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Distinct regulatory elements direct Delta1 expression in the nervous system and paraxial mesoderm of transgenic mice

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Abstract

The Delta1 gene encodes one of the Notch ligands in mice. Delta1 is expressed during early embryogenesis in a complex and dynamic pattern in the paraxial mesoderm and neuroectoderm, and is essential for normal somitogenesis and neuronal differentiation. Molecular components thought to act in response to ligand binding and Notch activation have been identified in different species. In contrast, little is known about the transcriptional regulation of Notch receptors and their ligands. As a first step to identify upstream factors regulating Delta1 expression in different tissues, we searched for *cis*-regulatory regions in the Delta1 promoter able to direct heterologous gene expression in a tissue specific manner in transgenic mice. Our results show that a 4.3 kb genomic DNA fragment of the Delta1 gene is sufficient in a *lacZ* reporter transgene to reproduce most aspects of Delta1 expression from the primitive streak stage to early organogenesis. Using a minimal Delta1 promoter we also show that this upstream region contains distinct regulatory modules that individually direct tissue-specific transgene expression in subdomains of the endogenous expression pattern. It appears that expression in the paraxial mesoderm depends on the interaction of multiple positive and negative regulatory elements. We also find that at least some regulatory sequences required for transgene expression in subdomains of the neural tube have been maintained during the evolution of mammals and teleost fish, suggesting that part of the regulatory network that controls expression of Delta genes may be conserved.

Keywords: Delta1; *Dll1*; Promoter analysis; Mouse; Development; Transgenesis; *LacZ* reporter gene; *deltaD*; Sequence conservation; *cis*-regulatory elements

1. Introduction

Cell-to-cell communication mediated by the evolutionary conserved Notch signalling pathway plays a pivotal role in regulating patterning and differentiation in various tissues during vertebrate embryogenesis. In mice, there are at least four genes encoding Notch receptors (del Amo et al., 1992; Lardelli and Lendahl, 1993; Lardelli et al., 1994; Uyttendaele et al., 1996), as well as two Delta and two Serrate genes that encode ligands for Notch (Bettenhausen et al., 1995; Lindsell et al., 1995; Shawber et al., 1996; Dunwoodie et al., 1997). These genes are expressed in partly overlapping and/or complementary patterns suggesting both unique and combinatorial interactions between ligands and receptors during development.

The mouse Delta1 (*Dll1*) gene has a complex and dynamic pattern of expression during embryonic development. The earliest expression is detected in mid-streak embryos (E7; Theiler Stage (TS) 10) in the embryonic mesoderm and primitive streak. Subsequently, in late-streak embryos (E7.5; TS 10b), its expression is confined to the posterior mesoderm and during presomitic stages (until TS 11) displays a sharp boundary of expression just anterior to the node. The strong expression in the presomitic mesoderm persists until the end of somitogenesis between day 14 (TS 22) and 15 (TS 23) of development. In somites Delta1 is expressed exclusively in posterior compartments and in the myotomes of differentiating somites (Bettenhausen et al., 1995). The targeted inactivation of the gene, in the $Dll1^{lacZ}$ knock-in allele, has demonstrated that the expression in the paraxial mesoderm is necessary for the transition of the

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mesenchymal presomitic mesoderm to epithelial somites, for the anterior-posterior patterning of somites, and the maintenance of somite borders (Hrabé de Angelis et al., 1997). In the central nervous system (CNS) Delta1 expression is first detected at day 8 of development (TS 12) in the anterior neural folds in the presumptive midbrain and forebrain. By day 9.5 of development (TS 15) expression extends throughout the neural tube, and later is also found in craniofacial ganglia, in neural crest cells, and spinal nerves and spinal ganglia (Bettenhausen et al., 1995). Consistent with the fine-grained pattern of Delta1 expressing and non-expressing cells in the neuroectoderm, Delta1 appears to be required for maintaining neuroepithelial progenitors and their coordinated differentiation into neuronal precursors (Hrabé de Angelis et al., unpublished results). This function of the Delta1 gene is reminiscent of the process known as 'lateral inhibition' originally described during neurogenesis in the neuroectoderm of flies (Campos-Ortega, 1995) and also known in neurogenesis of *Xenopus* (Chitnis et al., 1995) and teleost fish (Appel and Eisen, 1998). Outside the paraxial mesoderm and CNS, the mouse Delta1 gene is expressed early in the mesonephric mesoderm and tubules (Bettenhausen et al., 1995). Later, during organogenesis and foetal development (E13.5 to perinatal development) Delta1 is expressed, e.g. in the thymus and epithelial ducts of the metanephric kidney, developing bronchii, pancreas, and serous glands. Expression persists in skeletal muscle cells and the central nervous system and is also present in some sensory epithelia, such as the retina and the sensory patches of the inner ear (Beckers et al., 1999; Morrison et al., 1999). The loss-of-function of the Delta1 gene in the developing pancreas leads to an accelerated differentiation of pancreatic precursor cells into endocrine cells (Apelqvist et al., 1999). In mRNA extracts from the adult mouse, Delta1 transcripts were detected in the lung and the heart (Bettenhausen et al., 1995).

