. EcoR1 sites in the vector were used to release the insert to be used as a probe.

2.6 Testing the 3'UTR probes

To ensure that the 3'UTR probes were able to detect transcripts of their respective genes, each probe was tested on a multiple tissue Northern blot (MTN blot, Clontech). Each MTN blot

consists of 2 µg poly-A RNA from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. 50-100 ng of each probe was labelled with ^{32}P -dCTP (Amersham), using a High Prime DNA labelling kit, following manufacturer's instructions (Boehringer Mannheim). Probes were purified on a sephadex NICK column (Pharmacia Biotech). Membranes were pre-hybridized at 68°C for 30 minutes in ExpressHyb (Clontech). Labelled probes were boiled for 5 minutes, chilled on ice, then added to the pre-hyb solution at a concentration of 3×10^6 cpm/mL. Membranes were hybridized for one hour. Following hybridization, membranes were washed as per manufacturer's instructions. Each membrane was exposed to BioMax MR film (Kodak) using intensifier screens at -70°C for 1-3 nights. MTN blots were stripped, following manufacturer's instructions for re-probing.

2.7 Probing the Lineage Precursor Blot

The lineage precursor blot is a slot blot of amplified cDNAs made from precursors whose cell fate is known, maturing cell populations, and three fibroblast cell lines (slightly modified from Brady et al, 1995; kindly provided by Dr. N. Iscove). The source and culture conditions of the samples on the blots are described in Table 3A and Table 3B.

In brief, in generating amplified cDNAs from precursor cells (Table 3A), cells from different sources were plated in primary culture and allowed to divide a few times to generate a colony of a small number of cells. One cell was retained while the remaining cells from a particular colony were re-plated in a secondary culture which supported all the myeloid lineages.

Table 3A: Set of Pooled cDNA Samples From Hematopoietic Precursors

Slot #	Sibling Potential	# Samples Pooled	Source of Parent Cell	1 ^o Culture Cell
1	E/Meg/Mac/Neut/Mast	4	Sort	II
2	E/Meg/Mac/Neut	11	Sort	II
3	E/Meg/Mac	6	BM/TdR	I
		3	Sort	II
4	E/Meg	1	FL/Aa4.1 ⁺	I
		23	BM/TdR	I
5	Mac/Neut	7	BM/TdR	I
6	BFU-E	2	BM/TdR	I
7	CFU-E	2	FL/Aa4.1 ⁺	I
8	Mac	2	BM	III
		7	BM	IV
		2	FL/Aa4.1 ⁺	I
		2	Sort	II
9	Neut	5	BM/TdR	I
		2	BM	IV
10	Meg	7	BM/TdR	I

Potential:

E - erythroid; *Meg* - megakaryocyte; *Mac* - macrophage; *Neut* - neutrophil; *Mast* - mast cell; *CFU-E* - colony forming unit - E; *BFU-E* - burst forming unit - E

Source: (source of cells for primary culture)

BM - bone marrow

Sort - Rh^hSca^{hi} medium density bone marrow cells

BM/TdR - Bone marrow cells incubated with thymidine, to select for slowly cycling cells

FL/Aa4.1 - Day 12 fetal liver cells panned for Aa4.1 expression

Primary Culture Conditions: (conditions to generate colonies for sibling analysis)

I - methyl cellulose containing IL-1, IL-3, kit ligand (KL), erythropoietin (Epo), conditioned medium from human 5637 cells (CM5637)

II - methyl cellulose containing IL-1, IL-11, KL, CM5637

III - methyl cellulose containing G-CSF

IV - methyl cellulose containing IL-3

Secondary Culture Conditions: (conditions for sibling analysis that support myelopoiesis)

All sibling precursor cells from colony starts were plated in gridded plates in IMDM, 1.2% methyl cellulose, 4% FBS, 0.5% serum fraction V, lipids, 100 µg/mL transferrin, 1 ng/mL IL-1β, 15 U/mL IL-3, 3% KLCM, 30 ng/mL IL-11, 1 U/mL Epo, 10 µg/mL insulin, and 10% CM5637.

Table 3B: Set of cDNA Samples From Maturing Hematopoietic Populations

Slot #	Lineage	# Pooled Samples	Source	Culture
11	Erythrocytes	2	BM	V
		1	BM	VI
12	Neutrophils	2	BM	V
		3	BM	VI
13	Mast Cells	3	BM	VII
14	Megakaryocytes	2	FL/Aa4.1 ⁺	VIII
		4	BM	I
15	B cells	2	FL	IX
		3	FL (B/Mac)	IX
		3	BM	none
		3	BM	IX
16	Macrophages	3	BM	X
		3	BM	XI
		2	FL (B/Mac)	XI
17	T cells	2	Spleen (CD4 ⁺)	none
		2	Spleen (CD4 ⁺)	XII
		2	Spleen (CD4 ⁺)	XIII
		2	Spleen(CD8 ⁺)	none
		2	Spleen (CD8 ⁺)	XIV
		2	Spleen (CD8 ⁺)	XV
18	air	1	-ve control	none
19	NIH 3T3 fibroblasts	1	cell line	---
20	S17 fibroblasts	1	cell line	---
21	95/1.7 fibroblasts	1	cell line	---

Source: (source of cells for cultures)

BM - bone marrow

FL/Aa4.1 - Day 12 fetal liver cells panned for Aa4.1 expression

FL - Day 12 fetal liver cells, unseparated

FL (B/Mac) - Day 12 fetal liver cells panned for Aa4.1⁺/B220⁺/Mac-1⁺/Sca⁺

Spleen (CD4⁺) - spleen cell suspension

Culture Conditions: (conditions used to obtain maturing populations)

V - methyl cellulose containing IL-3, Epo

VI - methyl cellulose containing IL-1, IL-3, KL, IL-11, Epo

VII - Bulk cultures of marrow cells in IMDM containing IL-3, Concanavalin A (ConA), FBS, bovine serum fraction V, transferrin, insulin

VIII - methyl cellulose containing IL-1, IL-3, Epo, CM5637

IX - liquid culture, in IL-7

X - methyl cellulose containing IL-3, M-CSF

XI - methyl cellulose containing 20% LCM (L929 cell-conditioned medium as a source of M-CSF)

XII - spleen cells incubated for 24 hr with Con A, followed by sorting for CD4⁺CD8⁻

XIII - spleen cells incubated for 48 hours with Con A, followed by sorting for CD4⁺CD8⁻

XIV - spleen cells incubated for 24 hours with Con A, followed by sorting for CD4⁺CD8⁺

XV - spleen cells incubated for 48 hours with Con A, followed by sorting for CD4⁺CD8⁺

Negative Control:

'air' - no cell added for RNA extraction and subsequent cDNA amplification

RNA was isolated from the retained cell and reverse transcribed using an oligo (dT)₂₀ primer. A polyA-tail was added to the 3' end of the first strand using Terminal Deoxynucleotidyl Transferase. The cDNA was amplified by PCR using pGdT primers resulting in PCR products ranging in size from 200-600 bases of the most 3' untranslated region of mRNA transcripts.

For maturing cells, as described in Table 3B, populations of maturing cells from a particular lineage were used for cDNA amplification.

For the lineage precursor blot used here, independent amplified cDNAs from the same single cell precursors and the same maturing populations were pooled (described in Appendix D). 0.5 µl of amplified cDNA was taken from each individual sample, and pooled. 1 uL of the pooled material was used in subsequent PCR to generate enough material for the lineage blots (one blot per probe). 20 µL of the PCR material was used for each slot on the blot.

50-100 ng of each probe was labelled with ³²P-dCTP (Amersham) using a High Prime DNA labelling kit, following manufacturer's instructions (Boehringer Mannheim). Probes were purified on a sephadex NICK column (Pharmacia Biotech). The mJagged-1 probe was tested on the lineage blot using two methods. In Method 1, the lineage blot was pre-hybridized for one hour at 65°C in a solution of 2X SSC, 1 M NaCl, and 10% dextran sulphate. The labelled mJagged-1 probe was boiled for 5 minutes, chilled on ice, then added to the pre-hyb solution at a concentration of 3 X 10⁶ cpm/mL. The membrane was hybridized at 65°C overnight. Following hybridization, the membrane was washed in 2X SSC/0.1% SDS twice for 90 minutes at 65°C. The membrane was exposed to BioMax MR film (Kodak) using an intensifier screen at -70°C overnight. In Method 2, the lineage blot was pre-hybridized at 60°C for 1 hr in ExpressHyb solution (Clontech). The labelled mJagged-1 probe was boiled for 5 minutes, chilled on ice, then

added to the pre-hyb solution at a concentration of 3×10^6 cpm/mL. The membrane was hybridized for 1.5 hour at 60°C. Following hybridization, membranes were washed following manufacturer's instructions. Each membrane was exposed to BioMax MR film (Kodak) using an intensifier screen at -70°C overnight. A comparison of Method 1 and Method 2 demonstrated that Method 2 produced less background and greater signal. Thus, Method 2 was used to probe each lineage blot with the remaining probes. The membranes were exposed overnight, and for three days.

