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Mouse *Dll3*: a novel divergent *Delta* gene which may complement the function of other *Delta* homologues during early pattern formation in the mouse embryo

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SUMMARY

Mouse delta-like 3 *(Dll3)*, a novel vertebrate homologue of the *Drosophila* gene *Delta* was isolated by a subtracted library screen. In *Drosphila*, the Delta/Notch signalling pathway functions in many situations in both embryonic and adult life where cell fate specification occurs. In addition, a patterning role has been described in the establishment of the dorsoventral compartment boundary in the wing imaginal disc. *Dll3* is the most divergent *Delta* homologue identified to date. We confirm that *Dll3* can inhibit primary neurogenesis when ectopically expressed in *Xenopus*, suggesting that it can activate the Notch receptor and therefore is a functional *Delta* homologue. An extensive expression study during gastrulation and early organogenesis in the mouse reveals a diverse and dynamic pattern of expression. The three major sites of expression implicate *Dll3* in somitogenesis and neurogenesis and in the production of tissue from the primitive streak and tailbud. A careful comparison of *Dll3* and *Dll1* expression by double RNA in situ hybridisation demonstrates that these genes have distinct patterns of expression, but implies that together they operate in many of the same processes. We postulate that during somitogenesis *Dll3* and *Dll1* coordinate in establishing the intersomitic boundaries. We confirm that, during neurogenesis in the spinal cord, *Dll1* and *Dll3* are expressed by postmitotic cells and suggest that expression is sequential such that cells express *Dll1* first followed by *Dll3*. We hypothesise that *Dll1* is involved in the release of cells from the precursor population and that *Dll3* is required later to divert neurons along a specific differentiation pathway.

Key words: mouse embryo, *Delta*, neurogenesis, somitogenesis.

INTRODUCTION

The supply and patterning of tissue and cellular differentiation within that tissue are fundamental to the development of an organism. For example, during the initial stages of gastrulation in the mouse, the epiblast, a pluripotent and rapidly dividing epithelium, supplies tissue in the form of the newly induced mesoderm. The mesoderm is then patterned leading to a large diversity of differentiated tissue types. Throughout gastrulation and organogenesis these processes are repeated as the complexity of the embryo increases. These events are not restricted to the embryo but are also required in the adult during tissue replenishment. A signalling pathway first described in *Drosophila* has been implicated in tissue supply, patterning and cellular differentiation. The signal is supplied by Delta and received in the neighbouring cells by the receptor, Notch. The *Delta* gene encodes a transmembrane protein (Vassin et al., 1987; Heitzler and Simpson, 1991) which affects the development of adjacent cells displaying the transmembrane receptor, Notch (Wharton et al., 1985).

In *Drosophila*, a well-documented case where Delta/Notch signalling is required is during the production of neural precursors (see Artavanis-Tsakonas, 1995). A single neuroblast is produced from a precursor population (equivalence group) even though each cell in that population is capable of becoming a neuroblast. This production of individual neuroblasts occurs repeatedly from the equivalence groups until a full complement of neuroblasts has been derived. In each case, the cell that becomes the neuroblast signals via Delta to its neighbours and the activation of Notch in these cells prevents them from adopting a neural fate. This cell-cell dependent inhibitory signalling is referred to as lateral inhibition. Loss-of-function mutations in either of the corresponding genes, results in the production of as many neuroblasts as there are cells in the precursor population. In the peripheral nervous system (PNS), Delta/Notch signalling is required again to divert cell fate during differentiation (Hartenstein and Campos-Ortega, 1986; Hartenstein and Posakony, 1990; Spana and Doe, 1996; Guo et al., 1996).

In *Drosophila*, *Delta* and *Notch* are pivotal in numerous cell fate choices during both embryonic and adult life. In the embryo, homozygous null mutations for *Delta* or *Notch* result in the expansion of one cell type at the expense of another. In ectoderm discussed above, the embryonic nervous system is expanded while the embryonic epidermis is reduced (Lehman et al., 1983). In mesoderm, an expanded myoblast population

is apparent (Corbin et al., 1991). Similarly a phenotype exists in the endoderm lineage (Tepass and Hartenstein, 1995). In the adult, additional roles for *Delta* and *Notch* include specification of bristle precursors throughout the adult epidermis, cell type specification in the adult eye and differentiation of follicle cells during oogenesis (see Muskavitch, 1994). Genes homologous to *Delta* and *Notch* are present in *C. elegans* where *glp-1* and *lag-2* are required to allocate precursors of specific vulval fates (Greenwald et al., 1983). These pleiotropic functions appear to reflect the recurrent use of these genes in many scenarios where cell fate specification is occurring.

Vertebrate homologues of *Notch* have been identified in *Xenopus (X-Notch-1),* chick *(C-Notch-1)* and mouse *(Notch-1, Notch-2, Notch-3, Notch-4)* (Coffman et al., 1990; Henrique et al., 1995; Franco del Amo et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994; Uyttendaele et al., 1996). Like *Drosophila* and *C. elegans*, it is apparent that the Notch signalling pathway influences cell fate specification in vertebrates. Constitutively activated forms of Notch-1, which lack the extracellular portion of the protein, inhibit neurogenesis in *Xenopus* (Chitnis et al., 1995; Dorsky et al., 1995; Coffman et al., 1993). In addition, activated Notch inhibits muscle formation in *Xenopus* as well as in mouse cells in vitro and drives T lymphocyte lineage selection in the mouse (Kopan et al., 1994; Robey et al., 1996).

More recently, vertebrate homologues of *Delta* have been identified in *Xenopus (X-Delta-1, X-Delta-2),* chick *(C-Delta-1)* and mouse *(Dll1)* (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995; Jen et al., 1997). The *Delta-1* and *Dll1* genes appear to be orthologues due to conservation of amino acid sequence and gene expression. During embryogenesis, the predominant regions of *Delta-1/Dll1* gene expression are the neuroectoderm and the presomitic mesoderm. In the neuroectoderm, the punctate patterning of *C-Delta-1* expression foreshadows the spatiotemporal pattern of neuronal differentiation. Cells that have exited from the cell cycle transiently express C-*Delta-1* prior to differentiating into neurons (Henrique et al., 1995; Myat et al., 1996). Ectopic expression of *Delta-1* in *Xenopus* and chick inhibits the production of neurons in the neural plate and retina (Chitnis et al., 1995; Dorsky et al., 1997; Henrique, unpublished), presumably due to the ubiquitous activation of Notch-1. In addition, genetic analysis in the mouse demonstrates that Delta/Notch signalling is required during somitogenesis, since null mutant mice for *Notch-1* (Swiatek et al., 1994; Conlon et al., 1995) and *Dll1* (Hrabe de Angelis et al., 1997) exhibit defects in somite formation.

Here we report the identification of a novel vertebrate homologue of *Drosophila Delta*. This gene *Dll3* has been isolated in the mouse and is the third vertebrate *Delta* homologue to be identified. The *Dll3* gene, like *Dll1/Delta-1*, is predominantly expressed in the neuroectoderm and paraxial mesoderm during embryogenesis. We show that *Dll3*-expressing cells in the neuroepithelium, like those that express *Dll1* are postmitotic and that *Dll3*, like *X-Delta-1*, can inhibit primary neurogenesis in *Xenopus*. We suggest that, in the mouse, *Dll3* and *Dll1* cooperate in the formation of somite boundaries during segmentation of the paraxial mesoderm. We therefore argue that *Dll3* and *Dll1* function together in several contexts during mouse embryogenesis.