In *Drosophila*, some of the transcription factors that regulate *Delta* expression, are known. These include the 'proneural genes' of the *achaete-scute* complex (AS-C; i.e. *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*)) that act during neurogenesis. During specification of the neuroblasts, the expression of the proneural genes confers onto clusters of neuroectodermal cells the potential to become neuronal precursors (e.g. Ghysen and Dambly-Chaudiere, 1988; Campos-Ortega, 1995; Artavanis-Tsakonas et al., 1999). Subsequently, expression of these genes becomes restricted to a single neural progenitor cell, whereas their expression ceases in neighbouring cells that then become epidermal (Cubas et al., 1991; Skeath and Carroll, 1991). Inhibition of *achaete-scute* expression in epidermoblast cells involves cell-to-cell signalling mediated by the Notch receptor (expressed by the inhibited cell) and its ligand Delta (Artavanis-Tsakonas et al., 1995). In response to Notch activation, the products of genes in the *Enhancer of split* complex (E(spl)-C) accumulate in epidermal cells (Jennings et al., 1994). The *hairy* (*h*)-related bHLH transcription factors of the E(spl)-C are negative transcriptional regulators of the 'proneural genes' *ac* and *sc*, which are required for expression of *Delta* (Tietze et al., 1992; Oellers et al., 1994; Ohsako et al., 1994; Heitzler et al., 1996). Thus, it is thought that transcription of *Delta* in neuroblasts is activated by 'proneural genes', and suppression of 'proneural genes' in neighbouring cells through 'lateral inhibition' results in downregulation of *Delta*. Mutations in genes of the AS-C or their overexpression lead to loss or overexpression of the *Delta* gene, respectively (Kooh et al., 1993; Haenlin et al., 1994). The analysis of the *Delta* promoter in transgenic flies as well as in vitro assays have provided direct evidence that gene products of the *achaete-scute* complex (AS-C) activate *Delta* transcription by binding to specific sites in the *Delta* promoter (Haenlin et al., 1994; Kunisch et al., 1994). At least two homologues to the insect genes of the AS-C have been identified in mammals (Johnson et al., 1990). In the mouse, *Mash-2* (Mammalian *achaete-scute* homolog-2) has an important role in extraembryonic development (Guillemot et al., 1994; Nakayama et al., 1997) and *Mash-1* is required for proper neuronal differentiation in the peripheral and central nervous system (Guillemot and Joyner, 1993; Guillemot et al., 1993; Tomita et al., 1993; Hirsch et al., 1998). However, their role in the transcriptional regulation of the mammalian Delta genes remains elusive.

The dynamic expression of the mouse Delta1 gene in distinct tissues and cell types suggests that specific transcription factors individually or in combination direct the spatially and temporally regulated transcription of this gene. As a first step towards the identification of transcription factors that regulate Delta1 expression particularly in the paraxial mesoderm at early developmental stages, we have initiated a transgenic analysis to define *cis*-regulatory elements in the Delta1 promoter. Our analysis shows that distinct regulatory elements within 4.3 kb upstream the coding sequence are sufficient to specifically direct gene expression in subdomains of the endogenous expression pattern. Expression in the paraxial mesoderm appears to depend on multiple positive and negative regulatory elements. The sequences of two regions that are sufficient for transgene expression in distinct regions of the neural tube are highly conserved between the mouse Delta1 and zebrafish *deltaD* genes (following the gene nomenclature for the species) suggesting conserved regulatory pathways in the neuroectoderm.

2. Results

2.1. 4.3 kb promoter sequence reproduce most early aspects of Delta1 expression

We started the search for specific *cis*-regulatory regions in the Delta1 promoter by analyzing the expression of a reporter

transgene containing 4.3 kb of genomic sequence. This transgene (*Dll1*$^{tg4.3/lacZ}$; see Fig. 1) contains the 5′ untranslated *Dll1* sequence and the *E. coli lacZ* gene with the SV40 and PGK polyadenylation signals in its 3′ end fused in frame to the first codon of the Delta1 gene. To compare transgene expression with the endogenous expression we took advantage of the *Dll1*^{lacZ} knock-out allele, which expresses the *lacZ* reporter gene from the endogenous Delta1 locus (Hrabé de Angelis et al., 1997). We had shown previously that the histochemical detection of reporter gene expression in the *Dll1*^{lacZ} allele faithfully reproduces the endogenous expression in heterozygous mutants (Beckers et al., 1999). Expression of transgene *Dll1*$^{tg4.3/lacZ}$ was analyzed between E10.5 and E11.5 in three embryos with independent integration events and in two transgenic lines and was highly reproducible for these independent integration events.

Expression of the *Dll1*$^{tg4.3/lacZ}$ transgene and the *Dll1*^{lacZ} allele starts with the onset of gastrulation at the primitive streak stage (Theiler stage (TS) 10, E6.5–7; Fig. 2A). At this stage expression is seen in cells of the primitive streak and the mesoderm. Later, when the mesoderm starts being segmented (TS 12, E8) expression in the presomitic mesoderm and the somites is strong. In addition, both the transgene and the endogenous gene start to be expressed in the anterior neural fold (Fig. 2B), probably marking the onset of neuronal cell fate decisions. The transgene continues to reproduce the endogenous pattern rather faithfully at least through the early stages of organogenesis (TS 17–18, E10.5–11) where expression is consistently found in the presomitic mesoderm, the somites, throughout the central nervous system, as well as in spinal nerves and ganglia (Figs. 2D and 7A,E). Histological sections through the posterior tail region of transgenic embryos and mice that are heterozygous for the *Dll1*^{lacZ} allele show that reporter gene expression is restricted to the paraxial mesoderm. No expression was found in the neural tube, the notochord, the tailgut, or the embryonic ectoderm in this posterior region for either the transgene or the *Dll1*^{lacZ} allele (Fig. 2G). More anteriorly Delta1 is expressed in the neural tube. In the dorsal neural tube expression is predominantly restricted to marginal regions. In the ventral half of the CNS expression is broader and extends towards the lumen of the neural tube; Delta1 is not expressed in the floor plate. This distinct expression pattern is reproduced by the *Dll1*$^{tg4.3/lacZ}$ transgene (Fig. 2F). In contrast to the endogenous expression in somites, which is restricted to the posterior compartment of each segment, the transgene is expressed throughout somites and is not restricted to a compartment (Fig. 2C). At later stages, expression of the *lacZ* transgene reflects only a subset of the endogenous expression domains. For example, Delta1 is expressed in the vibrissae ('whisker') buds, the retina and skeletal muscles starting around E14 (TS 23), but these expression domains are absent in *Dll1*$^{tg4.3/lacZ}$ embryos (Fig. 2E).

