Notch1 is required for the coordinate segmentation of somites

Ronald A. Conlon1,†, Andrew G. Reaume1,‡ and Janet Rossant1,2

1 Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5
2 Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada

‡ Present address: Cephalon Incorporated, 145 Brandywine Parkway, West Chester, Pennsylvania 19380-4245, USA
† Author for correspondence (e-mail rac14@po.cwru.edu)
*Present address: Department of Genetics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4955, USA

INTRODUCTION

Many of the classical embryological phenomena such as induction and pattern formation are comprehensible today, at least in principle, in terms of cell-to-cell communication mediated by signaling molecules and their receptors. One such signaling pathway is that mediated by members of the highly conserved Notch family of proteins. These molecules are transmembrane receptors consisting of tandem EGF-type repeats, as well as tandem repeats specific to the family of proteins (NLR’s, for Notch/lin-12 repeats) in the extracellular domain, and, on the intracellular side, six tandem ankyrin or cdc10/SWi6 repeats that are essential for signal transduction (reviewed by Greenwald and Rubin, 1992). All the known ligands for the Notch family members are membrane-bound: the Delta and Serrate genes of Drosophila (Vässin et al., 1987; Fehon et al., 1990; Fleming et al., 1990), and the lag2 and apx1 genes of C. elegans (Tax et al., 1994; Mello et al., 1994) encode ligands that consist of multiple EGF-like repeats anchored to the cell surface by a membrane-spanning domain. These features imply that signaling through the Notch pathway is restricted to neighboring cells in contact with each other.

The members of the Notch family of receptors have been demonstrated to have essential roles in a large number of developmental processes in both Drosophila and C. elegans. In many instances, the Notch family members are necessary for correct implementation of cell fate decisions. In Drosophila, signaling through the Notch receptor plays a central role in the selection of fates of larval neuroblasts, larval muscle cells, photoreceptor cells, and adult peripheral sensory neurons (Lehman et al., 1983; Corbin et al., 1991; Cagan and Ready, 1989; Hartenstein and Posakony, 1990), to name just the best studied examples. In C. elegans, examples of processes requiring signaling through the Notch family member lin-12 include selection of the anchor cell fate in the gonad, and selection of cell fates in the developing vulva (Greenwald et al., 1983; Sternberg, 1988). In addition, there are a number of instances where Notch proteins are necessary for processes that are less clearly cell fate decisions. For example, in Drosophila, Notch is also required for many of the transitions to, and maintenance of, the epithelial state (Hartenstein et al., 1992).

The two apparently contrasting functions of Notch – in cell fate decisions and in generating epithelia – may in fact be two sides of the same coin (Greenspan, 1990). Changes in cellular organization and the selection of cell fates may be linked processes. For example, the selection of the fates of neuroblasts, muscle cells, photoreceptors and peripheral sensory neurons in Drosophila are accompanied by delamination or partial delamination of individual fated cells from an epithelium (reviewed by Greenwald et al., 1992). Loss of Notch function by mutation results in too many of the delaminated cell type forming, consistent with a requirement for Notch activity to maintain the epithelial state.

There has been considerable advance in the understanding of how individual cells might utilize Notch signaling with their immediate neighbors to select cell fates. The models propose that stochastic variations in the strength of signaling between cells become reinforced through feedback mechanisms such that one cell becomes a strong signaler, adopts the appropriate fate, and, through its strong signals, inhibits its immediate neighbors from adopting the same fate (Seydoux and Greenwald, 1989) – so-called lateral inhibition. This model has been convincingly applied to Drosophila neurogenesis.
(Heitzler and Simpson, 1991). Less is understood about the mechanism whereby groups of cells attain or maintain the epithelial state through the action of Notch.

There are many instances in vertebrate development where mechanisms that promote lateral inhibition and epithelialization would seem to be important and so the involvement of Notch family members in vertebrate development has been investigated. Vertebrates possess multiple genes related to the Drosophila Notch gene. There are at least three distinct Notch genes (Notch1 to Notch3) in the mouse (Franco del Amo et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994), as well as related genes in the rat (Weinstein et al., 1991, 1992), in humans (Ellisen et al., 1991; Stifani et al., 1992) and in Xenopus (Coffman et al., 1990). Consistent with the notion that the Notch proteins are important in multiple developmental events, Notch1 is expressed in a wide variety of tissues during early mouse development, including the primitive streak during gastrulation, the presomitic mesoderm during the process of somitogenesis, in differentiating endothelial cells, in the ectodermal placodes of the head when cells are delaminating to generate sensory neuron precursors, and in the ventricular zone of the brain when CNS neurons are being born (Franco del Amo et al., 1992; Reaume et al., 1992). Less is known about the expression of the Notch2 and 3 genes in the mouse, but the available evidence suggests that the three Notch genes are expressed in overlapping patterns (Franco del Amo et al., 1992; Reaume et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994).