MATERIALS AND METHODS

Generation of subtracted PS-(Ect+End) cDNA library and identification of cDNAs

The PS-(Ect+End) subtracted cDNA library was generated according to the procedure described in Harrison et al. (1995). Here, single-stranded DNA was generated from the Primitive Streak library and subtracted with biotinylated RNA generated from the Ectoderm and Endoderm libraries. The subtracted PS-(Ect+End) library was hybridised to 10,000 gridded clones from the Primitive Streak library according to Harrison et al. (1995). Hybridising clones were sequenced and those representing novel cDNAs were used to generate antisense riboprobes for RNA in situ hybridisation.

Northern blot analysis

Total RNA was isolated from embryonic tissue and ES cells according to Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated using the poly(A)Tract system (Promega). CGR8 ES cells (Mountford et al., 1994) were cultured according to Wilson et al. (1993). A DNA fragment representing 470 bp of *Dll3* 3′UTR was generated by PCR (1760-2225 bp), gel purified using QIAEX gel extraction (QIAGEN) and ^{32}PdCTP incorporated using a Random-Primed DNA Labelling Kit (Boehringer Mannheim). RNA was denatured and size fractionated (1% agarose, 2.2 M formaldehyde), transferred to a nylon membrane (Hybond-N+), cross linked to the membrane by exposure to UV light and hybridised according to Sambrook et al. (1989) using 1×10^6 cts/minute/ml of hybridisation buffer.

Embryo recovery

Embryos were collected from timed C57BL6 ×DBA matings. Noon on the day of appearance of the vaginal plug is designated 0.5 dpc. Embryos were dissected from the uterus and Reichert's membrane removed as described by Beddington (1987) in M2 medium (Hogan et al., 1994) containing 10% fetal calf serum (FCS, Advanced Protein Products) instead of bovine serum albumin. Embryos for whole-mount RNA in situ hybridisation were rinsed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS. Embryos for RNA isolation were immediately dissolved in denaturing solution (Chomczynski and Sacchi, 1987).

RNA in situ hybridisation to mouse embryos

Antisense riboprobes were transcribed according to Harrison et al. (1995). For *Dll3*, riboprobes were derived from PS93 (1075-2243 bp) or Delta-S (1-2102 bp). Whole-mount RNA in situ hybridisation was carried out according to Wilkinson (1992) using the hybridisation conditions of Rosen and Beddington (1993). The length of proteinase K treatment varied; 5.5-6.5 dpc (5 minutes), 7.0-8.5 dpc (10 minutes), 9.0 dpc and older (15 minutes.) Embryos were postfixed in 4% paraformaldehyde, 0.1% glutaraldehyde in PBS. RNA in situ hybridisation of cryosections were performed according to Myat et al. (1996). Double in situ hybridisation was achieved as follows. Synthesised riboprobes incorporating DIG-UTP or fluorescein-UTP were detected with alkaline phosphatase (AP)-conjugated anti-DIG or anti-fluorescein antibodies. The substrate for AP was either BCIP/NBT (blue) or BCIP/INT (brown; Boehringer Mannheim). Following the AP reaction in the first instance, the precipitate was fixed with 4% PFA in PBS, the AP activity destroyed with 0.1 M glycine in water, pH 2.2 (2×5 minutes, cryosection; 2×15 minutes, whole mount). The embryos or cryosections were blocked again prior to incubation with the next antibody.

Embryo sections

After whole-mount RNA in situ hybridisation, embryos were processed for paraffin sectioning by dehydration through an ethanol series, clearing in Histoclear (National Diagnostics) and embedding

Fig. 1. Nucleotide sequence and conceptual translation of *Dll3* cDNA. The cDNA consists of 2243 bp with 35 adenine residues at the 3′ end while the protein consists of 585 amino acids. The putative signal sequence (amino acids 1-32), the rotamase signature (amino acids 56-71) and the transmembrane domain (amino acids 489-513) are underlined. The DSL is heavily shaded, the six EGF-like repeats are lightly shaded and the stop codon is indicated (·). The EMBL accession number for *Dll3* is Y11895.

in paraffin wax (Histoplast, m.p. 56°C). Sections were dewaxed in Histoclear (5 minutes) and mounted under coverslips in DPX mountant (BDH). Embryos for cryosectioning were fixed with 4% PFA/PBS at 4°C overnight, cryoprotected with 30% sucrose/PBS at 4°C overnight.

BrdU labelling of mouse embryos and immunohistochemistry

10.5-13.5 dpc mice were injected with 100 μg BrdU per gram body weight according to Miller and Nowakowski (1988) for 30-120 minutes. Embryos were fixed in 4% PFA/PBS and cryosectioned as

above. RNA in situ hybridisation was performed on the cryosections using the Fast Red fluorescent alkaline phosphatase substrate (Boehringer Mannheim). After staining, slides were washed in PBS and processed for BrdU immunodetection (Biffo et al., 1992). Islet-1/2 protein was detected as follows. Following RNA in situ hybridisation and staining using the Fast Red substrate slides were washed in PBS and blocked. Anti-BrdU (1/50; Beckton Dickinson) and anti-islet-1/2 (1/50; Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, MD 21205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under contract N01-HD-2-3144 from the NICHD) were detected using FITC-conjugated goat anti-mouse secondary antibody (1/40; Cappel). Composite fluorescent images were obtained using a Bio-Rad MRC600 confocal microscope.

In vitro transcription, *Xenopus* embryos and microinjection

Dll3 cDNA was cloned into CS2+ vector (Turner and Weintraub, 1994). Capped RNA for injection was generated according to Kreig and Melton (1984). Capped *lacZ* RNA was generated from *pSP6nucbgal* (Smith and Harland, 1991). Synchronous embryos were obtained by artificial fertilisation, maintained in 10% Normal Amphibian Medium (NAM: Slack, 1984) at 14-18°C and staged according to Nieuwkoop and Faber (1975). Embryos for microinjection were transferred to 75% NAM containing 4% Ficoll Type 400 (Sigma). 200 pg of *Dll3* and 50 pg of *lacZ* RNA was injected in a volume of 10 nl into a single blastomere at the 2- to 4-cell stage. RNA was injected into an animal blastomere on the prospective dorsal side of the embryo in order to direct expression of the RNA into the neural tissue. 2 hours after injection embryos were returned to 10% NAM.

Xenopus X-gal staining and whole-mount RNA in situ hybridisation

At neural plate stage (stage 13-15), the vitelline membrane was removed and embryos fixed in MEMFA (Harland, 1991) for 1 hr at 4°C. Embryos were washed 3×20 minutes in wash buffer and stained at 37°C for 1 hour. X-gal wash and stain solutions were as described in Beddington et al. (1989). Embryos were postfixed in MEMFA at 4°C overnight and dehydrated. Whole-mount RNA in situ hybridisation was performed essentially as described by Harland (1991).

RESULTS

Isolation of *Dll3*

Following DNA sequencing and whole-mount RNA in situ hybridisation analysis of clones hybridising to the PS-(Ect+End) subtracted probe, several cDNAs corresponding to genes expressed in mesoderm and the primitive streak during gastrulation were identified. Of the six novel genes that were identified in this screen, one proved to be a homologue of the *Drosophila* gene *Delta*.