2.8 Cloning mJagged-1

A complete description of mJagged-1 cloning is given in Figure 4.

To clone the mJagged-1 cDNA by library screening, a probe was generated by Brenda Cohen in Dr. Phillip's lab. RT-PCR was performed on e14 embryonic liver using the upstream primer 5'-GACCAGAATGGCAACAAAACCTGC-3', and the downstream primer 5'-TCCAGCTGACAGAGGTTTCC-3', based on rat Jagged-1 and chicken Serrate-1 sequence alignment. The primers correspond to nucleotides 640-1577 of rat Jagged-1 to give a 937 base pair product. The PCR product was cloned into the pGEMT vector (Promega). Sequencing from both ends confirmed that the mouse clone was highly related to rat Jagged-1.

The 937 base pair probe was used to screen a mouse adult brain library. The library was oligodT and random primed, and cloned in the λ ZAPII vector (Stratagene). The probe was labelled with ^{32}P -dCTP (Amersham) using a High Prime DNA labelling kit (Boehringer

Rat Jagged-1 cDNA sequence

Mouse Jagged-1 Cloning Strategy

Fig 4: Description of Mouse Jagged-1 Cloning

Mannheim). The probe was purified on a sephadex NICK column (Pharmacia Biotech). The nylon membranes, on which the library was lifted, were first incubated at 55°C for two hours in a pre-hybridization solution consisting of 1%SDS, 1M NaCl, 10% dextran sulphate, 50 mM Tris pH 7.5, and 1X Denhardt's solution. The probe, at a final concentration of 1.3×10^6 cpm/mL, and sheared salmon sperm DNA, at a final concentration of 100 µg/mL, were boiled for 10 minutes, chilled on ice, and then added to the pre-hybridization solution. Membranes were hybridized overnight at 55°C. Following hybridization, membranes were rinsed in a wash solution of 2X SSC/0.5% SDS; washed at 55°C for 20 minutes in 2X SSC/0.5% SDS; washed at 55°C for 30 minutes in 1X SSC/0.5%SDS; and washed twice at 55°C for 30 minutes in 0.5X SSC/0.5% SDS. The filters were exposed to BioMax MR film (Kodak) using intensifier screens at -70°C, for three nights. Three positive clones were identified in the primary screen. A secondary screen was performed on the positive plaques, following manufacturer's instructions. On the secondary screen, only one clone was positive. A plasmid containing the insert was excised from the phage, following manufacturer's instructions. Sequencing from both ends of the insert confirmed the near identity of this mouse clone to rat Jagged-1. The cDNA clone (Clone 1) encompasses nucleotides 361-1704 of rat Jagged-1 sequence. This clone was sequenced in its entirety by ACGT Corporation of Toronto. The same library was re-probed with Clone 1, but did not yield any new clones.

The 5' end of mJagged-1, was obtained by RT-PCR. The upstream primer used was 5'-GGATCCATGGGGTCCCCACGGAC-3', and the downstream primer used was 5'-GTTTATCATGCCTGAGTGAGAAGCC-3', to give a 495 base product. The upstream primer was designed based on rat Jagged-1 sequence, and includes a BAMHI site at the 5' end of

the primer. The downstream primer was designed based on sequence from Clone 1. RT-PCR was performed on e16 mouse whole embryo. A PCR product was obtained, and cloned into the pCR 2.1 vector. End sequencing confirmed the near identity of a mouse clone (Clone 2) to the 5' region of rat Jagged-1. The clone was sequenced in its entirety by ACGT Corp. Clone 1 and Clone 2 have an overlapping StuI site at nucleotide 415.

To obtain the remainder of mJagged-1 cDNA, an e10 mouse embryonic library was screened. The library was oligodT and random primed, and cloned in the λ EXlox vector (Novagen). The library was lifted on nylon membranes (Qiabran, Qiagen). Two probes were used to screen this library: Clone 1, and the 641 base pair 3'UTR of mJagged-1 used to screen the lineage blot. The probes were labelled as previously described. The filters were pre-hybridized in the solution previously described for one hour at 50°C. Sheared salmon sperm DNA, and the probes were added to the pre-hybridization solution as previously described, with the Clone 1 probe at a final concentration of 1.9×10^6 cpm/mL, and the 3'UTR probe at a final concentration of 1.2×10^6 cpm/mL. Membranes were hybridized overnight at 50°C. Membranes were rinsed in a wash solution of 2X SSC/0.5% SDS; washed at 50°C for 25 minutes in 2X SSC/0.5%SDS; washed at 50°C for 30 minutes in 1X SSC/0.5% SDS; washed at 50°C for 45 minutes in 0.5X SSC/0.5% SDS; washed at 50°C for 40 minutes in 0.5X SSC/0.5% SDS; and washed at 50°C for 20 minutes in 0.2X SSC/0.5%SDS. Membranes were exposed to film as previously described. Eleven positive clones were identified on the primary screen. A secondary screen was performed on the positive plaques, following manufacturer's instructions. On the secondary screen, nine positive clones were identified. A tertiary screen was also performed on the nine positive plaques. These plaques were all positive. A plasmid containing the insert was excised from the phage,

following manufacturer's instructions. These clones were end sequenced and found to consist mainly of the mJagged-1 3'UTR. However, one clone (Clone 3) starts at nucleotide 2968 of the rat Jagged-1 sequence and extends to the polyA-tail, containing 695 nucleotides of coding sequence at the 3' end. This clone was sequenced in its entirety by ACGT Corp.

To obtain the remaining fragment of mJagged-1, RT-PCR was performed. The upstream primer used was 5'-TGGTTATCGCTGTATCTGTCCACCT-3', and the downstream primer used was 5'-CCACTGTTAAGACAGAGCTCAGCA-3'. The primers cover nucleotides 1410-3241 of the rat Jagged-1 sequence to give a 1831 base pair PCR product. Total RNA was obtained from mouse adult lung, a tissue found to express high levels of mJagged-1. RT-PCR was performed using the Advantage cDNA PCR kit, following manufacturer's instructions (Clontech). Advantage KlenTaq Polymerase Mix was used, which has proofreading abilities. The PCR product was cloned into pCR2.1. End sequencing confirmed the near identity of a mouse clone to rat Jagged-1 (Clone 4). The clone was sequenced in its entirety by ACGT Corp. Clone 4 and Clone 1 have an overlapping NheI site at nucleotide 1477. Clone 4 and Clone 3 have an overlapping SacI site at nucleotide 3226.

3.1 RT-PCR

The expression of the Notch receptors, Dll1, mJagged-1, and the Fringe genes in whole bone marrow was examined by RT-PCR. For each reaction a negative control consisting of non-reverse transcribed total RNA ensured there was no contamination from genomic DNA. A positive control using cDNA from a tissue known to express the gene of interest ensured that the PCR reaction amplified the correct product. A second positive control consisting of β -actin PCR on cDNA from whole bone marrow ensured that the reverse transcription reaction was effective. Because PCR was not done quantitatively, it was not possible to quantitate the relative levels of expression of the genes of interest. Results are shown in Figures 5A-C.

As shown in Figure 5A, mNotch1, mNotch2, mNotch3, and mNotch4 were all found to be expressed in whole bone marrow. Dll1 and mJagged-1 expression are shown in Figure 5B. There was a very weak band for the Dll1 PCR product from whole bone marrow, while mJagged-1 was found to be expressed in whole bone marrow. As shown in Figure 5C, Lunatic Fringe, Manic Fringe, and Radical Fringe were all found to be expressed in whole bone marrow.

Fig.5A RT-PCR Analysis of mNotch1, mNotch2, mNotch3, and mNotch4 Expression in Bone Marrow.