From these observations we conclude that the genomic region spanning 4.3 kb upstream the Delta1 coding region contains most of the regulatory sequences that are sufficient to reproduce the endogenous expression pattern in transgenic mice from the onset of expression at the primitive streak stage to early stages of organogenesis. Regulatory sequences, for example, for the restriction of expression to posterior somite compartments or expression at later developmental stages may be located either upstream or downstream the genomic region tested in transgene *Dll1*$^{tg4.3/lacZ}$.

2.2. Two regulatory regions for Delta1 expression in distinct domains of the neural tube are evolutionary conserved

Based on the assumption that part of the regulatory network of homologous genes may be conserved between different species, we searched for sequence conservations in the promoter regions of mouse Delta1 and zebrafish *deltaD* genes, the most closely related *Delta* homologue in the zebrafish. Similar to the mouse gene, the *deltaD* gene is expressed in the germ ring (the equivalent of the mammalian primitive streak) during epiboly, and the presomitic and somitic mesoderm during early organogenesis (Dornseifer et al., 1997). During primary neurogenesis *deltaD* is expressed in the epiblast (the future neuroectoderm), and later its expression foreshadows the onset of neuronal differentiation (Haddon et al., 1998), reminiscent of the expres-

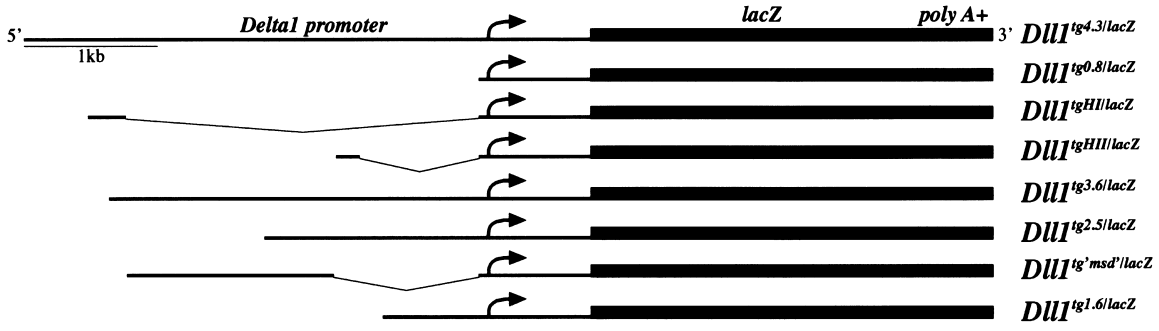

Fig. 1. Scheme of reporter transgenes. The arrow indicates the approximate position of the transcription start site in the *Delta1* promoter region. The bacterial *lacZ* reporter gene with the SV40 and PGK polyadenylation signals in the 3′ end (thick bar) was fused in frame into the start codon of the *Delta1* gene. The transgenes are arranged (from top to bottom) in the order of their description in this report.