Dll3 cDNA consists of 2243 bp and contains an open reading frame (ORF) of 1755 bp (Fig. 1). This ORF encodes a protein of 585 amino acids and contains several features that identify it as a homologue of *Drosophila Delta*. At the N terminus, a stretch of 32 hydrophobic amino acids indicate a signal sequence. Another hydrophobic region is located between amino acids 489-513 representing a membrane-spanning region of the protein. Like *Drosophila Delta*, the extracellular domain contains EGF-like repeats and a cysteine-rich domain, the DSL (Tax et al., 1994), which is required for binding to the Notch receptor (Fehon et al., 1990). There are six EGF-like repeats encoded by the *Dll3* cDNA with a gap consisting of 29 amino acids placed between the first and second repeats. This represents fewer repeats than are present in *Drosophila Delta* (9 repeats) (Kopczynski et al., 1988) and *Dll1* (8 repeats) (Bettenhausen et al., 1995). The Dll3 protein also contains a rotamase signature between amino acids 56 and 71 (PROSITE database, PDOC00426), a feature that is absent from *Drosophila Delta* and *Dll1* (Fig. 2). Although polyadenylation signals have not been identified in the 3′UTR, this sequence is derived from a full-length transcript as a poly(A) tail was present at the 3′ end of the cDNA. Northern blot analysis using a 3′UTR-derived probe of the *Dll3*, indicated that the gene produced a predominant transcript of 2.2 kb and a minor transcript of 0.8 kb. The 2.2 kb transcript is detected in the embryo (11.5 dpc), to a much lesser extent than in undifferentiated ES cells and is absent from the placenta (11.5 dpc) (Fig. 2).

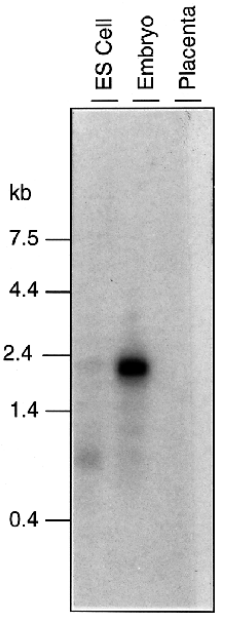

Fig. 2. Northern blot analysis of *Dll3* gene expression. 2.5 μg of Poly(A)+ RNA from undifferentiated ES cells, 11.5 dpc embryo and 11.5 dpc placenta was hybridised to a 3′UTR probe generated from the *Dll3* cDNA. A predominant transcript of 2.2 kb was detected in undifferentiated ES cell and embryo RNA. A minor transcript of 0.8 kb is visible.

An amino acid sequence comparison of Dll3 with *Drosophila* Delta and the mouse Dll1 (Fig. 3A) indicates that, overall, Dll3 shares 29% and 36% identity with *Drosophila* Delta and Dll1, respectively. Regions of greater identity exist between the EGF-like repeats, the greatest occurs between repeat 3 of Dll3 and repeat 5 of *Drosophila* Delta (47%), and between repeat 4 of Dll3 and repeat 6 of Dll1 (63%). The intracellular domains of these three proteins are highly dissimilar, the one from Dll3 being shorter. Fig. 3B illustrates a comparison of the DSL of Dll3 with the DSL of vertebrate and invertebrate Delta-like proteins. The DSL domain of Dll3 shows considerable divergence although some conservation of cysteine spacing is evident.

Dll3 inhibits primary neuron formation in *Xenopus*

In *Xenopus*, primary neurogenesis gives rise to three longitu-

dinal stripes of neurons either side of the dorsal midline. These neurons can be identified by the expression of neural-specific type II βtubulin referred to as *N-tubulin* (Oschwald et al., 1991; Chitnis et al., 1995). Ectopic expression of a constitutively active form of Notch-1 prevents the production of these primary neurons as judged by the loss of *N-tubulin*-expressing cells in the neural plate (Chitnis et al., 1995). Likewise, ectopic expression of *X-Delta-1* blocks neuron production indicating that it binds to and activates the Notch receptor (Chitnis et al., 1995). Given that Dll3 has such a divergent DSL and a reduced number of EGF-like repeats, it was possible that it might not be able to activate the Notch receptor. In order to test this, *Dll3* and *lacZ* RNA were coinjected into a single *Xenopus* blastomere at the 2- to 4-cell stage and the embryos allowed to develop until the neural plate stage (stage 13-15). Whole-mount RNA in situ hybridisation revealed a reduction or complete loss of *N-tubulin* expression in the neural plate on the injected side of the embryo, showing that ectopic expression of Dll3 can also inhibit primary neuron production. Even though *Dll3* is derived from the mouse, we observed inhibition of primary neurogenesis in 84% (16/19) of injected embryos, which is comparable with 79% (15/19) when *X-Delta-1* RNA is injected (Fig. 4).

Tissue-specific *Dll3* gene expression during gastrulation and early organogenesis

RNA in situ hybridisation either to intact embryos (5.5-13.5 dpc) or to cryosections (9.5-15.5 dpc) was used to determine the localisation of *Dll3* transcripts. *Dll3* transcripts are detected immediately prior to primitive streak formation (5.5-6 dpc) and are localised to the epiblast (data not shown). A low level of transcripts persist throughout the epiblast following the onset of gastrulation and up to mid-primitive streak stages (Fig. 5A,B). Later,