Fig. 5B RT-PCR Analysis of *mJagged-1* and *Dll1* Expression in Bone Marrow

Fig.5C RT-PCR Analysis of Lunatic Fringe, Manic Fringe, and Radical Fringe Gene Expression in Bone Marrow

3.2 3' Untranslated Region Probes

RT-PCR analysis of whole bone marrow demonstrated that all the Notch receptors, Dll1, mJagged-1, and the Fringe genes were expressed. To determine specifically which genes were being expressed in which cells of the bone marrow, a slot blot consisting of amplified cDNAs from precursor cells (whose fate is known), from maturing cells, and three fibroblast cell lines was probed. The three fibroblast cell lines examined were: NIH 3T3; S17, a marrow derived stromal cell line (Collins et al, 1987); and 95/1.7, a marrow derived fibroblastoid cell line (Iscove et al, 1988). The amplified cDNAs were constructed such that they consist of 200-600 bp of the most 3' untranslated regions (3' UTR) of the genes. A full description of the lineage blot can be found in Chapter 2.

To probe the lineage blot it was first necessary to obtain the appropriate probes. By using a combination of library screening, PCR, and 3' RACE techniques 3' UTR probes for mNotch1, mNotch3, mNotch4, Dll1, mJagged-1, Manic Fringe, and Radical Fringe were obtained. Probes for mNotch2 and Lunatic Fringe could not be obtained. 3' UTR sequence for mJagged-1, Manic Fringe and Radical Fringe are given in Appendix A, B, and C respectively.

Each probe was first tested on a multiple tissue Northern blot (MTN blot) to ensure that it detected the correct transcript. The MTN blot consists of 2 µg polyA RNA from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Results are shown in Figures 6A-G. Two MTN blots were needed to test the probes. One MTN blot was used to test probes for mJagged-1, Dll1, mNotch1, mNotch4, and mNotch3, in that order. A second MTN blot was used to test probes for Manic Fringe, and Radical Fringe, in that order.

Fig. 6A - mNotch1 3' UTR Probe Tested on MTN Blot

Fig. 6B - mNotch3 3' UTR Probe Tested on MTN Blot

Fig. 6C - mNotch4 3' UTR Probe Tested on MTN Blot

Fig. 6D - Dll1 3' UTR Probe Tested on MTN Blot

Fig. 6E - mJagged-1 3' UTR Probe Tested on MTN Blot

Fig. 6F - Manic Fringe 3' UTR Probe Tested on MTN Blot

Fig. 6G - Radical Fringe 3' UTR Probe Tested on MTN Blot

As shown in Figure 6A-G, each probe detected a transcript of the correct size in the appropriate tissues (refer to Table 1). The probe for Manic Fringe is shown in Fig. 6F. There are actually two Manic Fringe transcripts of 2.1 kb and 4.4 kb; however, the probe was only able to detect the 2.1 kb transcript. Radical Fringe shown in Fig. 6G, also has two transcripts of 2.8 kb and 3.7 kb, both of which were detected by the probe. The difference between the two transcripts in both cases is unknown.

3.3 Expression of the Notch Receptors, Notch Ligands, and the Fringe Genes in Lineage Precursor Cells, Maturing Cells, and Fibroblast Cell Lines

3' UTR probes labelled with ^{32}P were used to screen the lineage blots (one blot per probe). Two methods were first tested using the mJagged-1 probe. The first method involved a traditional overnight hybridization, while the second method involved a 90 minute hybridization using ExpressHyb solution (Clontech). Following hybridization, the membranes were washed and exposed to film overnight. Both methods gave the same hybridization pattern (overnight hybridization lineage blot not shown), however the ExpressHyb method was better: there was greater signal with less background. Thus, the ExpressHyb method was used to probe each lineage blot with the remaining probes.

Each lineage blot was exposed overnight, and for three days. The signal detected is proportional to the level of transcript in each sample. The probed lineage blots are shown in Figures 7A-H. Figures 7A-F are three day exposures, while Fig. 7G-H are overnight exposures.

Fig. 7A - Transcript Expression of mNotch1 in Various Hematopoietic Lineages

Fig. 7B - Transcript Expression of mNotch3 in Various Hematopoietic Lineages

Precursor populations:

- 1 - erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell
- 2 - erythrocyte/megakaryocyte/macrophage/neutrophil
- 3 - erythrocyte/megakaryocyte/macrophage
- 4 - erythrocyte/megakaryocyte
- 5 - macrophage/neutrophil
- 6 - BFU-E
- 7 - CFU-E
- 8 - macrophage
- 9 - neutrophil
- 10 - megakaryocyte

Maturing Populations:

- 11 - erythrocytes
- 12 - neutrophils
- 13 - mast cells
- 14 - megakaryocytes
- 15 - B cells
- 16 - macrophages
- 17 - T cells

Fibroblast Cell Lines:

- 19 - NIH 3T3
- 20 - S17
- 21 - 95/1.7

Negative Control:

Fig. 7C - Transcript Expression of mNotch4 in Various Hematopoietic Lineages

Fig. 7D - Transcript Expression of Dll1 in Various Hematopoietic Lineages

Precursor populations:

- 1 - erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell
- 2 - erythrocyte/megakaryocyte/macrophage/neutrophil
- 3 - erythrocyte/megakaryocyte/macrophage
- 4 - erythrocyte/megakaryocyte
- 5 - macrophage/neutrophil
- 6 - BFU-E
- 7 - CFU-E
- 8 - macrophage

Maturing Populations:

- 11 - erythrocytes
- 12 - neutrophils
- 13 - mast cells
- 14 - megakaryocytes
- 15 - B cells
- 16 - macrophages
- 17 - T cells

Fibroblast Cell Lines:

- 19 - NIH 3T3
- 20 - S17
- 21 - 95/1.7

Negative Control:

Fig. 7E - Transcript Expression of mJagged-1 in Various Hematopoietic Lineages

Precursor populations:

- 1 - erythrocyte/megakaryocyte/macrophage/neutrophil/
mast cell
- 2 - erythrocyte/megakaryocyte/macrophage/neutrophil
- 3 - erythrocyte/megakaryocyte/macrophage
- 4 - erythrocyte/megakaryocyte
- 5 - macrophage/neutrophil
- 6 - BFU-E
- 7 - CFU-E
- 8 - macrophage

Fig. 7F - Transcript Expression of Manic Fringe in Various Hematopoietic Lineages

Maturing Populations:

- 11 - erythrocytes
- 12 - neutrophils
- 13 - mast cells
- 14 - megakaryocytes
- 15 - B cells
- 16 - macrophages
- 17 - T cells

Fibroblast Cell Lines:

- 19 - NIH 3T3
- 20 - S17
- 21 - 95/1.7

Negative Control:

Fig. 7G - Transcript Expression of Radical Fringe in Various Hematopoietic Lineages

Precursor populations:

- 1 - erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell
- 2 - erythrocyte/megakaryocyte/macrophage/neutrophil
- 3 - erythrocyte/megakaryocyte/macrophage
- 4 - erythrocyte/megakaryocyte
- 5 - macrophage/neutrophil
- 6 - BFU-E
- 7 - CFU-E
- 8 - macrophage

Fig. 7H - Transcript Expression of L32 in Various Hematopoietic Lineages

Maturing Populations:

- 11 - erythrocytes
- 12 - neutrophils
- 13 - mast cells
- 14 - megakaryocytes
- 15 - B cells
- 16 - macrophages
- 17 - T cells

Fibroblast Cell Lines:

- 19 - NIH 3T3
- 20 - S17
- 21 - 95/1.7

Negative Control:

Figure 7H shows a lineage blot (loaded with only 5 μ l of PCR material, as opposed to 20 μ L for all other blots) probed for L32, a 'housekeeping gene', which encodes a ribosomal protein. The signal detected for L32 is indicative of the amount of cDNA loaded in each slot.

The assignment of the intensity of signal for each sample after a three day exposure is as follows: *no signal detected* above background; *possible signal* detected above background; *definite signal*; and *strong signal*, where the area of the signal is at least 4 times the area of the slot. Results are shown in Table 4.

Definite signal for mNotch1 transcripts was detected in macrophage precursors, maturing macrophages, NIH 3T3, S17, and 95/1.7.

Definite signal for mNotch3 transcripts was detected in all samples.