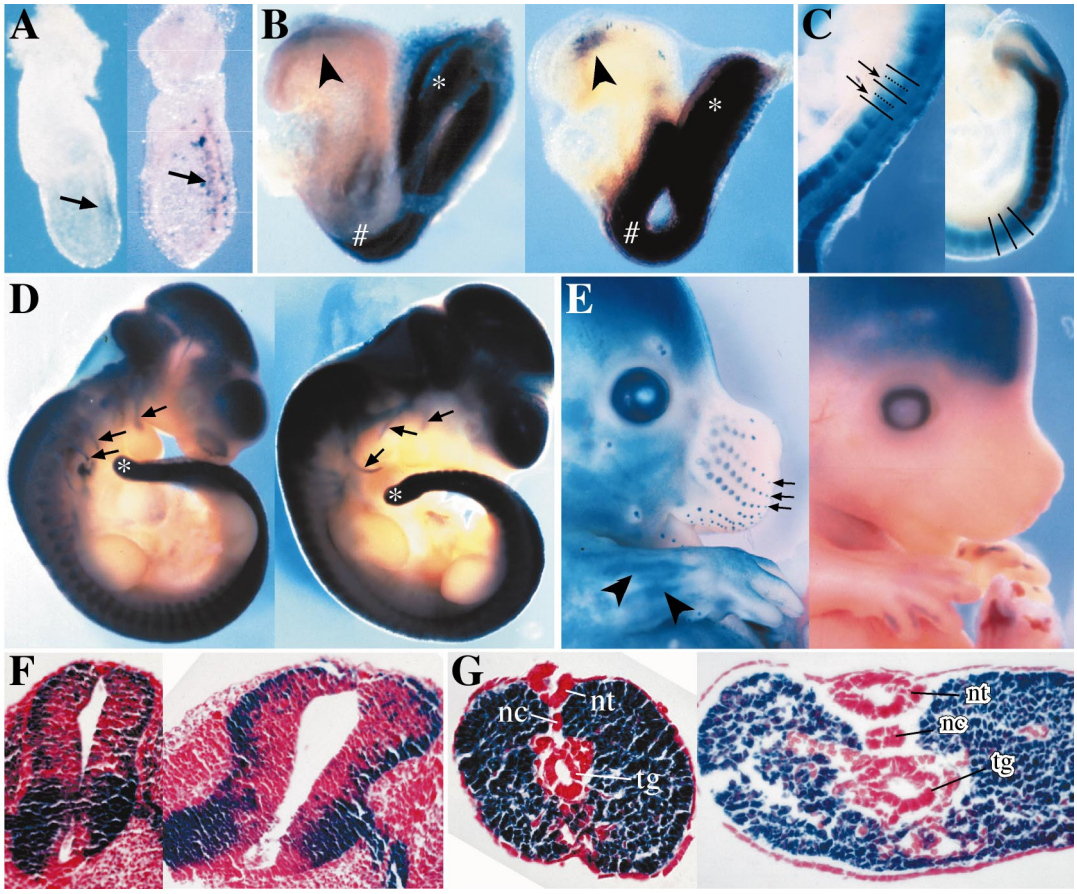

Fig. 2. Comparison of transgene expression (*Dll1*$^{tg4.3/lacZ}$) and endogenous *Dll1* expression (*Dll1*^{lacZ}) by detection of β-galactosidase activity; for each stage the endogenous expression is shown on the left and the transgenic expression is on the right. (A) Earliest expression at E6.5–7 (TS 10) in the primitive streak (arrow). (B) Strong expression in the presomitic (*) and somitic (#) mesoderm at E8 (TS 12). Expression also starts in the anterior neural fold (arrowhead). (C) Endogenous Delta1 expression (left) is restricted to posterior somite compartments (arrows). In contrast, the transgene (right) is expressed throughout somites without specific restriction to a compartment. The two embryos shown are at 9.5 days of development (TS 15) and were stained for β-galactosidase for only 30 minutes. (D) At E10.5–11 (TS 17–18) Delta1 and the transgene are expressed in the presomitic (*) and somitic mesoderm, in the central nervous system and in spinal nerves (arrows). (E) The endogenous Delta1 gene (left) is expressed in the retina, vibrissae precursors (whisker buds; arrows), and skeletal muscles (arrowheads) of E14 embryos (TS 23). At this stage, the transgene (right) is not expressed in these domains. Neural expression is maintained in both embryos. (F) Cross-sections through the neural tube, in the lumbar region, of E10.5–11 embryos (TS 17–18). Endogenous (left) and transgene (right) expression are similar. In the dorsal neural tube expression is restricted to marginal regions; in the ventral neural tube expression is broader and is found in the ventral horn and spinal nerves and ganglia. (G) Cross-section through the posterior tail at E10.5–11 (TS 17–18). In this region expression is restricted exclusively to the paraxial mesoderm; Delta1 and the transgene are not expressed in the neural tube (nt), notochord (nc), tail gut (tg) or ectoderm.

sion of Delta1 (Bettenhausen et al., 1995). Sequence comparison between 7.5 kb of mouse, and 5.3 kb of zebrafish genomic sequences upstream the Delta coding regions, revealed two highly conserved regions, located in the same relative positions and the same orientation (Fig. 3). The more 5′ located element, hereafter referred to as Homology I (HI), is approximately 270 bp long (279 bp in the mouse and 267 bp in the zebrafish) and has 80% sequence identity in an optimized alignment; the second region of homology, referred to as Homology II (HII), is approximately 165 bp in length (161 bp in the mouse and 172 bp in the fish) and shows 84% identity in an optimized alignment (Fig. 3).

To test the regulatory potential of each element, HI or HII, in transgenic mice, we first identified a minimal Delta1 promoter. We found that a transgene, *Dll1*$^{tg0.8/lacZ}$, extending from 40 bp upstream the major transcription start to the first codon 775 bp downstream (Fig. 1) is expressed in a way that is apparently not related to the expression of the Delta1 gene. Rather, expression was dependent on the genomic integration site ($n = 6$; Fig. 7B,I). We used this minimal promoter to test the regulatory potentials of HI and HII by individually cloning each of the conserved elements upstream of *Dll1*$^{tg0.8/lacZ}$, thus yielding transgenes *Dll1*$^{tgHI/lacZ}$ and *Dll1*$^{tgHII/lacZ}$ (Fig. 1).

HI in the context of the minimal Delta1 promoter (*Dll1*$^{tgHI/lacZ}$) directed reporter gene expression primarily to the ventral neural tube (Figs. 4A,C and 7D). Transgene expression was consistently found in the ventral horn of the neural tube and the ventral root in all nine embryos analyzed between E11.5 and E12.5 (TS 19–21), each of them representing an independent integration event of the transgene. In four of these embryos the transgene was also