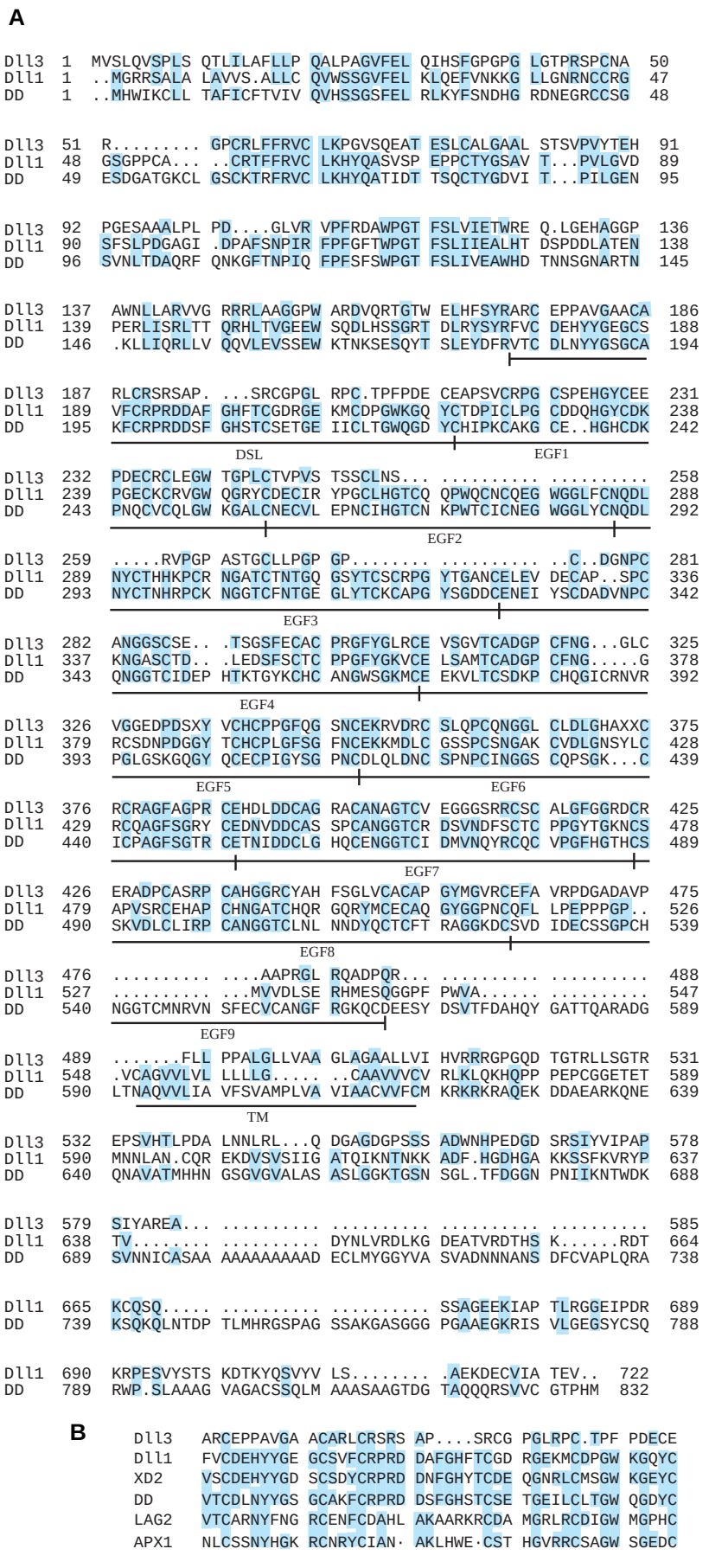

Fig. 3. Alignment of predicted protein sequence of Dll3 and other Delta-like proteins. (A) Protein alignment between Dll3, Dll1 and *Drosophila* Delta. The DSL, EGF-like repeats and transmembrane domain (TM) are underlined. The EGF-like repeats are numbered according to *Drosophila* Delta. The conserved amino acids are shaded. (B) Alignment of the DSL region from all Delta like proteins. The conserved amino acids are shaded. Dll3, mouse Delta-like 3; Dll1, mouse Delta-like 1 (Bettenhausen et al., 1996); XD2. *Xenopus* Delta-2 (Jen et al., 1997); DD, *Drosophila* Delta (Kopczynski et al., 1988); APX1 (Mello et al., 1994) and LAG2 (Tax et al., 1994; Henderson et al., 1994) represent Delta like genes in *C. elegans*.

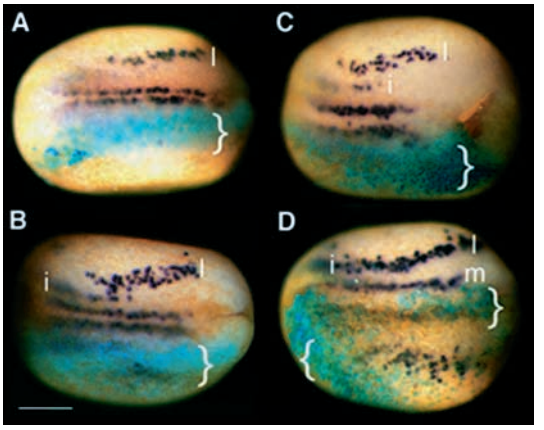

Fig. 4. Injection of *Dll3* RNA inhibits primary neuron formation in *Xenopus*. (A-D) Neural plate stage *Xenopus* embryos (dorsal view, posterior to the right) showing *N-tubulin* gene expression (purple) identifying primary neurons, in medial (m), intermediate (i) and lateral (l) locations. A single injection of 50 pg of *lacZ* RNA and 200 pg of *X-Delta-1* or *Dll3* RNA into a 2- or 4-cell embryo gave localisation of the transcripts to different regions of the neural plate as shown by X-gal staining (bracket). (A) Ectopic expression of *X-Delta-1* RNA supresses primary neuron formation. The lateral stripe of neurons (l) is absent in the region where *X-Delta-1* RNA is localised, indicated by Xgal-positive cells (bracket). (B-D) Ectopic expression of *Dll3* RNA supresses primary neuron formation. Lateral and intermediate neurons are reduced in number (B) or absent (C) where *Dll3* RNA is localised (bracket). (D) Medial, intermediate and anterior lateral neurons are absent where *Dll3* RNA is localised (bracket). Scale bar, 330 μm.

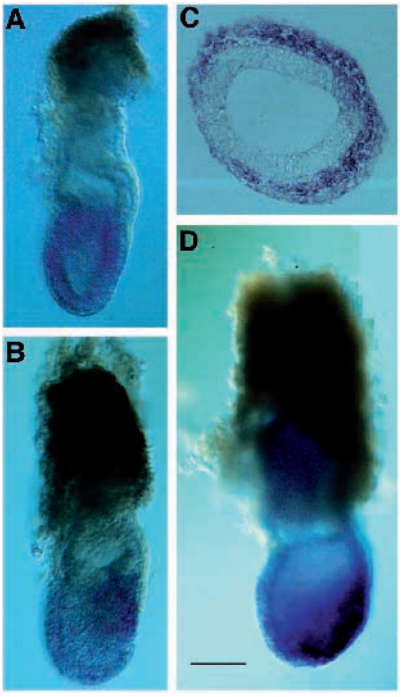

Fig. 5. *Dll3* gene expression during early gastrulation determined by whole-mount RNA in situ hybridisation. In each case, the posterior of the embryo is to the right. (A) Lateral view of an early-streak-stage embryo where *Dll3* transcripts are localised only to the epiblast. (B) Lateral view of a mid-primitive streak-stage embryo. *Dll3* expression persists in the epiblast and is seen in the nascent mesoderm as it emerges from the primitive streak. (C) Transverse section (7 μm) through the proximal region of full-length primitive-streak-stage embryo (slightly earlier stage than D), the primitive streak is to the right. *Dll3* is expressed in the primitive streak, the cells about to ingress through the primitive streak and the mesoderm. (D) Lateral view of a late-streak-stage embryo. *Dll3* expression persists in the primitive streak but is now restricted to the distal/posterior nascent mesoderm. Scale bar, 190 μm (A); 200 μm (B); 160 μm (C); 220 μm (D).

at the full length primitive streak stage, epiblast expression is only detected in the cells that are adjacent to the primitive streak (Fig. 5B,C). Transcripts are also localised to the primitive streak itself and extend along its entire length but are absent from the node. Expression of *Dll3* is also apparent in the nascent mesoderm as it emerges from the primitive streak (Fig. 5C). At first, this expression occurs throughout the entire wing of mesoderm but by the late primitive streak stage only the distal mesoderm which is fated to become paraxial mesoderm (Parameswaran and Tam, 1995) contains *Dll3* transcripts (Fig. 5D). At all stages, expression in the epiblast appears considerably lower than that in the mesoderm.

Dll3 transcripts continue to be localised to the primitive streak throughout the latter stages of gastrulation and also persist in the tail bud (Fig. 6A,F). At early somite stages the highest level of *Dll3* transcripts is seen in the paraxial mesoderm (Fig. 6A). Transverse sections through the posterior trunk confirm that the transcripts are localised to the paraxial mesoderm and are not present in axial (notochord), intermediate or lateral mesoderm (Fig. 6B). However, expression in paraxial mesoderm is only observed in presomitic mesoderm and nascent somites, and appears to cease as somites mature (Fig. 6C). The level of transcript accumulation is highest in the presomitic mesoderm, lower in the somite that is in the process of forming and in the most immature somites expression is detectable only at the anterior margin (Fig. 6C). This pattern of expression is maintained throughout somitogenesis indicating that *Dll3* may be involved only in the initial formation of somites and not their subsequent differentiation.