Definite signal for mNotch4 transcripts was detected in maturing macrophages, and S17.

Definite signal for Dll1 transcripts was detected in all samples.

mJagged-1 transcripts were detected in very distinct populations. Possible signal was detected in erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell precursors, erythrocyte/megakaryocyte/macrophage/neutrophil precursors, and megakaryocyte precursors; definite signal was detected in erythrocyte/megakaryocyte precursors, BFU-Es, maturing erythrocytes, maturing neutrophils, maturing B cells, maturing macrophages, S17, and 95/1.7; strong signal was detected in maturing mast cells, and maturing megakaryocytes.

Manic Fringe transcripts were detected in very distinct populations. Possible signal was detected in maturing T cells, NIH 3T3, and 95/1.7; definite signal was detected in erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell precursors, erythrocyte/megakaryocyte/macrophage/neutrophil precursors, BFU-Es, CFU-Es, megakaryocyte precursors, maturing erythrocytes, maturing neutrophils, maturing mast cells, maturing

Table 4: Expression of the Notch Receptors, Notch Ligands, and the Fringe Genes in Lineage Precursor Cells, Maturing Cells, and Fibroblast Cell Lines

<u>Slot #</u>	<u>Lineage</u>	<u>mNotch1</u>	<u>mNotch3</u>	<u>mNotch4</u>	<u>Dll1</u>	<u>mJagged-1</u>	<u>Manic Fringe</u>	<u>Radical Fringe</u>
<i>Precursor cells</i>								
1	E/Meg/Mac/Neut/Mast	0	++	0	++	+	++	+++
2	E/Meg/Mac/Neut	0	++	0	++	+	++	+++
3	E/Meg/Mac	0	++	0	++	0	+++	+++
4	E/Meg	0	++	0	++	++	+++	+++
5	Mac/Neut	0	++	0	++	0	+++	+++
6	BFU-E	0	++	0	++	++	++	+++
7	CFU-E	0	++	0	++	0	++	+++
8	Mac	++	++	0	++	0	+++	+++
9	Neut	0	++	0	++	0	+++	+++
10	Meg	0	++	0	++	+	++	+++
<i>Maturing Populations</i>								
11	Erythrocytes	0	++	0	++	++	++	+++
12	Neutrophils	0	++	0	++	++	++	+++
13	Mast cells	0	++	0	++	+++	++	+++
14	Megakaryocytes	0	++	0	++	+++	++	+++
15	B cells	0	++	0	++	++	+++	+++
16	Macrophages	++	++	++	++	++	++	+++
17	T cells	0	++	0	++	0	+	+++
<i>Negative Control</i>								
18	air	0	0	0	0	0	0	0
<i>Stromal Cell Lines</i>								
19	NIH 3T3	++	++	0	++	0	+	+++
20	S17	++	++	++	++	++	+++	+++
21	95/1.7	++	++	0	++	++	+	+++

Legend:

Abbreviations: E - erythrocytes; Meg - megakaryocytes; Mac - macrophages; Neut - neutrophils; Mast - mast cells; BFU-E - burst forming unit-E; CFU-E - colony forming unit-E

Assignment of intensity of signal:

- 0 - background levels in 'air' sample
- +
- ++ -definite signal
- +++ - strong signal; area of signal is at least 4X times the area of the slot

megakaryocytes, and maturing macrophages; strong signal was detected in erythrocyte/megakaryocyte/macrophage precursors, erythrocyte/megakaryocyte precursors, macrophage/neutrophil precursors, macrophage precursors, neutrophil precursors, maturing B cells, and S17.

Strong signal for *Radical Fringe* was detected in all samples. Low signal was also detected in the negative control. However, signal detected in sample slots were above this background.

3.4 mJagged-1 Cloning

To perform functional assays on the role of the Notch receptors, Notch ligands, and the *Fringe* genes in hematopoiesis it is necessary to clone the cDNAs for each of these genes. Our lab has cloned the cDNA for *Dll1* and *mNotch1*, while Dr. Sean Egan's lab has cloned the cDNA for *Lunatic Fringe*, *Manic Fringe*, *Radical Fringe*, and *Dll1*. Described here is the cloning of mouse *Jagged-1* cDNA. Mouse *Jagged-1* has not yet been described in the literature.

A complete description of *mJagged-1* cloning is given in Chapter 2. The cDNA for *mJagged-1* was obtained in four fragments. Clone 1 was obtained from a mouse adult brain library, Clone 2 was obtained by RT-PCR on e16 whole mouse embryo, Clone 3 was obtained from an e10 mouse embryonic library, and Clone 4 was obtained by RT-PCR on mouse adult lung. Figure 4 illustrates unique restriction enzyme sites that can be used to obtain the full length cDNA. Full length cDNA sequence is given in Appendix E.

4.1 Discussion of Results

The expression of Notch and its known role in cell-fate decisions in a wide range of systems suggests that the Notch signalling pathway may be an important regulator of the blood forming system. Indeed, RT-PCR analysis of whole bone marrow demonstrated the expression of transcripts for the Notch receptors, Dll1, mJagged-1, and the Fringe genes, suggesting that they are candidate genes to examine in hematopoiesis.

Whole bone marrow consists of hematopoietic cells as well as stromal cells and adipocytes. RT-PCR of whole bone marrow determines whether the transcript for a particular gene is expressed, but gives no indication as to which cell(s) are expressing the gene. Since Notch signalling occurs through cell-cell interactions via transmembrane receptors and ligands, it is critical to determine which cells in the bone marrow are expressing which candidate genes. Given this information one can surmise which genes are important in which cells, and specific functional experiments can then be designed.

To obtain expression data for the Notch receptors, Notch ligands, and the Fringe genes through hematopoiesis, a lineage blot consisting of amplified cDNAs from a hierarchy of hematopoietic precursor cells, from maturing hematopoietic cells, and three fibroblast cell lines was probed. The cDNA library consists of the most 3' untranslated regions (3' UTR) of mRNA transcripts in that cell. To screen the blot it was first necessary to clone the appropriate probe for each candidate gene. The lineage blots were screened for the expression of mNotch1, mNotch3,

mNotch4, Dll1, mJagged-1, Manic Fringe, and Radical Fringe transcripts. Probes for mNotch2 and Lunatic Fringe could not be obtained. To be complete, the expression of mNotch2 and Lunatic Fringe on the lineage blot needs to be examined.

Probed lineage blots are shown in Figures 7A-G, however caution must be taken in interpreting the results. Signal detected for different probes for a particular sample cannot be compared. One cannot be sure that two different probes will bind to their transcript with equal efficacy. Secondly, the lineage blot is not completely quantitative. Examination of the amplified cDNAs initially constructed from single cell precursors suggested that the relative abundance of transcripts was maintained (Brady et al, 1995). However, in constructing the amplified cDNAs for the lineage blots used in this study the initially constructed amplified cDNAs were manipulated and amplified two times by PCR. Due to these further manipulations it is not certain that the relative abundance of transcripts was faithfully maintained. Figure 7H shows a lineage blot probed for L32, a housekeeping gene. The signal detected in each of the samples was relatively equal, except for the erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell sample, erythrocyte/megakaryocyte/macrophage/neutrophil sample, neutrophil precursor sample, and maturing neutrophil sample, suggesting that the relative levels of L32 transcript was maintained. Again, it is difficult to be certain that relative abundance of transcripts was maintained, thus it is not possible to comment precisely on the relative levels of expression of a gene between samples.

It is interesting to examine the expression of transcript for a particular gene in cells through differentiation. The sample populations tested on the lineage blot can be arranged to give a hierarchical relationship between these samples through differentiation. With such a diagram one can follow the expression of a particular gene through differentiation in various lineages. The expression of transcript for mNotch1, mNotch3, mNotch4, Dll1, mJagged-1, Manic Fringe, and

Radical Fringe through a hierarchy of hematopoietic cells is given in Figures 8A-G. Each circle represents a stage in the hierarchy, with cells at the top being the most undifferentiated and cells at the bottom being the most differentiated. The shading in the circles correspond to the level of signal detected listed in Table 4.