It is important to test the role, if any, of the Notch1 gene in these tissues by functional analysis. Misexpression of activated Notch proteins in mammalian cells implicate the Notch signaling pathways in cell fate decisions (Kopan et al., 1994; Nye et al., 1994). A loss of function mutation in the Notch1 gene has been reported and has been found to be lethal at 10 days of gestation (Swiatek et al., 1994). However, the phenotype did not provide information on involvement of Notch in any particular developmental decisions. We report the generation of a different mutation in the murine Notch1 gene that shares a similar lethal phenotype. However, this mutation clearly affects the normal organization of the epithelial somites, implicating Notch1 in the cell interactions that coordinate the segmentation of the somites.

MATERIALS AND METHODS

Generation of the mutation

A positive/negative targeting vector (Mansour et al., 1988) was designed so as to delete a large portion of the Notch1 gene. Genomic DNA corresponding to the Notch1 locus was isolated from a 129/Sv genomic library. Regions of homology of 6.1 and 1.1 kb were cloned on either side of the neomycin resistance gene driven by the PGK1 promoter. The HSV thymidine kinase gene, also with a PGK1 promoter, was cloned 3’ of the shorter arm of homology. A successful recombination and replacement event would result in the acquisition of neomycin resistance, gancyclovir sensitivity, and the deletion of 10.4 kb of the Notch1 gene (Fig. 1). The ES cell line used for electroporation was R1-S3 (Nagy et al., 1993). The targeting event was verified by Southern analysis with probes from the Notch1 gene external to the targeting vector on both sides, and from within the deletion (Fig. 1 and not shown).

Chimeras were generated by blastocyst injection with two targeted lines. Chimeric males were bred to CD1, C57Bl/6 and 129/Sv female mice to establish F1 heterozygotes. Embryos from crosses between F1 heterozygotes were typed either by Southern analysis or by PCR of yolk sac DNA. PCR primers were designed for sites outside and inside the deletion, and in the introduced neomycin gene such that all three Notch1 genotypes could be unambiguously assigned (Fig. 1).

In situ hybridization, immunocytochemistry and histology

Whole-mount in situ hybridization was performed as described previously (Conlon and Rossant, 1992). The hybridization probes used were: Mox-1 (Candia et al., 1992), M-twist (Wolf et al., 1991) FGFRI (Yamaguchi et al., 1992), N-myc (Moens et al., 1992), SCG10 (Guillemot et al., 1993), myogenin (myogenin poly A: kindly provided by Dr Andre Schuh), brachyury (Herrmann, 1991), sonic hedgehog (Echelard et al., 1993), Mash-1 (Guillemot and Joyner, 1993), Hoxb-9 (#755: Conlon and Rossant, 1992), and Notch1 (Reaume et al., 1992).

Whole-mount immunocytochemistry was performed as described previously (Davis et al., 1991), except that the embryos were fixed in 4% paraformaldehyde in PBS for 2 hours. The monoclonal antibody 2H3 against the 160×10^3 M neurofilament protein (Developmental Studies Hybridoma Bank, Iowa) was used at 1:4 dilution. Peroxidase-coupled goat anti-mouse IgG (Boehringer Mannheim) was used as the secondary antibody at 1:500 dilution.

For histology, embryos were fixed overnight at 4°C in half strength Karnovsky’s fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M cacodylate, pH 7.5; Karnovsky, 1965). They were washed thoroughly in 0.1 M cacodylate, pH 7.5, and photographed in the same buffer, then dehydrated and embedded in paraffin wax.

Determination of cell death in whole-mounts

Apoptotic cells were labeled in whole embryos by a modification of the TUNEL procedure of Gavrieli et al. (1992). Embryos were dissected in cold PBS containing 1% BSA, and then fixed for 2 hours at 4°C in 4% paraformaldehyde in PBS. The embryos were washed with cold PBS (PBS containing 0.1% Tween-20), then transferred directly into 100% methanol and stored at ~20°C. Embryos were treated for 5-6 hours at room temperature in 5:1 methanol:30% hydrogen peroxide, followed by several washes in methanol. The embryos were either stored in methanol at ~20°C, or rehydrated through a methanol series into PBT. Digestion with 20 µg/ml protease K for 3 minutes at room temperature was followed by two quick washes with PBT and fixation for 20 minutes in 0.2% glutaraldehyde/4% paraformaldehyde in PBS. Three washes with PBT followed to remove the fixative. The embryos were treated with 0.1% sodium borohydride in PBT for 20 minutes to remove aldehydes, and then washed three times with PBT. The embryos were washed once with TdT buffer (30 mM Tris, 140 mM cacodylate pH 7.2, 1 mM CoCl2). The buffer was replaced with 200 µl of reaction mix (20 µM digoxigenin-dUTP, 20 µM dTTP, 0.3 U/µl terminal transferase in TdT buffer). The embryos were incubated at 37°C for 2 hours with occasional mixing. The embryos were washed through several quick changes of PBT, then incubated at 70°C for 20 minutes in the same buffer. Detection of the digoxigenin epitope was performed exactly as described by Conlon and Rossant (1992).