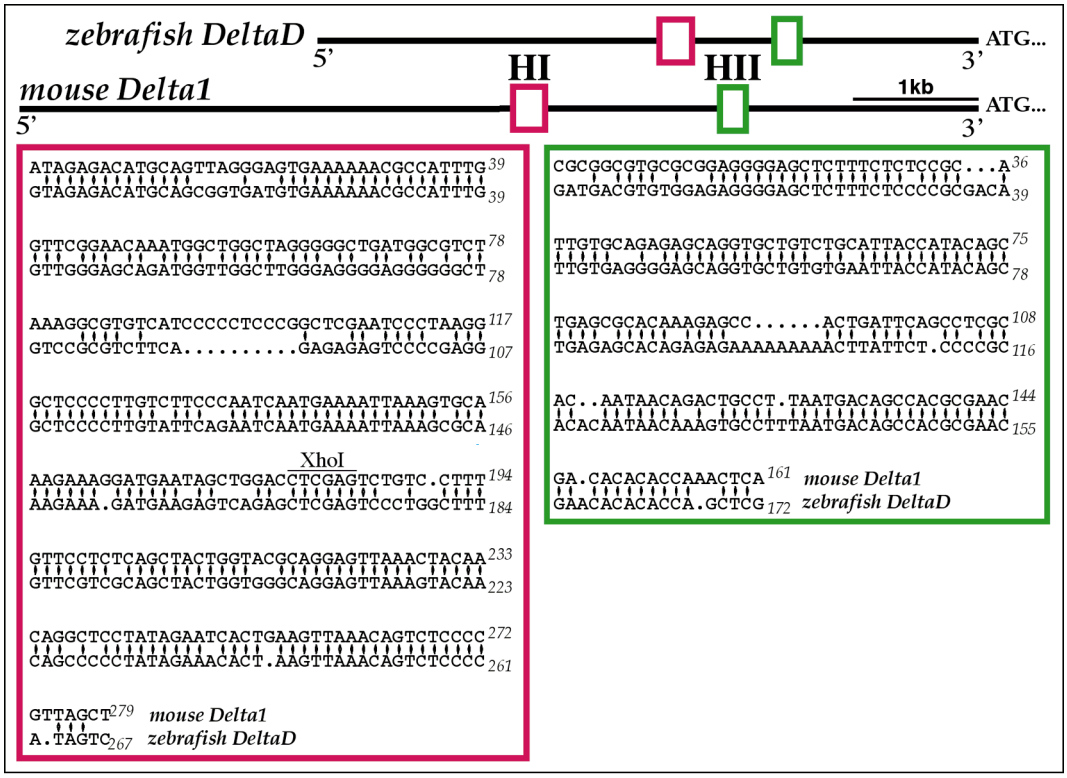

Fig. 3. Sequence comparison between 7.5 kb of the mouse Delta1 and 5.3 kb of the zebrafish *deltaD* promoter sequences. Two conserved regions, Homology I (HI, red) and Homology II (HII, green) are present in these two sequences. For each homology the optimized alignment is shown: Homology I (HI), is approximately 260 bp long (279 bp in the mouse and 267 bp in the zebrafish) and has 80% sequence identity; Homology II (HII), is approximately 165 bp in length (161 bp in the mouse and 172 bp in the fish) and shows 84% identity in the optimized alignment. The *XhoI* site in Homology I is the 5′ end in transgene Dll1$^{tg3.6/lacZ}$.

strongly expressed in at least some derivatives of migrating neural crest cells, such as the dorsal root and spinal ganglia (Figs. 4C and 7D). Similarly, Homology II in the context of the minimal Delta1 promoter (*Dll1*$^{tgHII/lacZ}$) directed reporter gene expression also into the neural tube, but with a clearly different specificity. In six independent integration sites expression of this transgene was primarily restricted to the marginal zone in the dorsal region of the neural tube (Figs. 4B,D and 7C). Neither of the two transgenes, *Dll1*$^{tgHI/lacZ}$ or *Dll1*$^{tgHII/lacZ}$, was significantly and reproducibly expressed in the paraxial mesoderm or any other tissue apart from the neural tube and its derivatives (Fig. 4A,B). In addition, we observed that the truncation of Homology I in transgene *Dll1*$^{tg3.6/lacZ}$ (see below and Fig. 1), leads to the loss of transgene expression in the ventral horn of the neural tube (Fig. 4E), indicating that HI is not only sufficient, but also necessary for expression in the ventral horn in the context of the tested promoter region.

2.3. A regulatory element for expression in the paraxial mesoderm

We continued our search for regulatory elements in the Delta1 promoter by generating a series of 5′ deletions from the 4.3 kb reporter construct (*Dll1*$^{tg4.3/lacZ}$). The smallest deletion truncates the promoter sequence within Homology I to 3.6 kb (*Dll1*$^{tg3.6/lacZ}$, Fig. 1). The expression of this transgene was analyzed in 11 embryos between E11 and E12.5 (TS 18–21) and two transgenic lines, each representing an independent integration of the reporter construct. The pattern of expression of this transgene, *Dll1*$^{tg3.6/lacZ}$, was highly reproducible and, with the exception of the expression in the ventral neural tube (see above, Fig. 4E), was comparable to the pattern of transgene *Dll1*$^{tg4.3/lacZ}$ (compare Fig. 2B,D with Fig. 5A). In particular, it was expressed in the presomitic and somitic mesoderm, throughout the length of the neural tube, and in spinal nerves and ganglia (Figs. 5A and 7F).

The consequent deletion of an additional 1.1 kb yielded a reporter construct with 2.5 kb Delta1 promoter sequence, *Dll1*$^{tg2.5/lacZ}$, which has its 5′ end upstream of Homology II (Fig. 1). The expression of this transgene was analyzed in five independent transgenic embryos between E11.5 and E12.5 (TS 19–21). In these embryos the transgene was neither expressed in the presomitic nor in the somitic mesoderm (Figs. 5C and 7G). Each of them, however, showed expression in the neural tube (probably controlled by the HII region contained in this transgene) and some additional tissues (not shown). Since the latter expression domains were not reproducible and were not observed in transgenic embryos with