Again, caution must be taken in interpreting these results. As mentioned earlier, the lineage blot is not completely quantitative thus it is difficult to comment precisely on the dynamics of transcript expression through differentiation. Furthermore, the lineage blot only provides information on the expression of mRNA transcripts. The presence of transcript for a particular gene in a cell however, does not necessarily signify the presence of protein or the levels of protein, as mRNA transcripts are regulated at the level of translation and degradation. To be complete, the cell-surface protein expression of the Notch receptors, Dll1, mJagged-1, and the secretion of the Fringe proteins should be determined. However, assuming that the presence of transcript reflects the presence of protein, there are a number of interesting observations and comments that can be made.

mNotch1 expression was detected for macrophage precursors, maturing macrophages, and the three fibroblast cell lines on the lineage blot (Fig. 8A). A study of a population of human CD34⁺ cells, enriched for early precursors and stem cells, was found to express Notch1 (TAN-1) by RT-PCR (Milner et al, 1994). A known role for Notch is to inhibit differentiation. One could hypothesize that early precursors expressing Notch1 could receive a signal to inhibit differentiation, thus maintaining a population of undifferentiated precursors. Given these observations, it was expected that multipotent precursors on the lineage blot would express mNotch1. In addition, studies of T cells demonstrated mNotch1 protein expression at intermediate levels in mature

Figure 8A: Expression of mNotch1 Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8B: Expression of mNotch3 Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8C: Expression of mNotch4 Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8D: Expression of Dll1 Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8E: Expression of mJagged-1 Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8F: Expression of Manic Fringe Transcript Within a Hierarchy of Sampled Hematopoietic Cells

Figure 8G: Expression of Radical Fringe Transcript Within a Hierarchy of Sampled Hematopoietic Cells

CD4⁺CD8⁻ and CD4⁺CD8⁺ T cells sorted from thymus (Hasserjian et al, 1996). However, CD4⁺CD8⁻ and CD4⁺CD8⁺ T cells from spleen on the lineage blot were not found to express mNotch1.

There are a number of possibilities for the observed results: relative abundance of transcripts was not maintained in the amplified cDNAs, mNotch1 was truly not expressed in those samples that have no signal, or that the mNotch1 probe was not efficient enough to detect transcript. The latter possibility may reflect the true situation. Hybridization of the lineage precursor blot with probes for cytokine receptors was often not detected, despite the fact that the samples expressed the receptor since the cells were able to respond to the corresponding cytokine (Dr. Iscove, personal communication). Analysis of the 'initially negative' amplified cDNAs by specific secondary PCR demonstrated that the receptor transcripts were indeed present. Thus, the lineage blot has a limit of sensitivity for detecting transcripts. A sample that does not seem to express a particular gene as determined by the lineage blot, may be found to express the gene by secondary PCR.

mNotch3 expression was detected in all samples on the lineage blot (Fig. 8B). In this case if mNotch3 is important for inhibiting differentiation, specific interactions between a cell expressing mNotch3 (which is to receive the inhibition of differentiation signal) and a cell expressing the ligand are important. As previously mentioned, the four mouse Notch receptors are not redundant. It could be hypothesized that each Notch receptor has a particular function in different cells, and that each Notch receptor becomes activated by different ligands. Alternately, it is possible that the probe may be binding non-specifically. It is difficult to determine if this is the case, since the blot is a slot blot as opposed to a Northern blot. However, the mNotch3 3'

UTR probe only hybridized to an appropriate sized transcript on the MTN blot, and no signal was seen in the negative control sample on the lineage blot, suggesting that this is not the case.

mNotch4 expression was detected at low levels in maturing macrophages, and S17 on the lineage blot (Fig. 8C). It is interesting to find the expression of mNotch4 in these populations, since mNotch4 has been found to be typically expressed in endothelial cells of embryonic mice, and also likely in endothelial cells of adult tissues (Uyttendaele et al, 1996).

Dll1 expression was detected in all samples on the lineage blot (Fig. 8D). It was surprising that there was no distinct pattern of Dll1 expression in precursors, maturing populations, or the stromal cell lines. Again, a known role of Notch is to inhibit differentiation. It could be expected to find Notch ligand expression in committed precursors and maturing populations such that they could signal back to early Notch-expressing progenitors to remain in an undifferentiated state. Another possibility is that Dll1 signals through Notch to cause a cell to differentiate along a particular pathway instead of another. Such a model would require specific cell-cell interactions. Hematopoietic cells are known to be in contact with the stroma of the bone marrow. It is interesting that Dll1 was found to be expressed in NIH 3T3, S17, and 95/1.7 cells. Such cell-cell interactions may be a source of Notch signalling.

There are a number of factors to explain the ubiquitous expression of Dll1 in hematopoietic cells. One possibility is that Dll1 does not play a significant role in Notch signalling in hematopoiesis. In fact, additional mouse Delta homologs exist, although they have yet to be characterized (Nye et al, 1995). It is possible that other mouse Delta ligands may play a role in hematopoiesis. A second possibility relates to an example of Notch-Delta signalling. The classic example of Notch signalling in cell-fate determination is during neurogenesis in the CNS of *Drosophila*. As described earlier, a cluster of cells, all expressing Delta and Notch, have equal

potential to become neuroblasts or epidermoblasts. A natural fluctuation in the levels of expression of Delta and/or Notch occurs, which becomes further enhanced by a feedback mechanism. This results in cells expressing high levels of Delta to be surrounded by cells expressing high levels of Notch. A Delta expressing cell sends a signal to the Notch expressing cell to not become a neuroblast, and later become an epidermoblast. In this system the relative levels of expression and the dynamics of expression of Notch and Delta are important for signalling. The lineage blot does not provide this information, and it is these subtle differences that may be important in Notch-Delta signalling. Alternately, as mentioned for mNotch3, it is possible that the probe may be binding non-specifically. However, the Dll1 3' UTR probe only hybridized to an appropriate sized transcript on the MTN blot, and no signal was seen in the negative control sample on the lineage blot, suggesting that this is not the case.

mJagged-1 expression was quite varied (Fig. 8E). Strong expression was detected in maturing mast cells, and maturing megakaryocytes; expression was detected in erythrocyte/megakaryocyte precursors, BFU-Es, maturing erythrocytes, maturing neutrophils, maturing B cells, maturing macrophages, S17, and 95/1.7; and there was possible expression in erythrocyte/megakaryocyte/macrophage/neutrophil/mast cell precursors, and erythrocyte/megakaryocyte/macrophage/neutrophil precursors. As for Dll1, one could hypothesize that mJagged-1 should be particularly expressed in committed precursors, maturing populations, and stromal cells if it plays a role in inhibiting differentiation of Notch expressing precursors. However, the expression of mJagged-1, as determined on the lineage blot, is not exclusive to committed precursors nor do all committed precursors express mJagged-1. One could also hypothesize, as for Dll1, that mJagged-1 signals through Notch to direct differentiation

along a particular pathway since mJagged-1 was found to be expressed in S17, and 95/1.7 cells. In both models, mJagged-1 signalling may be important in particular lineages.

Expression of Manic Fringe was also found to be quite varied (Fig. 8F). Strong expression was detected in erythrocyte/megakaryocyte/macrophage precursors, erythrocyte/megakaryocyte precursors, macrophage/neutrophil precursors, macrophage precursors, neutrophil precursors, maturing B cells, and S17 on the lineage blot. In *Drosophila* it has been demonstrated that the secreted protein Fringe inhibits Notch activation by Serrate (Jagged), but may facilitate Notch activation by Delta (Irvine et al, 1994; Egan et al, 1997). The mechanism by which this occurs is not understood. From the lineage blot there is no apparent correlation between Dll1, mJagged-1, and Manic Fringe expression.

Manic Fringe may regulate whether a particular Notch expressing cell receives a signal through Dll1 or mJagged-1. The source of the Notch signal may be important for the outcome of that cell. In *Drosophila*, it has been demonstrated that Delta and Serrate (Jagged) are not entirely redundant ligands. In *Drosophila*, the Serrate protein can substitute for Delta during neuroblast segregation (Gu et al, 1995). During wing development however, Delta can only activate Notch in the dorsal compartment of the wing disc, while Serrate can only activate Notch in the ventral compartment of the wing disc, although Notch functions in both these compartments (Doherty et al, 1996; Couso et al, 1995; Jonsson et al, 1996; Kim et al, 1995; Speicher et al, 1994).