RESULTS

In order to begin a functional study of the mouse Notch1 gene, a deletion was generated in the gene by homologous recombination in ES cells. The region deleted accounts for the extra-cellular EGF repeats 28 to 36, the three Notch/in-12 repeats, the transmembrane domain and CDC10/SWl6 repeats 1 to 5 (amino acids 1056 to 2049 of the protein numbered according to the Genbank entry Z11886, Fig. 1). The CDC10/SWl6
Notch family members are required for Notch activity (Lieber et al., 1993; Struhl et al., 1993; Rebay et al., 1993; Roehl and Kimble, 1993; Fortini and Artavanis-Tsakonas, 1994), so we expect the Notch1Δ1 mutation to be a null allele.

Two cell lines arising from independent targeting events were successfully introduced into the germ line of mice. No difference was discerned in the two mutations, so data from the two have been combined. The mutant genes were crossed into two inbred backgrounds (129/Sv and C57Bl/6) and one outbred background (CD1). In crosses to wild-type mice, the Notch1Δ1 mutation was transmitted to the expected number of offspring (101 of 200 mice). The phenotype associated with the mutant Notch1 gene was essentially indistinguishable on the different backgrounds, and remained associated through as many as 5 generations of outcrosses to wild-type mice of the given strains.

General aspects of the Notch1Δ1/Notch1Δ1 phenotype

No homozygous mice were obtained from heterozygous intercrosses of Notch1Δ1/+ mice. However, prior to day 11 of...
gestation, homozygous mutant embryos were recovered in about the expected numbers (Table 1), indicating that embryonic loss did not occur prior to day 11. However, at day 11, all mutant embryos were dead and being resorbed (not shown).

The first expression of Notch1 that we have detected is on embryonic day 7 in the posterior mesoderm of midstreak embryos (Reaume et al., 1992). The early mesodermal expression of Notch1 condenses at later stages into a strong domain of expression within the presomitic mesoderm – the mesoderm from which somites arise. The earliest mutant embryos we examined were in the early stages of somitogenesis on embryonic day 8, when normally from 1 to 16 somites have condensed from the presomitic mesoderm. At these stages, the homozygous Notch1Δ1 embryos were similar in size, stage, and overall appearance to their heterozygous and wild-type littermates (but see below). However, homozygous mutant embryos harvested on embryonic day 9 were almost always readily distinguishable from their littermates. Mutant embryos were smaller, had distended pericardia, and showed a deficit in posterior development (for example, the mutant embryos shown in Fig. 3B, 3G, 6F and 6L). On embryonic day 10, most homozygotes were dead, or had large patches of necrotic tissue (visible at the time of dissection as opaque white cells). From day 9 to 10 the mutant embryos had not grown any larger and many of the changes in appearance appeared to be degenerative in nature. Therefore we concentrated our more detailed analysis on embryos from embryonic days 8 and 9.

Table 1. Genotypes of embryos recovered from Notch1Δ1/+ intercrosses

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Fig. 2. Apoptotic cell death is not altered in Notch1Δ1 homozygotes prior to developmental arrest. The sheared nuclear DNA characteristic of apoptotic cells was labeled with terminal transferase in situ and detected immunohistochemically in intact embryos. Individual apoptotic nuclei appear as darkly stained dots at the magnification shown. A wild-type 14 somite embryo is shown in A,C and E, and a homozygous mutant embryo at approximately the same stage in B,D and F. In A and B, apoptotic cells are present along the ridges of the closing neural tube (between arrows) at the level of the hindbrain, seen in dorsal view. In C and D, comparable levels of apoptosis are seen in wild-type and mutant embryos dorsal to the eye (e), within the first branchial arch (b1) and dorsal to the heart (h). In E and F, embryos of both genotypes display comparable amounts of apoptosis along the neural tube (between arrows) at the level of the spinal cord (between arrows), seen in dorsal view.
Cell death in Notch1Δ1 embryos