Dll3 expression in the presumptive brain region is first detected at 8.25 dpc at a low level (Fig. 6A). High levels of transcript are seen only in a punctate pattern, and are first seen in the presumptive midbrain at 8.75 dpc (Fig. 6D), which then extends posteriorly into the hindbrain and spinal cord regions (Fig. 6E,F). This peppered expression pattern appears to coincide with the formation of neurons which, in the mouse, arise in the brain (8.5 dpc) before the spinal cord (9.5 dpc) (Mastick and Easter, 1996; Nornes and Carry, 1978). By the 25-somite stage, the anterior neuropore has closed and expression in the midbrain region has extended laterally. In addition, *Dll3* transcripts are particularly prominent in the nasal pits, sensory ganglia (V, VII, IX, X), the sympathetic chain and the dorsal root ganglia (Fig. 6E,F).

Proliferative status of *Dll3*-expressing cells in the CNS

Neural precursors proliferate within a pseudostratified columnar epithelium (ventricular zone) and their nuclei migrate between the basal and lumenal surfaces in a cell-cycle-dependent manner. S-phase nuclei lie basally while

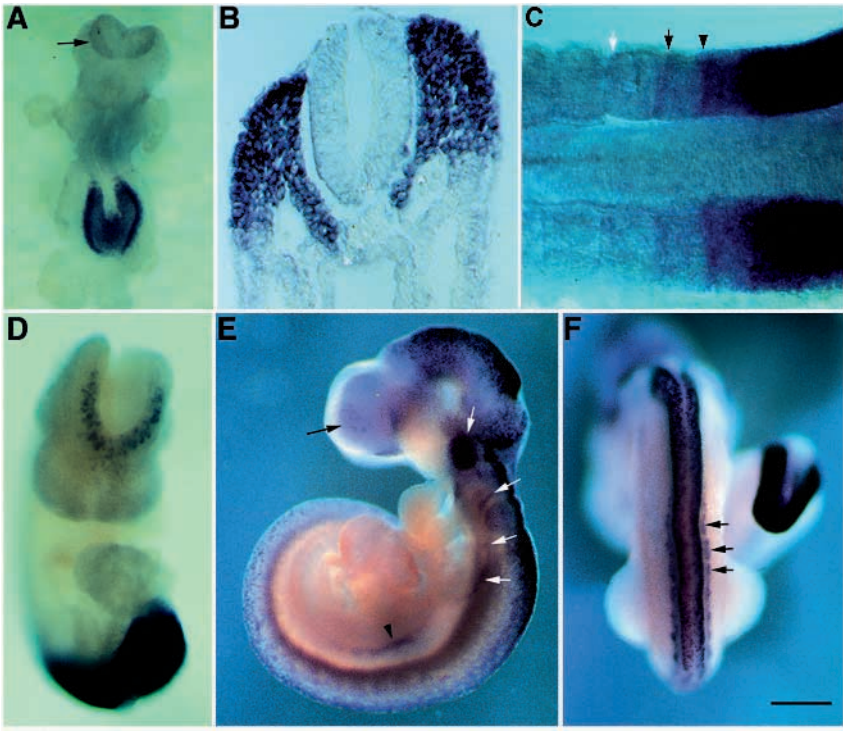

Fig. 6. *Dll3* gene expression in paraxial mesoderm and neuroectoderm determined by whole-mount RNA in situ hybridisation. (A) Ventral view, flat-mounted 8-somite embryo. *Dll3* transcripts are localised to the primitive streak, presomitic mesoderm, nascent somites and the anterior neuroectoderm (arrow). (B) Transverse section through posterior trunk of 12-somite embryo shows *Dll3* expression in the paraxial (presomitic) mesoderm. (C) Flat mount of an 8-somite embryo viewed dorsally (surface ectoderm and endoderm removed) shows that *Dll3* expression is high in presomitic mesoderm and greatly reduced upon somite formation. A band of expression defines the anterior boundary of the nascent somite (white arrow) and of the somite which is in the process of forming (black arrow). At the anterior of the presomitic mesoderm, a thicker band of expression defines the anterior of the next somite that is to form (black arrowhead). (D) Anterior view of an 11-somite embryo shows punctate *Dll3* gene expression in the dorsal region of the presumptive midbrain. *Dll3* gene expression in a 25-somite embryo viewed laterally (E) and dorsally (F). (E) Expression is detected superficially in the midbrain and hindbrain, the nasal pits (black arrow), the sensory cranial ganglia (V, VII, IX, X) (white arrows), the sympathetic chain (black arrowhead) and along the length of the spinal cord. (F) *Dll3* transcripts are also localised to the dorsal root ganglia (arrows) and expression persists in the primitive streak/tail bud. Scale bar, 380 μm (A); 35 μm (B); 80 μm (C); 280 μm (D); 400 μm (E,F).

mitosis occurs at the lumenal surface. As the neural tube closes, cells at the basal surface start to lose their cytoplasmic connection with the lumenal surface and become postmitotic. These neuroblasts reside in the newly established intermediate layer (marginal zone) and will differentiate into neurons. Neurons migrate laterally into the mantle layer before elaborating axons. As neural differentiation progresses the mantle layer broadens initially in the ventral half of the neural tube (the basal plate) and then dorsally in the alar plate. *Dll3* is expressed in the spinal cord along its dorsoventral extent (Fig. 7A) until 15.5 dpc when transcripts are no longer detected. This loss of expression is preempted by reduced expression at 14.5 dpc (data not shown). In order to clarify the proliferative status of the *Dll3*-expressing cells, RNA in situ hybridisation was performed in conjunction with BrdU labelling. This showed that *Dll3*-expressing cells are postmitotic since they do not incorporate BrdU (Fig. 7B). In general, the nuclei that had incorporated BrdU lie adjacent to the lumen, while those that express *Dll3* lie lateral to this (Fig. 7C). Since the cells that express *Dll3* are postmitotic, we determined whether they had started terminal differentiation. *Islet-1* is a LIM homeobox gene expressed by motor neurons soon after they have left the cell cycle and by dorsal ipsilateral interneurons (Ericson et al., 1992). An antibody that detects islet-1 and islet-2 (also present in motor neurons; Tsuchida et al., 1994) was used in double labelling experiments and shows that virtually all cells that express *Dll3* are devoid of the islet-1/2 protein (Fig. 7E), although coexpression was detected in occasional cells adjacent to the basal plate (Fig. 7F). Taken together these data show that *Dll3* gene expression occurs after neuroepithelial cells have ceased proliferating but generally before terminal differentiation.

The relationship of *Dll3* expression with that of *Dll1*

It has been demonstrated in the chick (Henrique et al., 1995; Myat et al., 1996) and we have confirmed in the mouse (data not shown) that *C-Delta-1*- and *Dll1*-expressing cells of the spinal cord are also postmitotic. Therefore, in the mouse, *Dll1* and *Dll3* may be expressed in the same cells. RNA in situ hybridisation shows that the distribution of *Dll3* and *Dll1* transcripts differs within the spinal cord (Fig. 8A,B), such that *Dll1*-expressing cells are localised to the ventricular zone while *Dll3*-expressing cells lie more laterally. Double RNA in situ hybridisation confirms this and shows very little overlap in gene expression (Fig. 8C). In three small patches, some overlap of expression is observed and this normally corresponds with the expression of *Dll3* extending towards the lumen and 'invading' the *Dll1* domain. While a more or less continuous rim of *Dll3*-expressing cells extends dorsoventrally, the expression of *Dll1* is discontinuous and creates two gaps adjacent to the lumen that are devoid of *Delta* gene expression. These gaps in *Dll1* expression coincide with the expression of *Serrate-1*, another Notch ligand (data not shown). In the hindbrain, similar domains of coincident and separate expression exist for *Dll3* and *Dll1* (Fig. 8D,E). Furthermore, whole-mount RNA in situ hybridisation demonstrates that *Dll3* is only expressed in a subset of the regions of the hindbrain containing *Dll1* transcripts. Thus at 8.5 dpc, only *Dll1* appears to be expressed in patches in the lateral midbrain and in the forebrain while both *Dll3* and *Dll1* are co-localised in the dorsal midbrain (Fig. 8F). By 9.5 dpc, *Dll3* expression is observed in the forebrain but unlike *Dll1*, in a very defined patch of cells in the ventral telencephalon (Fig. 8G). In addition at this stage, *Dll3* transcripts appear to outline rhombomere 4 and mark the ventral aspect of rhombomere 2.