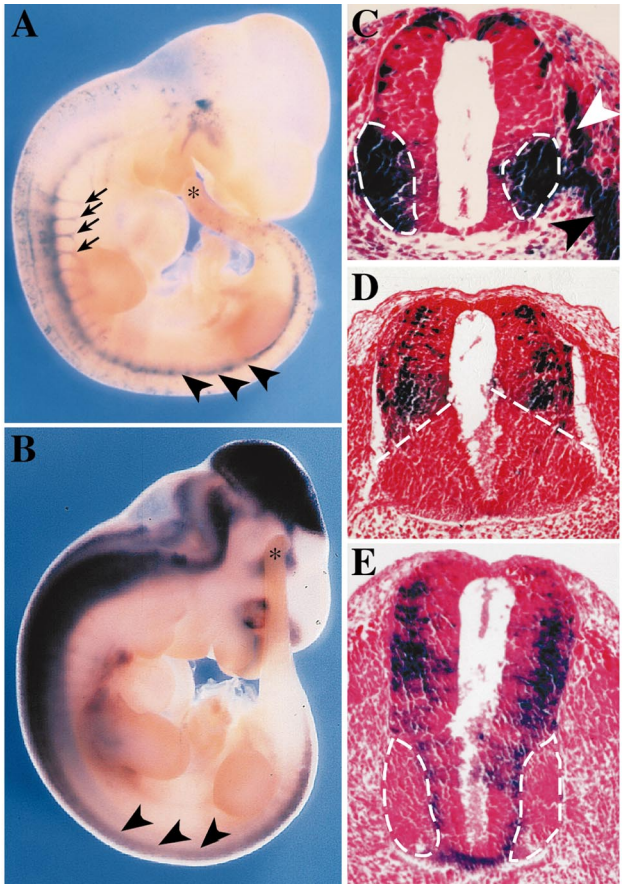

Fig. 4. Two neural enhancers in the Delta1 promoter region. (A,B) E11.5 (TS 19) transgenic embryos stained for β-galactosidase. (A) Embryo transgenic for construct *Dll1*$^{tgHI/lacZ}$ showing expression in the ventral neural tube (large arrowheads) and in spinal ganglia (small arrows). No expression was found in the paraxial mesoderm (*). (B) *Dll1*$^{tgHII/lacZ}$ transgenic embryo showing expression in the dorsal neural tube (large arrowheads); the transgene is not expressed in the paraxial mesoderm (*). (C–E) Cross-section through the neural tube of E11.5 embryos stained for β-galactosidase. (C) Section from an embryo transgenic for *Dll1*$^{tgHI/lacZ}$; expression is strong in the ventral horn and is also found in dorsal (white arrowhead) and ventral (black arrowhead) spinal ganglia. (D) Section from an embryo that is transgenic for *Dll1*$^{tgHII/lacZ}$; expression is restricted to the dorsal region of the neural tube. (E) Section through the neural tube of an embryo that is transgenic for *Dll1*$^{tg3.6/lacZ}$. This transgene has its 5′ end within Homology I (XhoI site in Fig. 3) and is not expressed in the ventral horn.

the larger transgenes (*Dll1*$^{tg4.3/lacZ}$ or *Dll1*$^{tg3.6/lacZ}$) this expression is likely due to regulatory influences of the respective integration site. In order to test whether *Dll1*$^{tg2.5/lacZ}$ may be transiently activated in the paraxial mesoderm at earlier stages or later during embryonic development we generated two expressing transgenic mouse lines. In contrast to the embryos analyzed in the transient assay, one transgenic line expressed the reporter gene in the presomitic but not in the somitic mesoderm whereas the second line expressed the transgene only in somites but not in the presomitic mesoderm (not shown). Since these expression domains were not found in the other five embryos carrying this transgene (*Dll1*$^{tg2.5/lacZ}$), these expression patterns may be the result of regulatory influences from regions flanking the integration site. Alternatively, the variable expression in the paraxial mesoderm could be indicative of a mesodermal enhancer in the 2.5 kb promoter sequence which, in the configuration of transgene *Dll1*$^{tg2.5/lacZ}$, manifests itself only with low frequency and with variable expressivity (see also below: *Dll1*$^{tg1.6/lacZ}$).

Since transgene *Dll1*$^{tg3.6/lacZ}$ was reproducibly and strongly expressed in the paraxial mesoderm and the smaller transgene *Dll1*$^{tg2.5/lacZ}$ was not we concluded that an enhancer for expression in this tissue should be present in the 5′ region of the 3.6 kb promoter region. To test this we isolated the region between the two conserved neuronal enhancer regions, HI and HII, and analyzed the regulatory potential of this DNA fragment in the context of the Delta1 minimal promoter (*Dll1*$^{tg'msd'/lacZ}$; Fig. 1). This transgene was expressed exclusively in the presomitic and somitic mesoderm in two independently generated transgenic embryos at E10.5 (TS 17–18). At this stage expression was strong in at least the eight to nine most recently formed somites (Figs. 5D and 7J). No significant expression was detectable in the neural tube or any other tissue outside the paraxial mesoderm. We concluded from these observations that it is possible to isolate distinct regulatory regions from the Delta1 promoter that are sufficient to drive reporter gene expression specifically to either neural or mesodermal domains.

2.4. The regulatory function of the mesodermal enhancer is specific from gastrulation to organogenesis

To further analyze the regulatory function of the mesodermal enhancer ('msd') over time, we established two expressing mouse lines with transgene *Dll1*$^{tg'msd'/lacZ}$ (Fig. 1). The onset of transgene expression coincides with the appearance of the paraxial mesoderm at the primitive streak stage (E7, TS 10; Fig. 6A). At E8.75 (TS 13) expression persists in the presomitic mesoderm and is also found in somites (Fig. 6B). Short stainings for β-galactosidase revealed that expression of this transgene in somites, similar to *Dll1*$^{tg4.3/lacZ}$, was not confined to posterior somite compartments but was detected throughout segments (Fig. 6C; compare with Fig. 2C). During organogenesis, transgene expression was also found in tissues derived from the paraxial mesoderm, such as the primordia of the ribs and precursors of the limb skeletal muscles at E11.5 (TS 20; Fig. 6D) and the dermis of the trunk region at E13.5 (TS 22; Fig. 6E).

To further confirm that the observed β-galactosidase activity throughout somites truly reflects the transcriptional regulation of the transgene in anterior and posterior somite halves, whole-mount in situ-hybridizations with a probe specific for the bacterial *lacZ* gene were performed. Similar to the β-gal activity, *lacZ* mRNA was not restricted to posterior somite compartments in *Dll1*$^{tg'msd'/lacZ}$ transgenic embryos (Fig. 6F). However, in contrast to β-gal activity, *lacZ* transcripts were not detected contiguously in the paraxial mesoderm: transcripts of the reporter gene were present in the presomitic mesoderm and in mature somites, but no

lacZ mRNA was detected in the nascent somites, suggesting that additional regulatory elements are required for correct Delta1 expression in the transition zone between presomitic and somitic mesoderm. The apparently contiguous expression of transgene *Dll1*$^{tg'msd'/lacZ}$ in the paraxial mesoderm as detected by X-gal staining is therefore most likely due to the perdurance of the β-galactosidase enzyme in nascent somites (compare Fig. 6C with Fig. 6F).

From these observations we conclude, that the expression directed by regulatory elements in the 'msd' region coincides with the appearance of the paraxial mesoderm at the primitive streak stage and remains restricted to this tissue and derived structures at least until early organogenesis.