Between embryonic days 8 and 9, the Notch1Δ1 homozygotes fell behind their littermates in their growth. One means by which the difference in growth of the embryos might be accounted for is by a difference in the amount of apoptotic cell death. Cells undergoing apoptosis characteristically fragment their nuclear DNA. This feature forms the basis of a technique for labeling apoptotic cells in situ with terminal transferase (Gavrieli et al., 1992). We adapted this procedure to whole embryos so that the embryos could be easily staged, and a complete overview of cell death throughout the embryos could readily be obtained. The patterns of cell death that we observed with this procedure on wild-type embryos were consistent with what has been seen in chick and mouse embryos by other techniques (Graham et al., 1993; Jeffs et al., 1992; Jeffs and Osmond, 1992). Accordingly we observed cell deaths in the cranial and branchial arch mesenchyme, in the neural ridge of the hindbrain, in the ectoderm along

![Image](https://via.placeholder.com/150)

**Fig. 3.** Early neurogenesis, neural patterning, and notochord formation is largely normal in Notch1Δ1 mutants. Embryos derived from heterozygous intercrosses were analyzed for the expression of markers for neurogenesis, neural patterning and notochordal development by whole-mount immunocytochemistry (A,B) or whole-mount in situ hybridization (C-I). (A) A 9.5-day wild-type embryo stained for neurofilament protein shows extensive axonal tracts in the midbrain (short arrow) and in the cranial ganglia (long arrows) in this lateral view. (B) A homozygous mutant embryo harvested on the tenth day of gestation and stained for neurofilament protein shows a pattern of staining similar to the 9.5-day wild-type embryo shown in A. Axonal tracts in the midbrain (short arrow), and in the cranial ganglia (long arrows) are evident. The expanded pericardium (arrowheads) characteristic of homozygotes at this stage was preserved in this specimen. (C) Comparable numbers of Mash-1-expressing neuroblasts are evident in wild-type and mutant embryos. Littermates from a heterozygous intercross were probed by whole-mount in situ hybridization for expression of the neuroblast marker Mash-1. Expression is restricted to the dorsal midbrain at this stage (arrows). (D) Neural patterning, as assayed by Hoxb-9 expression in the neural tube, is normal in Notch1Δ1 mutants. Hoxb-9 mRNA expression was assayed by in situ hybridization. The intensity and anterior-posterior extent of staining for Hoxb-9 is similar in embryos of both genotypes. (E-F) The notochord is initially normal in mutant embryos on day 8 as revealed by analysis of sonic hedgehog and T expression assayed by in situ hybridization. The embryos in E are shown in dorsal view; the embryos in F, in lateral view. (G-I) The notochord becomes discontinuous on the ninth day of gestation in Notch1Δ1 mutants. The disparity in size between normal and mutant littermates is evident in G. The expression of T was analyzed by whole-mount in situ hybridization. Discontinuities in the staining for T are evident along much of the length of the notochord in the mutant embryo shown in G and I. The notochord of the wild-type embryo, in contrast, displays strong continuous staining for T (G,H).
were not disturbed in the truncated embryos. Indicating that mechanisms of anterior-posterior patterning of a posterior patterning gene, branchial arches, the cranial ganglia etc. However, expression appeared to be more severely affected than that of the head. The posterior axis development of Notch1Δ embryos typically did not grow larger than a 16 somite wild-type embryo. Accordingly, we looked for cell deaths at and before this time. No excess of cell death was observed by this technique in 4 pre-16 somite stage homozygotes, and in 2 embryos at about the 16 somite stage (Fig. 2). Nor was there a noticeable difference in frequencies of pyknotic nuclei when similar embryos were subjected to conventional histological analysis (not shown). After the 16 somite stage, however, both techniques detected an abundance of dying cells increasing with developmental age (not shown). Thus we conclude that the homozygotes are subject to growth arrest, followed by cell death, but that an increase in apoptotic cell death is not the main cause of embryonic growth arrest.

**Neurogenesis and Notch1Δ**

Loss of Notch function in *Drosophila* leads to an overproduction of neuroblasts in the larva and in the adult. In the mouse, Notch1 is expressed in regions where peripheral and CNS neurons are born (Reaume et al., 1992; Franco del Amo et al., 1992). Although the Notch1 mutant embryos arrest in their growth at about the time neurogenesis begins, we felt that if the Notch1Δ mutation was strongly neurogenic we still might be able to detect an effect. Therefore embryos were examined with markers for mature neurons (neurofilament and SCG10, Fig. 3 and not shown). Cells positive for these markers were detected in mutant embryos in the midbrain, and in condensations at the normal positions of the fifth and seventh cranial ganglia. These are the first cells to stain for these markers in wild-type embryos at about the 20 somite stage. Mutant embryos harvested on embryonic day 10 had not produced any more SCG10 or neurofilament-positive cells than they had a day earlier. To test whether an excess of neuroblasts, rather than neurons, might be present in the Notch1 mutants, we assayed the expression of Mash-1, the mammalian *achaete-scute* homologue, which is expressed on days 8 and 9 in the midbrain region from which the first neurons of the CNS emerge. On days 8 and 9 of development we saw no difference in the numbers of Mash-1-positive cells between mutant and sibling wild-type embryos (Fig. 3C and not shown). Thus, we were unable to detect an effect on neurogenesis in Notch1 mutant embryos.