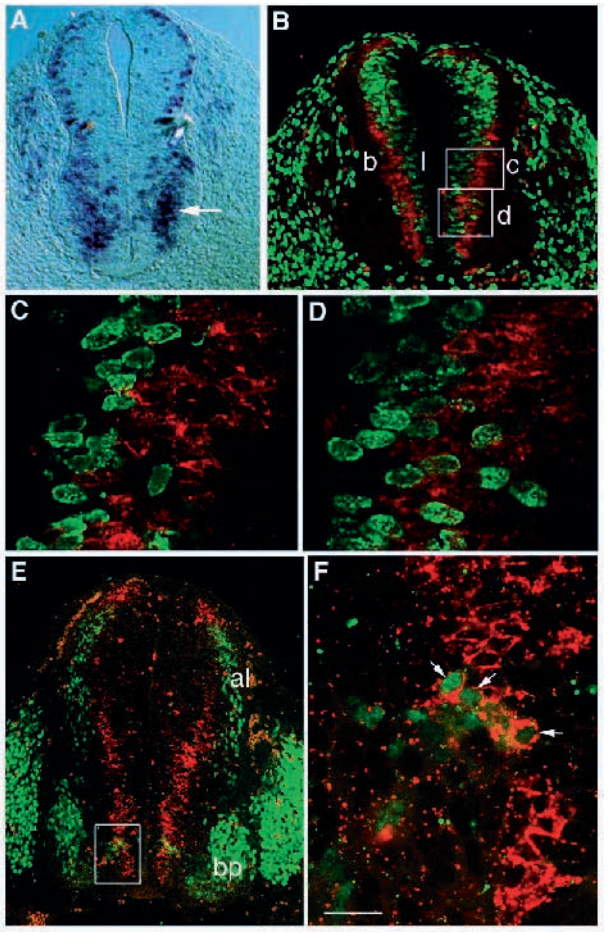

Fig. 7. Localisation of *Dll3* transcripts, BrdU-labelled cells and islet-1/2 protein in the spinal cord. Cryosections (12-14 μm) of 9.5 to 12.5 dpc spinal cord subjected to RNA in situ hybridisation and antibody detection of BrdU and islet-1/2. (A) At 9.5 dpc, *Dll3* transcripts are localised to the basal surface of the ventricular zone and the dorsal root ganglia and are absent from the roof and floor plates. An active region of neurogenesis abuts the expanding basal plate and here a broader band of transcripts accumulation is evident (arrow). (B) *Dll3* transcript (red) and BrdU-labelled cells (green) at 11.5 dpc. BrdU-labelled cells reside within the ventricular zone while *Dll3* transcripts accumulate more laterally. This is the case along most of its dorsoventral extent (box c). In the ventricular zone, a region opposite the basal plate (box d) exists where cells expressing *Dll3* and cells labelled with BrdU are scattered between the basal (b) and lumenal (l) surfaces. (C,D) Higher magnification of boxes c and d, respectively. One small region opposite the basal plate shows *Dll3*-expressing cells interspersed with those that have incorporated BrdU (D). (E) *Dll3* transcript (red) and islet-1/2 protein (green) at 11 dpc. *Dll3* transcripts are localised to the venricular zone while islet-1/2-positive cells (subset of differentiated motor neurons) are localised to the mantle zone and the dorsal root ganglia. Note the build up of islet-1/2-positive neurons in the basal plate (bp) compared to the alar plate (al). In the ventral part of the spinal cord, opposite the basal plate, *islet-1/2*-positive cells are found within the ventricular zone. (F) Higher magnification of box in E shows that, within this region, a few cells appear to coexpress *Dll3* and *islet-1/2* (arrows). Scale bar, 65 μm (A); 200 μm (B); 30 μm (C,D); 150 μm (E); 35 μm (F).

Both *Dll3* and *Dll1* transcripts are abundant in paraxial mesoderm but double RNA in situ hybridisation revealed differences in their patterns of expression. Within the presomitic mesoderm, the levels of *Dll1* transcripts are not constant resulting in two regions where expression is relatively higher. In contrast, *Dll3* transcripts were more uniformly distributed along the presomitic mesoderm but extended more rostrally than *Dll1* and this rostral limit appeared to correspond to the anterior of the somite just about to form (Fig. 8H). During formation of a somite, a broad band of *Dll3* expression was evident anteriorly while *Dll1* was restricted to a posterior domain. In the most recently formed somite, these bands were refined to give a thinner stripe of *Dll1* expression at the posterior and a comparable anterior stripe of *Dll3* (Fig. 8I,J). The anterior stripe of *Dll3* expression was only evident in the most recently formed somite whereas *Dll1* expression in the posterior persisted in mature somites.

DISCUSSION

Identification of a divergent vertebrate homologue of Delta

The mouse *Delta* homologue, *Dll3*, which we identified from a subtractive cDNA screen, is significantly divergent from *Drosophila Delta* and its other homologues (Fig. 3A). In the DSL region, the spacing of cysteine residues, which is conserved in all other *Delta* homologues, is only partly conserved in *Dll3*. Since the DSL is required for the binding of Delta to Notch (Fehon et al., 1990), this divergence suggests that Dll3 may preferentially activate a different Notch receptor to Dll1/Delta-1. The identification of a rotamase signature indicates that Dll3 has the potential to bend proteins (Fischer and Schmid, 1990), suggesting additional protein interactions.

Ectopic expression of *X-Delta-1* or a constitutively active form of *X-Notch-1* inhibits primary neuron formation in *Xenopus* (Chitnis et al., 1995). Despite the divergence of *Dll3*, it is also capable of inhibiting primary neurogenesis (Fig. 4). This strongly suggests that Dll3 can bind and activate Notch-1 or another Notch receptor and therefore is a true functional homologue of *Drosophila Delta*.

Potential functions of *Dll3* during mouse development

The epiblast, primitive streak and tail bud

One function of the Delta/Notch signalling pathway is to control the release of individual cells from an initial population where cells are equivalent. A putative stem cell population resides within the primitive streak and its descendant, the tail bud, in the mouse (Tam and Beddington, 1987; Lawson et al., 1991; Wilson and Beddington, 1996; Wilson and Beddington, unpublished) which contributes to the supply of mesoderm required for axial elongation. Since *Dll3, Dll1* and *Notch-1* are all expressed in the primitive streak and tail bud (Figs 5C, 6A,F) (Bettenhausen et al., 1995; Franco del Amo et al., 1992; Reaume et al., 1992), they may influence the balance between the stem cell pool and the rate at which progeny for differentiation are supplied. One might predict that loss-of-function mutations in members of the Delta/Notch signalling pathway would cause a decrease in the size of the stem cell population and hence axis truncations. However, such a phenotype has not been observed for either *Notch-1, Dll1* or *RBP-Jκ* (a component of the Notch signal transduction pathway) null mutant embryos (Swiatek et al., 1994; Conlon et al., 1995; Hrabe de Angelis et