2.5. Positive and negative regulatory interactions for expression in the mesoderm

We completed our series of 5′ truncations by deleting another 850 bp to yield transgene *Dll1*$^{tg1.6/lacZ}$ having its 5′ end downstream of Homology II (Fig. 1). In contrast to the larger transgene *Dll1*$^{tg2.5/lacZ}$, this construct gave rise to consistent *lacZ* expression in the paraxial mesoderm of three independent transgenic embryos between E10.5 and E11.5 (TS 17–19) and one transgenic line (Fig. 5E). Analysis of the dynamic expression of this transgene revealed that expression in the presomitic mesoderm was detected as early as E8.5 (TS 13). At around the ten somite stage (E8.75, TS 13.5) the first somitic expression was visible in the three most recently formed segments (Fig. 5B). From the analysis of this transgene (*Dll1*$^{tg1.6/lacZ}$) we conclude that the 1.6 kb proximal promoter region contains (an) additional mesodermal regulatory element(s), msdII, sufficient to reproducibly direct transgene expression into the presomitic and somitic mesoderm (Fig. 7H). Since transgene *Dll1*$^{tg2.5/lacZ}$ was not significantly expressed in the paraxial mesoderm we conclude that the region from 1.6 to 2.5 kb upstream the

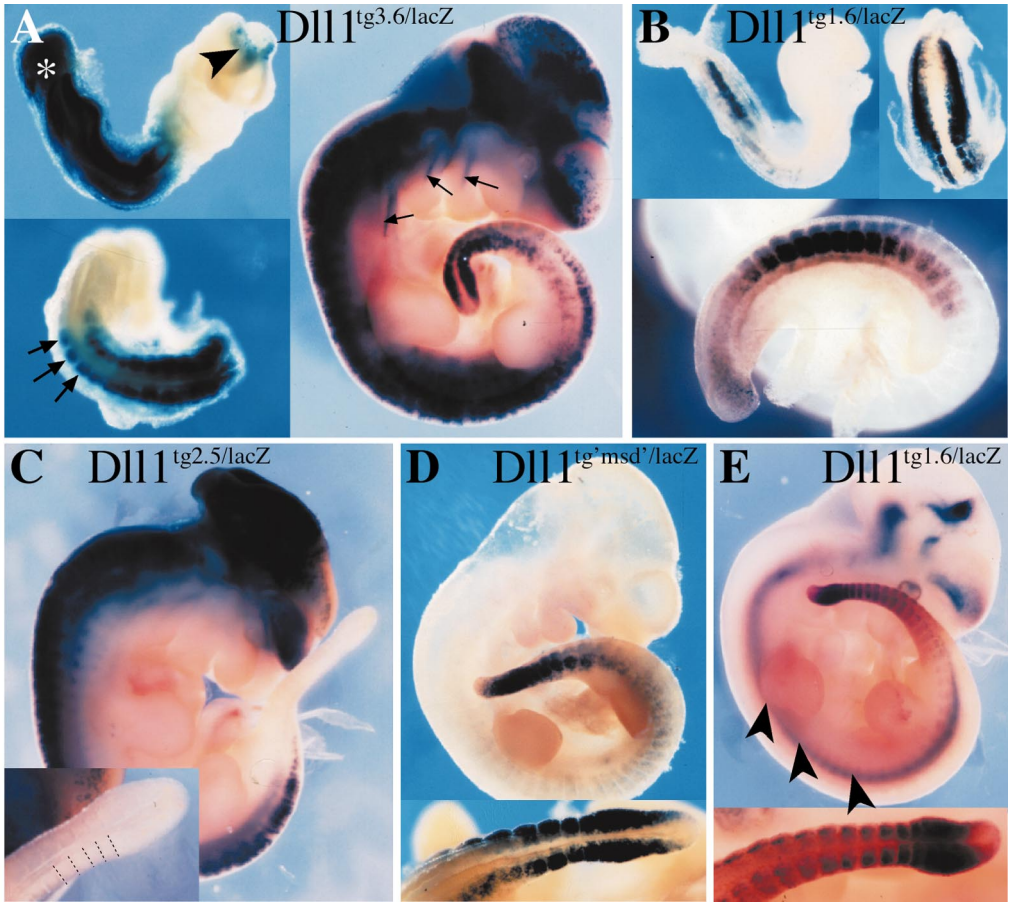

Fig. 5. Expression of *lacZ* reporter constructs in transgenic embryos detected by β-galactosidase staining. (A) E9 (left) and E11.5 (right) embryos transgenic for *Dll1*$^{tg3.6/lacZ}$. Expression is detected in the presomitic mesoderm (*), the somites (arrows), and the anterior neural fold (arrowhead) at E9. At E11.5 expression is persistent throughout the neural tube, the paraxial mesoderm, and spinal ganglia (arrows). (B) Embryos transgenic for transgene *Dll1*$^{tg1.6/lacZ}$. At E8.5 (top left) expression is seen in the presomitic mesoderm, shortly afterwards at E8.75 (top right) also in the most recently formed somites. At E9.5 (below) expression is found in the presomitic and somitic mesoderm but not yet in the neural tube. (C) E11.5 embryo transgenic for *Dll1*$^{tg2.5/lacZ}$. Expression is found throughout the neural tube but not in the presomitic and somitic mesoderm (see enlargement of posterior tail at the bottom). (D) E11.5 embryo transgenic for *Dll1*$^{tg'msd'/lacZ}$; the transgene is expressed exclusively in the presomitic and somitic mesoderm (enlargement of the posterior tail at the bottom). (E) E11.5 embryo transgenic for *Dll1*$^{tg1.6/lacZ}$. Expression is detected in the presomitic and somitic mesoderm (enlargement of posterior tail at the bottom) and is at this stage also found in the ventral neural tube (arrowheads).

Delta1 coding region contains a regulatory element that suppresses the function of the proximal mesodermal element for expression in the mesoderm at least in the context of transgene *Dll1*$^{tg2.5/lacZ}$.

In addition to the mesodermal expression we found that transgene *Dll1*$^{tg1.6/lacZ}$ was weakly expressed in a ventral (not further characterized) region of the neural tube. This expression was first detectable at around E10 (TS 16) in the transgenic line and was also present in the three embryos analyzed in the transient assay (Fig. 5E). This suggests that the proximal 1.6 kb promoter region (in addition to the proximal mesodermal enhancer) also contains (an) additional enhancer(s) for neural expression.