**Posterior patterning and the notochord in Notch1 mutant embryos**

The posterior axis development of Notch1Δ mutant embryos appeared to be more severely affected than that of the head region. The patterning of the head appeared normal, given the normal disposition of the various morphological specializations such as eyes, otic vesicles, sulci of the neural tube, the branchial arches, the cranial ganglia etc. However, expression of a posterior patterning gene, Hoxb-9, was normal (Fig 3D), indicating that mechanisms of anterior-posterior patterning were not disturbed in the truncated embryos.

A feature common to two other mutations that result in a deficit in posterior development is a notochord that is absent or interrupted (*Sd* and *T*: Chesley, 1935; Dunn et al., 1940; Herrmann, 1991; Koseki et al., 1993). Mutant day 8 and 9 embryos were found to have notochords that were normal in appearance in histological sections (Fig 5F and not shown). In addition, on embryonic day 8, embryos had normal patterns of expression of *T* and *shh*, two genes expressed in the notochord, suggesting that notochordal gene expression was normal, and that the notochord was continuous (Fig. 3E,F). On embryonic day 9 however, discontinuities in the staining for *T* were evident along the length of the notochord (Fig. 3G,H,I), suggesting a secondary degeneration.

**Somitogenesis in Notch1 mutants**

The most prominent domain of Notch1 gene expression in early embryos is within the presomitic mesoderm. Notch1 is expressed at high levels in a somite-sized domain at the anterior end of the presomitic mesoderm (Reaume et al., 1992). The expression of Notch1 appears to anticipate the formation of each somite, since it is these cells that will epithelialize to form the next somite in the cranial-caudal series. Notch1 expression is sharply down-regulated once a somite forms. Because of the strong expression of Notch1 in presomitic mesoderm, we undertook a detailed study of somitogenesis in Notch1 mutant embryos. To do so, we examined intact fixed embryos by differential interference contrast (DIC) microscopy, as well as sectioned material, and we looked at an array of markers expressed in somites and in subdivisions of somites.

At the earliest stages of somitogenesis when wild-type embryos had from 3 to 4 somite pairs, their mutant littermates of similar stage typically had continuous unsegmented strips of condensed mesoderm on either side of the neural tube where somites should have been (Figs 4A,B, 6C). The absence of segmentation in the paraxial mesoderm of the mutant embryos was transient, however. When wild-type embryos had 5 or more somites, similarly staged Notch1Δ embryos did display somites at the cranial end of the condensed paraxial mesoderm (Fig. 4C-H). Thus it appeared that, in the mutants, the mesoderm exiting the presomitic mesoderm was condensing at the appropriate time, but the process of generating separate epithelial somites was delayed. This delay persisted through the early stages of somitogenesis prior to the developmental arrest of the mutants. The appearance of transverse fissures was not coordinate on the two sides of the embryo, so that condensed, undivided mesoderm was often present near the presomitic mesoderm on one side only (Fig. 4F). To analyze the cellular organization of the forming somites, we examined sections of mutant and wild-type embryos.

Sections of wild-type embryos showed the loose mesenchymal nature of the presomitic mesoderm, the tighter packing of cells and epithelialization of cells in the somites, the large intersomitic clefts, and the strict pairing of somites as discussed above (Fig. 5A-C). In mutant embryos, the most recently formed somites were not as tightly packed as those of the wild-type and the epithelialization sometimes appeared incomplete (Fig. 5D). A lack of coordination across the midline in the segmentation of somites was also evident in frontal sections where segmentation was sometimes present on one side, but not the other (Fig. 5E,F). However, the mutant somites, once formed, appeared to show normal dispersion of
the sclerotome (and maintenance of the dermamyotome) at the cranial end of the embryo (Fig. 5F). These observations again pointed to a defect in the transition from presomitic mesoderm to somite, and in addition showed that subsequent developmental events in the somites were not affected. An analysis of gene expression in somites was performed to confirm and extend these conclusions.