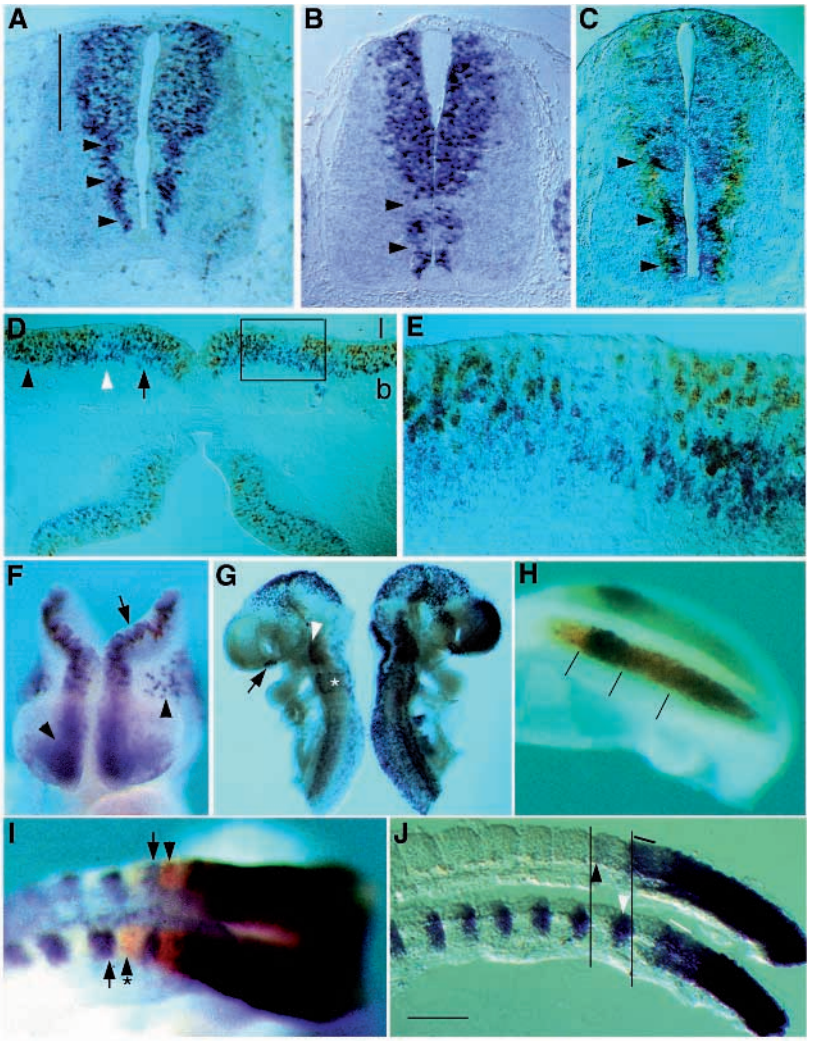

Fig. 8. Colocalisation of *Dll3* and *Dll1* in neuroectoderm and paraxial mesoderm determined by double RNA in situ hybridisation. (A) Transverse cryosection (12 μm) of 12.5 dpc spinal cord. *Dll3* is expressed in the ventricular zone but distribution of expression is not even throughout. Expression is highest at the basal surface and this is seen along its dorsoventral extent. Expression is also observed extending mediolaterally within the ventricular zone, this is most evident opposite the alar plate (line) and in narrower domains ventrally (arrowheads). Transcripts are excluded from the roof and floor plates. (B) Transverse cryosection (12 μm) of a 12.5 dpc spinal cord. *Dll1* is expressed in the ventricular zone extending mediolaterally along its dorsoventral extent. Two gaps in this expression domain exist (arrowheads). Transcripts are excluded from the roof and floor plates. (C) Transverse cryosection (14 μm) of 10.5 dpc spinal cord. *Dll3* transcripts are localised to the basal surface of the ventricular zone (brown) while *Dll1* transcripts (blue) are detected within the ventricular zone medial to *Dll3*. Regions are evident where *Dll3* and *Dll1* transcripts have co-localised (arrowheads). (D) Transverse cryosection (12 μm) of 12.5 dpc hindbrain. *Dll3* (blue) and *Dll1* (brown) are expressed in the ventricular zone in domains spreading medial to lateral. Distinct domains exist where *Dll1* is expressed adjacent to the lumen (l) and *Dll3* adjacent to the basal surface (b) (arrow) and overlapping domains of Delta gene expression exist (black arrowhead). There is a gap adjacent to the lumen where *Dll1* is not expressed (white arrowhead). (E) Similar to boxed region of D; expression domains identified as in D. (F) Anterior view of 10-somite embryo. *Dll3* (brown) and *Dll1* (blue) transcripts colocalise to the dorsal region of the presumptive midbrain (arrow). *Dll1* transcripts alone are detected laterally in the presumptive midbrain and in the forebrain (arrowhead). (G) Internal lateral view of the head region of a 9.5 dpc embryo. The head region was dissected in half, the *Dll3* riboprobe hybridised to the left half and the *Dll1* probe hybridised to the right half. *Dll1* transcripts are localised to much of the forebrain while *Dll3* transcripts are restricted to a small region in the ventral forebrain (arrow). *Dll3* transcripts outline a rectangular domain opposite the second branchial arch (*), the ventral side of the rectangle is thicker and above this opposite the first branchial arch a similar expression domain exists (arrowhead). (H) Lateral view of presomitic mesoderm from 8-somite embryo (posterior to right). *Dll3* (brown) and *Dll1* (blue) are both expressed in the presomitic mesoderm. *Dll3* is expressed in a domain at the anterior of the presomitic mesoderm. Posterior to this *Dll3* and *Dll1* are coexpressed with different relative levels of expression creating 4 domains (marked with lines). (I) Dorsal caudal view of 10-somite embryo (same as F, posterior to right). *Dll3* (brown) and *Dll1* (blue) transcripts are colocalised within the presomitic mesoderm but not the somites. *Dll3* transcripts are localised to the anterior boundary of the forming somite (black arrowhead) and the nascent somite (black arrowhead*). *Dll1* transcripts are localised to the posterior somite boundaries (black arrow). (J) Lateral view of caudal part of 9 dpc embryo split along the midline (fine vertical lines align somites, posterior to right). The embryo was dissected in half, the *Dll3* riboprobe hybridised to the top half and the *Dll1* probe hybridised to the bottom half. *Dll3* is expressed in a broad band of cells at the anterior of the forming somite (short thick black line), this expression is refined to a faint narrow band at the anterior of the nascent somite (black arrowhead). *Dll1* is expressed in a broad band presumably at the posterior of the forming somite (white line), this domain narrows to the posterior boundary of the somites (white arrowhead). The relative differences in expression of *Dll3* and *Dll1* observed in the presomitic mesoderm in H are not evident in J as the colorimetric reaction was extended to best visualise the somite expression. Scale bar, 300 μm (A,B); 110 μm (C); 100 μm (D); 40 μm (E); 200 μm (F); 800 μm (G); 250 μm (H); 200 μm (I);160 μm (J).

al., 1977; Oka et al., 1995), but this failure to disrupt gastrulation could be due to compensation by other family members. Although speculative, the uniform expression, albeit apparently low, of *Dll3* in pre- and early gastrulation epiblast may help to maintain pluripotency of this tissue.