Strong Radical Fringe expression was detected in all samples on the lineage blot (Fig. 8G). The difference in the ability of Lunatic Fringe, Manic Fringe, and Radical Fringe to inhibit Notch activation by mouse Jagged ligands and enhance that by mouse Delta ligands is not known. It is interesting that Radical Fringe was found to be expressed in all samples, while Manic Fringe was found to be expressed in distinct populations. This result suggests that Radical Fringe does not

play a role in regulating the Notch signal in a cell-specific manner, or that there is another mechanism for cell specificity. Alternately, it is possible that the probe may be binding non-specifically. The Radical Fringe 3' UTR probe slightly cross-reacted with other transcripts on the MTN blot, and a signal was seen in the negative control sample on the lineage blot. To confirm that the signal is real, one could perform PCR on the pooled precursor cDNA samples.

The Wnt family of proteins also regulate Notch signalling. In mouse, there are at least 12 members of the Wnt gene family (reviewed in Nusse et al, 1992). This family of proteins is involved in regulating cell proliferation, differentiation, and survival. It has been demonstrated that the secreted protein Wingless, the *Drosophila* homolog of the Wnts, inhibits Notch activation by Delta and may facilitate the activation of Notch by Serrate (Jagged) (Axelrod et al, 1996; Couso et al, 1995; Jonnson et al, 1996). It seems that *Drosophila* Fringe and Wingless act antagonistically to determine whether a Notch expressing cell will receive a signal by Delta or Serrate (Jagged). In mouse, the different members of the Fringe and Wnt families may interact to regulate Notch signalling by different ligands.

4.2 Interpretation of Results

There was no apparent expression of the candidate genes in distinct populations (ie. expression only in pluripotent precursors or committed cells), or overlapping expression patterns (two or more genes expressed in the same populations) seen on the lineage blot. Thus it is difficult to surmise which cell populations and genes could potentially be interacting.

From information on the expression of transcripts of candidate genes in samples within a hierarchy of hematopoietic differentiation, it is difficult to predict a model for their role. Even if one accepts the limitations of interpretation of the lineage blot, it is not possible to infer function from patterns of transcript expression. Specifically, the lineage blot gives no indication of cell-cell interactions occurring during growth and differentiation in the *in vitro* cultures. Cell-cell interactions are required for Notch signalling, thus it is difficult to predict which cell types might be involved in signalling if it is not known which Notch expressing cells are adjacent to Notch ligand expressing cells.

In addition, samples on the lineage blot were obtained from *in vitro* cultures which may not reflect *in vivo* conditions in bone marrow. Artificial *in vitro* conditions may result in aberrant up-regulation or down-regulation of transcription of various genes. It would be interesting to perform immunohistochemical analysis on bone marrow sections to determine protein expression of the various candidate genes. Theoretically, one would like to identify cells at specific stages along the differentiation hierarchy and determine which proteins they are expressing. This could provide information on adjacent cells expressing receptor and ligand that could potentially be involved in Notch signalling. Such an analysis would be difficult for a number of reasons. Unlike vertebrate CNS development for example, where different zones of the neural tube correspond to particular cell types at distinct stages of differentiation, there is no such correspondence in bone marrow. Additionally, individual markers to identify cells at a particular stage in the differentiation pathway do not exist for all cells, particularly for early precursors.

4.3 Future Experiments

To elucidate the role of the Notch receptors, Notch ligands, and the Fringe genes in hematopoiesis it is necessary to perform functional experiments. Transcript expression data obtained from the lineage blots provides information to direct future experiments.

mJagged-1 and Manic Fringe were found to have the most distinct expression pattern suggesting that they may be important in regulating differentiation through Notch signalling. An *in vitro* system could be designed to examine the functional role of mJagged-1 and Manic Fringe in myelopoiesis or lymphopoiesis, for example. Whole bone marrow (or various sorted cells, as described in Table 3A) could be plated on a layer of stromal cells under conditions that fully support myeloid differentiation or lymphoid differentiation. One set of samples would be plated under 'wild type' culture conditions, while another set of samples would be plated under 'modified' culture conditions. The 'modified' culture conditions would consist of stromal cells expressing cell-surface mJagged-1 protein (cDNA cloning described here), stromal cells expressing secreted Manic Fringe protein (cDNA cloned in Dr. Sean Egan's lab), or stromal cells expressing both. A comparison of the lineages represented and the size of the colonies obtained from each of the culture conditions can provide information on the role of Notch signalling in regulating differentiation and proliferation.

An *in vivo* experiment could also be designed to look at the role of mJagged-1 and Manic Fringe in hematopoiesis. A study by Pear *et al* used a retroviral construct consisting of the promoter elements of the Moloney murine leukemia virus long terminal repeats (MoMLV LTR; which drives expression in most hematopoietic cell types), to over-express various forms of activated human Notch1 (TAN-1). The retroviral constructs were used to infect mouse bone

marrow cells *in vitro*, which were then used to reconstitute lethally irradiated syngeneic mice (Pear et al, 1996). It was found that activated Notch alleles resulted in the exclusive development of T cell neoplasms. Similar experiments could be performed using the same retroviral construct to overexpress mJagged-1 or Manic Fringe in hematopoietic cells used to reconstitute lethally irradiated mice. A thorough analysis of the number of cells, types of lineages, and the stages of differentiation represented in the peripheral blood, thymus, spleen, and lymph nodes of the reconstituted mice could indicate the lineage these genes are important in, and what their effect is.

4.4 Summary

Hematopoiesis is a complex process of differentiation and proliferation in which totipotent stem cells are able to give rise to all the mature blood cell types throughout the lifetime of an organism. Differentiation and cell cycling must be controlled to ensure that proliferation is controlled, that stem cells do not become extinguished, and that systemic demands for particular blood cell types are met. Considering the degree of regulation required to meet these criteria it seems likely that the hematopoietic microenvironment plays a role in the process of hematopoiesis, and that hematopoiesis is not purely stochastic.

Cell-fate decisions of the type made during the process of hematopoiesis also occur in a wide range of tissues of all organisms throughout development. Surprisingly, the Notch signalling system has been found to be a common element in cell-fate determination throughout a diverse range of tissues. Notch signalling has been found to inhibit differentiation of Notch-activated cells, but has also been found to play a role in determining one cell-fate over another.

Described here is an analysis of the expression of the Notch receptors, Notch ligands, and the Fringe genes in cells of the blood system. The expression of transcript for mNotch1, mNotch3, mNotch4, Dll1, mJagged-1, Manic Fringe, and Radical Fringe through a hierarchy of hematopoietic precursors, maturing blood cell populations, and three fibroblast cell lines were examined. Distinct transcript expression patterns were found for mJagged-1 and Manic Fringe. These expression data can be used to guide future functional experiments.

A small number of studies have examined aspects of Notch in hematopoiesis, and Notch signalling has indeed been found to play a role in a number of systems. The distinct expression of mJagged-1 and Manic Fringe through a hierarchy of hematopoietic precursors described here also seems to suggest a role for Notch in hematopoiesis. Given the role of Notch in cell-fate decisions in numerous other systems, it is very likely that Notch signalling plays a role in regulating the proliferation and differentiation of cells of the blood system.

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CAGGACAACAGAGACTTGGAAAGTGCCAGAGCTTGAACCGGATGGAATACAT
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TTGTATGTAAAAA

italics - 3' RACE primer

bold - stop codon

underline - mJagged-1 3' UTR probe

TCAGCTACGGTGTCTTTGAGGGGAAACTGAATGCCATCAAGCTACCGGGCCCC
TTTCTCCCATGAAGAGGACCCCTCCAGATTCCGCTCCCTCCATTGTCTCCTCTAC
CCAGACACACCCTGGTGTCCGCTGCTGGCAGCGCCCTGA