We analyzed the expression of a number of marker genes specific for different aspects of somitogenesis. The homeobox gene *Mox-1* is expressed in the anterior presomitic mesoderm and in the somites and serves as an excellent marker for the development of the somites. *Mox-1* was normally expressed in mutant embryos (Fig. 6A-I-N), but the clear delineation of somites by the staining for *Mox-1* in whole mounts highlighted the defects in somite organization. A lack of clear transverse clefts between somites proximal to the presomitic mesoderm was evident in day-8.5 embryos (Fig. 6A). On embryonic day 9, it can be seen that the somites are not aligned across the midline in some regions (Fig. 6N). Within the somites, on day 9, *Mox-1* expression is stronger in the posterior half of the somites (Fig. 6J). In the mutant embryos, this anterior/posterior difference can be seen in some, but not all somites (Fig. 6M). The proto-oncogene N-*myc* is also expressed differentially in anterior and posterior half somites (Fig. 6D,E). Mutant embryos, on day 8, showed stronger N-*myc* expression in posterior half somites, similar to wild-type embryos (Fig. 6D,E). From the expression of N-*myc* and *Mox-1* we conclude that the subdivision into anterior and posterior halves occurs normally in many of the somites of *Notch1* mutants. An analysis of the expression of *myogenin*, a bHLH transcription factor specific to the myogenic lineage, showed that mutant somites initiated the myogenic program appropriately, consistent with our histological observations that the mutant somites could differentiate into dermamyotome and sclerotome (Fig. 7).

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**Fig. 4.** Somitogenesis is delayed and uncoordinated in *Notch1*Δ/*Notch1*Δ embryos. Embryos from heterozygous matings were fixed in half-strength Karnovsky’s fixative and photographed intact in PBS with differential interference contrast optics. Wild-type embryos are shown in A,C,E and G and homozygous *Notch1*Δ embryos in B,D,F and H. Early in somitogenesis (A,B) the wild-type embryo has four distinct somite pairs, whereas the age-matched mutant has none. Unsegmented condensations of mesoderm are visible on either side of the neural tube (bracketed). At slightly later stages (C,D), the mutant embryo (D) has formed 2 pairs of somites toward the anterior end of the condensed paraxial mesoderm, but, posteriorly, segmentation lags relative to the wild-type embryo (C). At later stages, increasing numbers of somites are found in the mutant embryos, but asymmetries in segmentation are evident (F,H) whereas in littermates segmentation is strictly coordinated across the midline (E,G).
Fig. 5. The paraxial mesoderm of Notch1Δ1 mutants condenses and reorganizes into dermamyotome and sclerotome normally; however, the timing and coordination of somite segmentation appear affected. Sectioned wild-type embryos (A-C) display the increase in cell density that takes place during the transition from presomitic mesoderm (psm) to somites. In C, wild-type somites are seen to be in tight register across the midline. The sectioned mutant embryo in D also shows apparently normal condensation of the presomitic mesoderm as it matures at the anterior end. In E and F, abnormal blocks of unsegmented mesoderm (usm) are seen opposite arrays of somites. The somites of the mutant embryos mature to form dermamyotome (dmm) and sclerotome (scl). In F, a long stretch of notochord (noto) of normal appearance is evident. All embryos are oriented with anterior to the left.

Fig. 6. Analysis of gene expression in the mesoderm of Notch1Δ1 mutant embryos. Gene expression was assayed in mutant embryos and their littermates by whole-mount in situ hybridization. (A) The paraxial mesoderm-specific homeobox gene Mox-1 is appropriately expressed in day 8.5 mutant embryos. Despite the normal pattern of expression of Mox-1, the mutant embryo mesoderm just anterior to the presomitic mesoderm is unsegmented (indicated by bracket). Somite borders are indicated by arrowheads. (B,C) The expression of the FGF receptor-1 gene is not altered in Notch1Δ1 mutants. In the wild-type embryo shown in B, the FGFR1 mRNA is strongly expressed in the presomitic mesoderm (bracket). The same pattern of expression is evident in the mutant embryo in C (small bracket), even though the segmentation of somites has not occurred in this embryo (large bracket). (D,E) The subdivision of somites into anterior and posterior domains appears to occur normally in Notch1Δ1 mutants as shown by the expression of N-myc transcripts in the posterior half of the somites of both wild-type and mutant 8.5-day embryos (arrows). (F) The different types of mesoderm appear to be present in relatively normal proportions on day 9 in Notch1Δ1 homozygotes as revealed by in situ hybridization with a probe for the M-twist gene. Note that the head of the mutant embryo is relatively well-developed, but that elongation of the posterior of the embryo is deficient. (G,H) Head and head mesoderm development of the mutant embryos proceeds relatively normally, with grossly normal development of the eye (e), first branchial arch (b1), and otic vesicle (ov). The head and first branchial arch of the mutant become populated by M-twist-expressing cells (H). (I-K) On day 9.5, Mox-1 transcripts are strongly expressed in wild-type somites (I), and anterior-posterior differences in expression levels within each somite are evident (arrows in J). (L-N) In the mutant on day 9.5, Mox-1 expression in the somites (L) serves to highlight regions where somite segmentation has failed to occur (bracket in M) and regions where somites are out of register across the midline (compare K and N). However, the anterior-posterior differences in intensity seen in wild-type embryos are also seen in the mutant (compare K and N). (O) In wild-type embryos, Notch1 is strongly expressed in the presomitic mesoderm (bracket) on day 8.5.
The normal expression of these transcription factors indicates that cell-type specification of somites and somitic subpopulations is not disturbed in the mutants, but rather that the overall allocation and organization of the somites is affected. Likewise, the normal expression of M-twist in the different mesodermal cell types in the mutants (Fig. 6F-H) suggests that mesodermal patterning in general is normal in the Notch1Δ1 mutant.