Somitogenesis

Segmentation of paraxial mesoderm proceeds in a rostrocaudal sequence with the newly generated epithelial somite forming at the anterior of the presomitic (unsegmented) mesoderm. A periodicity is apparent in the presomitic mesoderm in the form of somitomeres; whirls of mesenchyme thought to presage the formation of epithelial somites (Meier, 1979; Tam et al., 1982). The distribution of *Dll3* and *Dll1* transcripts in the presomitic mesoderm clearly indicates that this tissue has explicit anterior-posterior polarity (Fig. 8H). Rotation of the presomitic mesoderm through 180° in the chick results in the original sequence of somite formation being

maintained so that they now form in a caudal-to-rostral sequence (Menkes and Sandor, 1977), arguing that this expression profile reflects a stable 'prepattern' within the presomitic mesoderm. However, if the cells are disaggregated prior to grafting into a host, then a normal rostrocaudal sequence results (Menkes and Sandor, 1969; Stern and Keynes, 1986). It is possible that the Delta genes play a role here by maintaining cell-cell interactions required for stable anterior-posterior polarity.

Ultimately the production of a somite requires the formation of a boundary and the final transition from mesenchyme to epithelium necessitates an increase in cellular adhesion (see Keynes and Stern, 1988). *Dll3* and *Dll1* (Bettenenhausen et al., 1995) are expressed in the presomitic mesoderm and during the birth of nascent somites. Despite this overlap of expression in the paraxial mesoderm, *Dll1* null mutant embryos display a somite phenotype (Hrabe de Angelis et al., 1997). This suggests that *Dll3* cannot compensate for the absence of *Dll1* and that *Dll1* and *Dll3* do have different functions during somitogenesis. Mutant analysis shows that *Dll1* is required for the formation of epithelial somites and in establishing their anterior-posterior polarity. Notch signalling is also implicated in somitogenesis since null mutations for both *Notch-1* and *RBP-Jκ* exhibit a somite phenotype (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995). In contrast to *Dll1* mutants, *Notch-1* and *RBP-Jκ* mutants form epithelial somites, but these are irregular in size and shape. Despite this irregularity, there is evidence that anterior-posterior and dorsal-ventral pattern is established.

Dll3 and *Dll1* are expressed in nascent somites as well as in the somite that is in the process of forming. In the nascent somite, *Dll3* is expressed at the anterior boundary while *Dll1* is expressed at the posterior boundary. Given the expression of *Dll3* and *Dll1* on either side of the nascent somite boundary, it is possible that one of their roles may be to reinforce segmentation by homotypic protein interaction. In *Drosophila*, Delta can bind to itself as well as to Notch (Fehon et al., 1990) and therefore one could envisage an adhesive function whereby the two mutually exclusive Delta molecules serve to maintain nascent somite integrity. This also assumes that Dll1 and Dll3 do not bind to one another, which is supported by the fact that they are considerably divergent. That like and unlike somite halves exist is demonstrated by grafting experiments in the chick, which show that when the anterior (A) of one somite and the posterior (P) of another somite adjoin the integrity of the somite is maintained. But when two like halves (A and A or P and P) abut then cell mixing occurs and the boundary is lost (Stern and Keynes, 1987). In addition, it is also possible that *Dll3* and *Dll1* are involved in the formation of the somite boundary itself. As the somite forms, a broad band of *Dll3* expression is evident in the anterior half. Thus the anterior boundary of the somite in the process of forming (which expresses *Dll3*) abuts the posterior aspect of the somite that has just formed (which expresses *Dll1*). Perhaps formation of the somite boundary is analogous to the establishment of the dorsoventral boundary during *Drosophila* wing development (see Brook et al., 1996). Notch is required to set up the dorsoventral wing boundary but activation of Notch requires two different ligands expressed in mutually exclusive compartments. In the ventral compartment, Notch is activated by Serrate expressed on the surface of dorsal cells. In the dorsal compartment, Notch is activated by Delta which is expressed on the surface of ventral cells. Thus, at the prospective somite boundary, Dll3 may activate Notch on one side and Dll1 may activate Notch on the other. *Serrate-1* may also play a role since, in the mouse, it is expressed in a thin stripe of cells corresponding to the site of somite boundary formation (Mitsiadis et al., 1997). However, it remains to be resolved whether the activation of Notch by different ligands (Dll3 and Dll1) produces a differential signal or whether Notch is activated by different ligands simply because the ligands are differentially expressed due to prior patterning constraints. It may be relevant that during *Drosphila* wing development, *Notch*-expressing cells in a given compartment have different responses to Delta and Serrate (Doherty et al., 1996). Therefore, during wing development in *Drosophila*, appropriate signalling via Notch does appear to be dependent on activation by two different ligands. A similar sensitivity to different ligands may exist in mouse somitogenesis although it is also possible that different Notch receptors may be activated on either side of the somite boundary.

Neurogenesis

Dll3 and *Dll1* are extensively expressed during neurogenesis in both overlapping and distinct domains. For example, in the midbrain overlapping gene expression occurs dorsally with exclusive *Dll1* expression extending more laterally (Fig. 8F). Likewise, *Dll1* is expressed throughout much of the forebrain while *Dll3* is restricted to a small ventral patch (Fig. 8G). Overlapping expression is evident in the dorsal root ganglia and much of the CNS extending from the midbrain to the caudal spinal cord. In the CNS, the cells that express *Dll1* and *Dll3* are postmitotic cells (Fig. 7B,C,D). These genes are however not coexpressed, as double RNA in situ hybridisation reveals almost mutually exclusive domains of gene expression (Fig. 8C-E). The *Dll1*-expressing cells lie within the ventricular zone while cells expressing *Dll3* reside more laterally but are negative for islet-1/2 protein, a very early marker of motor neuron differentiation. Therefore the *Dll3*-expressing cells appear to represent a population on the verge of differentiating.

The different domains of *Dll1* and *Dll3* expression could represent two distinct postmitotic populations destined for different neuronal fates. However, while it is clear that different neuronal cell types emerge from different dorsoventral levels of the CNS, there is no evidence for distinct mediolateral neuronal populations (see Tanabe and Jessell, 1996). Therefore, it is more likely that the different mediolateral expression profiles of *Dll1* and *Dll3* represent sequential gene expression and reveal an additional layer of complexity in the sequence of events leading to the birth of a neuron. That the occasional cell can coexpress *Dll3* and islet-1/2 (Fig. 7E,F) supports this hypothesis since coexpression of *Dll1* and islet-1/2 was not observed (data not shown).

In *Drosophila*, distinct basic helix-loop-helix (bHLH) proteins contribute to the specification of different neurons (Jarman et al., 1993; Skeath and Doe, 1996). In vertebrates, non-overlapping dorsoventral domains of *neurogenin*, *Mash-1* and *Math-1* (Ma et al., 1996; Lo et al., 1991; Akazawa et al., 1995) gene expression exist within the ventricular zone of the spinal cord (see Tanabe and Jessell, 1996). These genes probably act at the head of the neurogenic patterning program

and lie upstream of other bHLH genes such as *NeuroD* (Lee et al., 1995; Ma et al., 1996). In a comparable fashion, the progression from *Dll1* to *Dll3* may represent increasing commitment to terminal differentiation. If neuronal diversification involves the sequential activation of bHLH proteins, a control mechanism is required to release cells from one bHLH-expressing state to the next. This is where lateral inhibition via Delta/Notch signalling may again have a role. The utilisation of more than one Delta ligand in a stereotyped spatiotemporal sequence, as cells leave the progenitor pool and approach differentiation, may allow for finer control of neuronal supply. Therefore the progression from *Dll1* to *Dll3* expression may reflect a stepwise increase in commitment to terminal differentiation of distinct neuronal types during the relatively prolonged process of vertebrate neurogensis.

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