GCTCTGAACTGCCGCAAAGGATGATGGATGCTACTGCCTGACCACTCCCTCAC
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CATTGGGAGAAGGCCAGGGTCTGTGACCTGCGGCTGTGGAACCTGGTCTCAGCT
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GTGAGGAGCTGCCATGTGACCCAGTGGAACCTGAGCAAAGCCAATAGAAGGGT
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AATCTAAAGTTGTTCTCCCTAACACCTCATCTGTAAGAAATTAAACCTTTATTTAA
GTCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

italics - 3' RACE primer

bold - stop codon

Note: Entire clone was used as the Manic Fringe 3' UTR probe

AACATACAGCAGGACCCTACACGGTTTCAGTCTGTGCACTGCCTTCTCCTACCC
AGACACCCACTGGTGTCTATGAAGAACAGGGTTGAGGGAGCTTTCAGTAA

 CTGCCGACTCCTGGTGGCTGCTCTACCCGGCTCCCTCGGGCCTTAGATCATGG
 CGGTCAGCAGAAGCTGCTTGAAGGCCTTGACCTCGGTCCCAGTTGGACACCAG
 GTACCTCTGGAAAATGAGACAACCGGGCTGTGCGTGCCGAGACCGTCCAGGA
 AGAGAGAAAGGCCAAAGATGCTGAGTGCCTGATGCCTTTAGCCCAGCCACAGGT
 CCTTCCCTGGACAAAGTCTACCACCCCAGCTACCCCAGAGACAGTGGTTCCCA
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 GGCTGCTGTTCTCCATTACCTCCATCTGAGCCCTCCCAGAGACACTGCTGGGA
 GACTTCCTTCTCTAACGTGGTGTTCAGGATGCTTCCCTCTGAGCTTCCCATCG
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 TTTACGGGGAGGCCGAGGGGACCGGTTGCAATGTTGAGTAAAGGCTTCCTTGT
 CATTCGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

italics - 3' RACE primer

bold - stop codon

Note: Entire clone was used as the Radical Fringe 3' UTR probe

Appendix D: Sample Number (by Iscove lab) of Pooled Samples

Slot #	Potential/Lineage	Sample Number
1	E/Meg/Mac/Neut/Mast	1, 158, 159, 160
2	E/Meg/Mac/Neut	2, 3, 4, 5, 6, 161, 162, 7, 8, 9, 10
3	E/Meg/Mac	13, 14, 15, 16, 17, 18, 19, 20, 21
4	E/Meg	22, 23, 24, 25, 26, 27, 28, 29, 30, 33, 34, 36, 37, 39, 41, 163, 164, 165, 31, 32, 35, 38, 40, 42
5	Mac/Neut	43, 44, 45, 46, 47, 48, 166
6	BFU-E	51, 52
7	CFU-E	89, 90
8	Mac	53, 54, 55, 56, 57, 58, 59, 60, 65, 66, 67, 69, 74
9	Neut	75, 76, 77, 78, 79, 80, 81
10	Meg	82, 83, 84, 85, 86, 87, 88
11	E	91, 92, 93
12	Neut	98, 99, 100, 101, 102
13	Mast	103, 104, 105
14	Meg	107, 108, 109, 110, 111, 112
15	B	113, 114, 115, 116, 117, 120, 121, 122, 123, 124, 125
16	Mac	128, 129, 130, 132, 133, 134, 135, 136
17	T	140, 141, 143, 144, 146, 147, 149, 150, 152, 153, 155, 156
18	Air	I40-4
19	NIH 3T3	---
20	S17	---
21	95/1.7	---

Appendix E: Full Length Coding Sequence of Mouse Jagged-1

ATGGGGTCCCCACGGACGCGCGGCCGGCCCGGGCGCCCCCTGAGTCTTCTGC
 TCGCCCTGCTCTGTGCCCTGCGAGCCAAGGTGTGCGGGGCTCGGGTCAGTTT
 GAGCTGGAGATCCTGTCCATGCAGAACGTGAATGGAGAGCTACAGAATGGGA
 ACTGTTGTGGTGGAGTCCGGAACCCTGGCGACCGCAAGTGCACCCGCGACGA
 GTGTGATACGTACTTCAAAGTGTGCCTCAAGGAGTATCAGTCCCGCGTCACTGC
 CGGGGGACCTGCAGCTTCGGCTCAGGGTCTACGCCTGTCAATCGGGGGTAAC
 ACCTTCAATCTCAAGGCCAGCCGTGGCAACGACCGTAATCGCATCGTACTGCCT
 TTCAGTTTCGCCTGGCCGAGGTCTACACTTTGCTGGTGGAGGCCTGGGATTC
 CAGTAATGACACTATTCAACCTGATAGCATAATTGAAAAGGCTTCTCACTCAGG
 CATGATAAACCTAGCCGGCAATGGCAGACACTGAAACAAAACACAGGGATTG
 CCCACTTCGAGTATCAGATCCGAGTGACCTGTGATGACCACTACTATGGCTTTG
 GCTGCAATAAGTTCTGTGCTCCAGAGATGACTTCTTTGGACATTATGCCTGTG
 ACCAGAACGGCAACAAAACCTTGCATGGAAGGCTGGATGGGTCTGTATTGCAAC
 AAAGCTATCTGCCGACAGGGCTGCAGTCCCAAGCATGGGTCTTGTAACACTTCC
 AGGTGACTGCAGGTGCCAGTACGGTTGGCAGGGCCTGTACTGCGACAAGTGC
 ATCCCGCACCCAGGATGTGTCCACGGCACCTGCAATGAACCTCGGCAGTGCCT
 CTGTGAGACCAACTGGGGTGGACAGCTCTGTGACAAAGATCTGAATTACTGTG
 GGACTCATCAGCCCTGTCTCAACCGGGGAACATGTAGCAACACTGGGCCTGAC
 AAATACCAGTGCTCCTGCCCAGAGGGCTACTCGGGCCCCAACTGTGAAATTGC
 TGAGCATGCTTGTCTCTCTGACCCCTGCCATAACCGAGGCAGCTGCAAGGAGA
 CCTCCTCAGGCTTTGAGTGTGAGTGTCTCCAGGCTGGACTGGCCCCACGTGTT
 CCACAAACATCGATGACTGTTCTCCAAATAACTGTTCCCATGGGGGCACCTGCC
 AGGATCTGGTGAATGGATTCAAGTGTGTGTGCCCCGCCCACTGGACTGGCAAG
 ACTTGTCAGTTAGATGCAAATGAGTGCGAGGCCAAACCTTGTGTAATGCCAG
 ATCCTGTAAGAATCTGATTGCCAGCTACTACTGTGATTGCCTTCCTGGCTGGAT
 GGGTCAGAACTGTGACATAAATATCAATGACTGCCTTGGCCAGTGTGAGAATG
 ACGCCTCCTGTGCGGGATTTGGTTAATGGTTATCGCTGTATCTGTCCACCTGGCT
 ATGCAGGCGATCACTGTGAGAGAGACATCGATGAGTGTGCTAGCAACCCCTGC
 TTGAATGGGGGTCACTGTGAGAATGAAATCAACAGATTCCAGTGTCTCTGTCCC
 ACTGGTTTCTCTGGAACCTCTGTGAGTGGACATCGATTACTGCGAGCCCAAC
 CTTGCCAGAATGGCGCCCAGTGCTACAATCGTGCCAGTGACTATTTCTGCAAG
 TGCCCCGAGGACTATGAGGGCAAGAACTGCTCACACCTGAAAGACCACTGCCG
 TACCACCACCTGCGAAGTGATTGACAGCTGCACTGTGGCCATGGCCTCCAACG
 ACACGCCTGAAGGGGTGCGGTATATCTCTTCTAACGTCTGTGGTCCCCATGGG
 AAGTGCAAGAGCCAGTCCGGAGGCAAATTCACCTGTGACTGTAACAAAGGCTT
 CACCGGCACCTACTGCCATGAAAATATCAACGACTGCGAGAGCAACCCCTGTA
 AAAACGGTGGCACCTGCATCGATGGCGTTAACTCCTACAAGTGTATCTGTAGTG
 ACGGCTGGGAGGGAGCGCACTGTGAGAACAACATAAATGACTGTAGCCAGAA
 CCCTTGTCATAACGGGGTACATGTGAGACCTGGTCAATGACTTTTACTGTGA
 CTGCAAAAATGGCTGGAAAGGAAAGACTTGCCATTCCCGTGACAGCCAGTGTG
 ACGAAGCCACGTGTAATAATGGTGGTACCTGCTATGATGAAGTGGACACGTTT
 AAGTGCATGTGTCCCGGTGGCTGGGAAGGAACAACCTGTAATATAGCTAGAAA
 CAGTAGCTGCCTGCCGAACCCCTGTCATAATGGAGGTACCTGCGTGGTCAATG
 GAGACTCCTTCACCTGTGTCTGCAAGAAGGCTGGGAGGGGCCTATTTGTA
 CAAAATACCAACGACTGCAGTCCCCATCCTTGTACAATAGCGGGACCTGTGTG
 GACGGAGACAACTGGTATCGGTGCGAATGTGCCCCGGGTTTTGCTGGGCCAG
 ACTGCAGGATAAACATCAATGAGTGCCAGTCTTCCCTTGTGCCTTTGGGGCCA
 CCTGTGTGGATGAGATCAATGGCTACCAGTGTATCTGCCCTCCAGGACATAGT
 GGTGCCAAGTGCCATGAAGTTTCAGGGCGATCTTGATCACCATGGGGAGAGT