Another signaling pathway acting through the FGF receptor 1 (FGFR1) protein has recently been shown to be important for normal formation of somites (Yamaguchi et al., 1994). We therefore analyzed the expression of FGFR1 in Notch mutant embryos to see if these pathways were independent. The expression of the FGFR1 gene marks the anterior portion of the presomitic mesoderm (Yamaguchi et al., 1992). FGFR1 was expressed normally in the presomitic mesoderm of day-8 mutant embryos (Fig. 6B,C). In the mutant embryo shown in Fig. 6C, FGFR1 is expressed in the loosely packed mesoderm.
DISCUSSION

**Notch1** lethality and neurogenesis

Embryos homozygous for a mutation in Notch1, which deletes a large portion of both extracellular and intracellular domains of the protein, show a gross phenotype similar to that reported for the insertional mutation in the Notch1 gene reported by Swiatek et al. (1994). It is not possible to ascertain from the available information whether or not the phenotypes of Notch1Δ and Notch1m32 (Swiatek et al., 1994) are identical in all respects: only further investigation can determine that. By examining cell death prior to embryonic arrest, as well as after, we were able to show that the excess of cell death first seen by Swiatek et al., (1994) is likely to be a secondary effect of general embryonic morbidity. Embryos developed to the 14 somite stage and then underwent growth arrest. After growth arrest, the notochord degenerated, an excess of cell death occurred and pericardial edema developed. The development of the head appeared to be less severely affected than that of the posterior. The cause of the growth arrest and eventual degeneration and death of the embryo are still unknown, although the generalized lethality and the stage of death is indicative of a defect in the embryonic circulatory system or in placentation. No obvious defects in these systems were apparent in our mutant embryos, or those of Swiatek et al. (1994).

Clearly further investigation into the cause of the generalized growth arrest and cell death in Notch1 mutant embryos is required. However, specific neuronal developmental events in which Notch signaling might be involved occur prior to growth arrest.

The neurogenic role of the Drosophila Notch gene, and the expression of the vertebrate Notch1 genes in regions of neurogenesis suggested that Notch1 might have a neurogenic function. In mammalian cells in culture, misexpression of Notch protein fragments has an inhibitory effect on neurogenesis (Nye et al., 1994). However, we could detect no evidence for a neurogenic function of Notch1 in mice • i.e. no hypertrophy of neurons or neuroblasts was observed. It is possible that Notch1 is a neurogenic gene, but that we failed to observe neural hypertrophy because of our choice of markers. However we used two different markers of postmitotic neurons and one marker for neuroblasts. Therefore it is probable that Notch1 is not required for early neurogenesis. It should be noted however, that embryonic arrest and degeneration begins in the Notch1Δ embryos at about the same time that neurogenesis begins. A truer test of the neurogenic capacity of Notch1 will require averting the early embryonic lethality associated with gross alterations to the gene.

**Notch1** and somitogenesis

Because of the striking pattern of expression of Notch1 in the presomitic mesoderm, and because of the role that Notch gene family members play in mediating cell-cell interactions, we had proposed that Notch1 may be involved in promoting the cell associations that lead to somite formation (Reaume et al., 1992). Detailed examination of the mutants reported here provides the first evidence that this is indeed the case.

In Notch1Δ homozygotes there is a delay and lack of coordination in segmentation of the somites, leading to variations in somite size, and to misalignment of the somites across the midline of the embryo (summarized in Fig. 8A). These defects in somitogenesis have their origin at the transition between presomitic mesoderm and somite. In wild-type embryos there is an abrupt change in cellular organization from the loose mesenchymal cells of presomitic mesoderm to the tightly packed epithelial cells of the somites, whereas in the Notch1Δ mutants there is a disordered transition from presomitic mesoderm to somite (Fig. 8). The somites that do form in the mutant are subdivided into anterior and posterior halves and subsequently into dermamyotome and sclerotome. They express transcription factor genes such as Mox-1, N-myc and Myogenin, that are suspected or known to be involved in cell type specification within the somites, suggesting that Notch1 signaling is not involved in somite cell specification. Recently, it has been shown that two other signaling pathways, involving FGFs and Wnt-3a, are involved in normal somite generation (Takada et al., 1994; Yamaguchi et al., 1994; Deng et al., 1994). However, in these cases, mutations affecting these pathways affect the initial formation or continued production of somites, not the organization of the somites itself. FGFR1 expression is normal in Notch1 mutants, suggesting that these signaling pathways...
play different roles in somite development. Thus we conclude that *Notch1* signaling is not required for somite formation per se, but is required for the coordination of somite formation.