GATACTTGATGGGGCCAAGTGGGATGATGACTGTAACACCTGCCAGTGCCTGA
ATGGACGGGTGGCCTGCTCCAAGGTCTGGTGTGGCCCGAGACCTTGCAGGCT
CCACAAAAGCCACAATGAGTGCCCCAGTGGGCAGAGCTGCATCCCGGTCCTGG
ATGACCAGTGTTTTCGTGCGCCCCTGCACTGGTGTGGCGAGTGTCCGGTCCTCC
AGCCTCCAGCCAGTGAAGACCAAGTGACATCTGACTCCTATTACCAGGATAAC
TGTGCAAAACATCACTTTCACCTTTAACAAAGAGATGATGTCTCCAGGTCTTACCA
CCGAACACATTTGCAGCGAATTGAGGAATTTGAATATCCTGAAGAATGTTTCTG
CTGAATATTCGATCTACATAGCCTGTGAGCCTTCCCTGTCAGCAAACAATGAAA
TACACGTGGCCATCTCTGCAGAAGACATCCGGGATGATGGGAACCTGTCAAG
GAAATTACCGATAAAATAATAGATCTCGTTAGTAAACGGGATGGAAACAGCTCA
CTTATTGCTGCGGTGCGAGAAGTCAGAGTTCAGAGGCGTCCTCTGAAAAACAG
AACAGATTTCTGGTTCCTCTGCTGAGCTCTGTCTTAACAGTGGCTTGGGTCTG
TTGCTTGGTGACAGCCTTCTACTGGTGTGTACGGAAGCGGCGGAAGCCCAGCA
GCCACACTCACTCCGCCCCCGAGGACAACACCACCAACAATGTGCGGGAGCAG
CTGAACCAAATCAAAAACCCCATCGAGAAACACGGAGCCAACACGGTCCCAT
TAAGGATTACGAGAACAAAACTCCAAAATGTCAAAAATCAGGACACACAACTC
GGAAGTGGAGGAGGATGACATGGATAAACACCAGCAGAAAGTCCGCTTTGCC
AAACAGCCAGTGTATACGCTGGTAGACAGAGAGGAGAAGGCCCCCAGCGGCA
CGCCGACAAAACACCCGAACTGGACAAATAAACAGGACAACAGAGACTTGGA
AGTGCCCAAGAGCTTGAACCGGATGGAATACATCGTATAG

Top Line: Mouse Jagged-1 Amino Acid Sequence

Bottom Line: Rat Jagged-1 Amino Acid Sequence

1	MGSPRTRGRPGRPPLSLLLALLCALRAKVCASGQFELEILSMQNVNGELQ	50
1	MRSFPRTRGRPGRPPLSLLLALLCALRAKVCASGQFELEILSMQNVNGELQ	50
51	NGNCCGGVRNPGRKCTRDECDTYFKVCLKEYQSRVTAGGPCSFGSGSTP	100
51	NGNCCGGARNPGRKCTRDECDTYFKVCLKEYQSRVTAGGPCSFGSGSTP	100
101	VIGGNTFNLKASRGNDRNRIVLPFSFAWPRSYYTLLVEAWDSSNDTIQPDS	150
101	VIGGNTFNLKASRGNDRNRIVLPFSFAWPRSYYTLLVEAWDSSNDTIQPDS	150
151	IIEKASHSGMINPSRQWQTLKQNTGIAHFEYQIRVTCDDHYYGFGCNKFC	200
151	IIEKASHSGMINPSRQWQTLKQNTGIAHFEYQIRVTCDDHYYGFGCNKFC	200
201	RPRDDFFGHYACDQNGNKTCEGWMGPDCKNAICRQGCSPKHGSKLPDGD	250
201	RPRDDFFGHYACDQNGNKTCEGWMGPECNKAICRQGCSPKHGSKLPDGD	250
251	CRCQYGWQGLYCDKCIPHPGCVHGTCEPWPQCLCETNWWGQLCDKDLNYC	300
251	CRCQYGWQGLYCDKCIPHPGCVHGTCEPWPQCLCETNWWGQLCDKDLNYC	300
301	GTHQPCLNRGTCSENTGPDKYQCSCPEGYSGPNCEIAEHACLSDPCHNRGS	350
301	GTHQPCLNRGTCSENTGPDKYQCSCPEGYSGPNCEIAEHACLSDPCHNRGS	350
351	CKETSSGFEECECPGWTGPTCSTNIDDCSPNNCSHGGTCQDLVNGFKVC	400
351	CKETSSGFEECECPGWTGPTCSTNIDDCSPNNCSHGGTCQDLVNGFKVC	400
401	PPQWTGKTCQLDANECEAKPCVNARSKNLIASYYCDCLPGWMGQNCIDIN	450
401	PPQWTGKTCQLDANECEAKPCVNARSKNLIASYYCDCLPGWMGQNCIDIN	450
451	INDCLGQCQNDASCRDLVNGYRCICPPGYAGDHCERDIDECASNPCNLGG	500
451	INDCLGQCQNDASCRDLVNGYRCICPPGYAGDHCERDIDECASNPCNLGG	500
501	HCQNEINRFQCLCPTGFSGNLCQLDIDYCEPNPCQNGAQCYNRASDYFCK	550
501	HCQNEINRFQCLCPTGFSGNLCQLDIDYCEPNPCQNGAQCYNRASDYFCK	550
551	CPEDYEGKNCSHLKDHCRITTCVIDSCTVAMASNDTPEGVRYISSNVCG	600
551	CPEDYEGKNCSHLKDHCRITTCVIDSCTVAMASNDTPEGVRYISSNVCG	600
601	PHGKCKSQSGGKFTCDCNKGFTGTYPHENINDCESNPCKNGGTCTIDGVNS	650
601	PHGKCKSESGGKFTCDCNKGFTGTYPHENINDCEGNPCTNGGTCTIDGVNS	650
651	YKCICSDGWEGAHCENNINDCSQNPCHYGGTCRDLVNDIFYCDCKNGWKVK	700
651	YKCICSDGWEGAHCENNINDCSQNPCHYGGTCRDLVNDIFYCDCKNGWKVK	700

Amino Acid Sequence Comparison of Mouse Jagged-1 to Rat Jagged-1 (cont.)

701	TCHSRDSQCDEATCENGGTCYDEVDTFKCMCPGGWEGTTCNIARNSSCLP	750
701		750
751	NPCHNGGTCVVNGDSFTCVCKEGWEGPICTQNTNDCSPHPCYNSGTCVDG	800
751		800
801	DNWYRCECAPGFAGPDCRININECQSSPCAFGATCVDEINGYQCICPPGH	850
801		850
851	SGAKCHEVSGRSCITMGRVILDGAKWDDDCNTCQCLNGRVACSKVWCGR	900
851		900
901	PCRLHKSHNECPGQSCIPVLDDQCFVRPCTGVGECRSSSLQPVKTKCTS	950
901		950
951	DSYYQDNCANITFTFNKEMMSPGLTTEHICSELRLNINILKNVSAEYSIYI	1000
951		1000
1001	ACEPSLSANNEIHVAISAEDIRDDGNPVKEITDKIIDLVSKRDGNSSLIA	1050
1001		1050
1051	AVAEVRVQRRPLKNRTDFLVPLLSSVLTVAWVCLVTAFYWCVRKRR-KP	1100
1051		1100
1101	SSHHSAPEDNTTNNVREQLNQIKNPIEKHGANTVPIKDYENKNSKMSKI	1150
1101		1150
1151	RTHNSEVEEDMDKHQQKVRFAKQPVYTLVDREKAPSGTPTKHPNWTNK	1200
1151		1200
1201	QDNRDLESAQSLNRMEYIV*.....	1250
1201		1250
1201	QDNRDLESAQSLNRMEYIV*.....	1250

APPLIED IMAGE . Inc
 1653 East Main Street
 Rochester, NY 14609 USA
 Phone: 716/482-0300
 Fax: 716/288-5989

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