**A model for the regulation of somitogenesis by Notch1**

A sophisticated model for fate determination of individual cells by signaling through Notch family members has been proposed and refined over the years (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991; Greenwald and Rubin, 1992; Ghysen et al., 1993; Heitzler and Simpson, 1993). In this model, physiological fluctuations in Notch-Delta signaling between neighboring cells become amplified by feedback mechanisms to establish strong signaling cells (high Delta activity) surrounded by signal-receiving cells (high Notch activity). Strong signalers prevent receivers from becoming signalers; signalers and receivers subsequently follow alternate fates. A remarkable feature of this model is that spatial periodicity is generated without recourse to oscillators or a preexisting periodicity. Unfortunately, no comparable model exists for the coordinate regulation of groups of cells by Notch signaling despite the fact that Notch is involved in many processes of this type.

We would like to propose a model for the regulation of somitogenesis by Notch and its ligand(s) (Fig. 8B). The present model differs from the one cited above in that the expression of Notch is downregulated in the absence of external promotion, and as a result, cell adhesion mechanisms are upregulated, leading to the epithelialization of a cohort of cells that formerly centered on a signaling cell.
to those cells: signals from another tissue cause Notch expression to be promoted only in receiving cells in a somite-sized domain of the presomitic mesoderm, and cause Notch to be down-regulated at the anterior boundary. (3) The high level of expression of Notch and its ligand(s) in the anterior of the presomitic mesoderm promotes the physical association of these cells through interaction of the membrane-bound receptor Notch and its membrane-bound ligand. These strengthened interactions lead to a physical separation of this group of cells from those posterior to it. (4) The ligand-receptor interactions of Notch and its ligand(s) are transduced as signals within these cells. (5) Down-regulation of Notch at the anterior limit of the presomitic mesoderm results in the cessation of Notch signaling, and thus the activation of cell adhesion and epithelialization. The primitive streak is a candidate source for a diffusible factor that modulates the expression of Notch in the presomitic mesoderm.

In this model, the mesodermal cells participate in three different processes as they progress through the presomitic mesoderm and into the somites (Fig. 8B). In the first phase, cells compete to establish which cells are net signalers or receivers. Through this process, the periodicity of the somites is generated. In the next phase, the organization of the cohorts of signaling and receiving cells is strengthened by upregulation of Notch in the receiving cells. This upregulation is controlled by factors extrinsic to the cells themselves and results in condensation of the cells within the cohort, and thus their separation from the following cohort. The last phase occurs in the somites where Notch expression is abruptly extinguished, again by factors extrinsic to those cells, resulting in epithelialization of the cohort of cells. In sum, through this mechanism, it is ensured that the flow of cells is unidirectional, and cells of the presomitic mesoderm cannot associate with the last-formed somite (or vice versa) because their adhesion mechanisms differ: a sharp transition between the somitic and presomitic mesoderm is ensured. In addition, somitogenesis on both sides of the midline is coordinated because both sides rely on signals from a common source. Lastly the periodicity of somite segmentation is generated without reliance on a pre-existing periodicity, and this periodicity is first expressed as a prepattern in the presomitic mesoderm. (A summary of other models of somitogenesis is presented by Keynes and Stern, 1988.)

This model is consistent with much of what is known of both Notch signaling and somitogenesis. We have proposed a dual role for Notch and its ligand(s) in signaling and cell adhesion. The molecular genetics of Notch in Drosophila, and of lin-12 and glp-1 in C. elegans, indicate that Notch proteins act as ligand-activated receptors capable of influencing gene expression. But in addition, investigations of Drosophila Notch in vitro have indicated that interactions of Notch with its ligands Delta and Serrate are sufficient to cause adhesion of cells (Fehon et al., 1990; Lieber et al., 1992). Also, the prepattern for somites that we have suggested is consistent with the serially repeated radial arrangements of cells (somitomeres) that can be observed in the presomitic mesoderm of vertebrate embryos, including the mouse (Tam et al., 1982; reviewed by Meier, 1984).

The model is consistent with the phenotype that we observe in the Notch1 mutant mice: the model predicts that the role of the Notch1 protein in somitogenesis is in the coordination of somite formation, but that cell-type specification and other aspects of somite maturation are independent of Notch1 function. It is of interest to note that the Notch2 gene is also expressed in the anterior presomitic mesoderm (Swiatek et al., 1994). One might expect that the coordination of somite formation would be more severely disrupted in the double mutant.

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