3. Discussion

The transgenic analysis of *cis*-regulatory elements in the Delta1 (*Dll1*) promoter is a first step towards the identification of transcription factors that are required for the spatially and temporally regulated expression of this gene during development. Our initial analysis has shown that 4.3 kb of genomic sequence upstream the Delta1 coding region are sufficient in transgenic mice to reproduce most aspects of the endogenous expression from gastrulation to the onset of organogenesis. This suggested that this sequence contains essential *cis*-regulatory elements required for the expression during early development and that it should be possible to isolate tissue specific enhancers from this region.

3.1. Distinct cis-regulatory regions direct Delta1 expression in the paraxial mesoderm and specific regions of the neuroectoderm

Our transgenic analysis has shown that the Delta1 promoter contains regulatory elements which can individually direct gene expression in subdomains of the early endogenous expression pattern, and the transcriptional regulation of expression in the neuroectoderm and paraxial mesoderm can be separated. The analyzed upstream sequence contains at least two neuronal enhancer regions (Homology I and II), two regions that direct expression to the paraxial mesoderm (msd and msdII), as well as one element that is apparently able to suppress the function of msdII at least in the context of *Dll1*$^{tg2.5/lacZ}$ (Fig. 7).

In our experiments both neuronal and the positive regulatory elements for expression in the paraxial mesoderm were able to interact directly with the proximal promoter

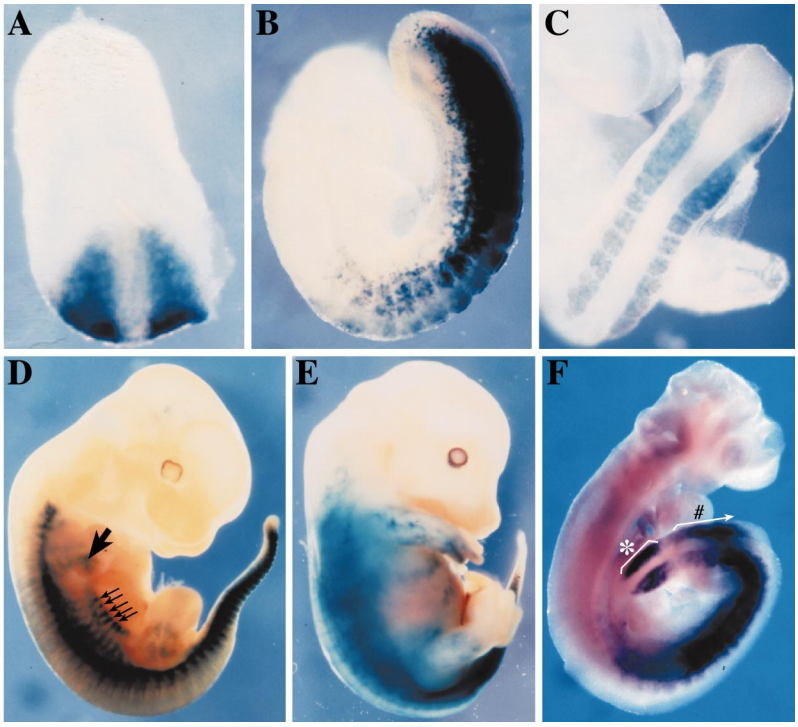

Fig. 6. Expression of *Dll1*$^{tg'msd'/lacZ}$ from gastrulation to organogenesis. (A–E) β-galactosidase activity, (F) whole-mount in situ-hybridization with a probe specific for the bacterial *lacZ* gene. (A) Transgenic embryo at E7 with expression in the paraxial mesoderm. (B) At E8.75 transgene expression is detected in the presomitic and somitic mesoderm. (C) Short staining for β-galactosidase activity (30min) in a transgenic embryo at E9.25. Expression is not restricted to posterior compartments of somites (compare Fig. 2C), and expression at the β-galactosidase protein level appears continuous between presomitic and somitic mesoderm (compare F). (D) Transgenic embryo at E11.5 expressing the reporter gene also in derivatives of the paraxial mesoderm: e.g. the rib primordia (small arrows), and muscle precursors in the developing limb (large arrow). (E) Transgene expression in the dermis and subcutaneous tissues at E13.5. (F) Whole-mount in situ-hybridization of a transgenic embryo at E9.5 with a probe specific for *lacZ* expression. At the mRNA level, expression is evident in the presomitic mesoderm (*), and an independent activation of the transgene is seen in the somitic mesoderm (#).

region when tested in the context of a minimal Delta1 promoter (Fig. 7C,D,H,J). However, it is not clear whether in the context of the larger transgenes (i.e. *Dll1*$^{tg4.3/lacZ}$ and *Dll1*$^{tg3.6/lacZ}$) each of the positive and negative regulatory elements (respectively, the transcription factors that bind to these regulatory elements) interact directly and individually with the proximal promoter region or whether they also interact with each other (Fig. 7F). In transgene *Dll1*$^{tg2.5/lacZ}$, for example, the mesodermal suppresser may either block the function of the proximal mesodermal enhancer (msdII) by preventing its interaction with the minimal promoter region, or it may interact directly with msdII, or both (Fig. 7G). Likewise, factors that associate with the more upstream located mesodermal enhancer region (msd) may also interact with the mesodermal suppresser and/or the more proximal second mesodermal enhancer element, msdII, present in the larger transgenes (i.e. *Dll1*$^{tg4.3/lacZ}$ and *Dll1*$^{tg3.6/lacZ}$; see Fig. 7E,F). However, the factors binding to the msd region are able to directly interact with the proximal promoter region (*Dll1*$^{'msd'/lacZ}$) and activate gene expression in the paraxial mesoderm (Fig. 7J). Regardless of their function and relation in their endogenous genomic location in vivo, our transgenic analysis clearly demonstrates that individual 'modules' of *cis*-regulatory elements are sufficient to direct

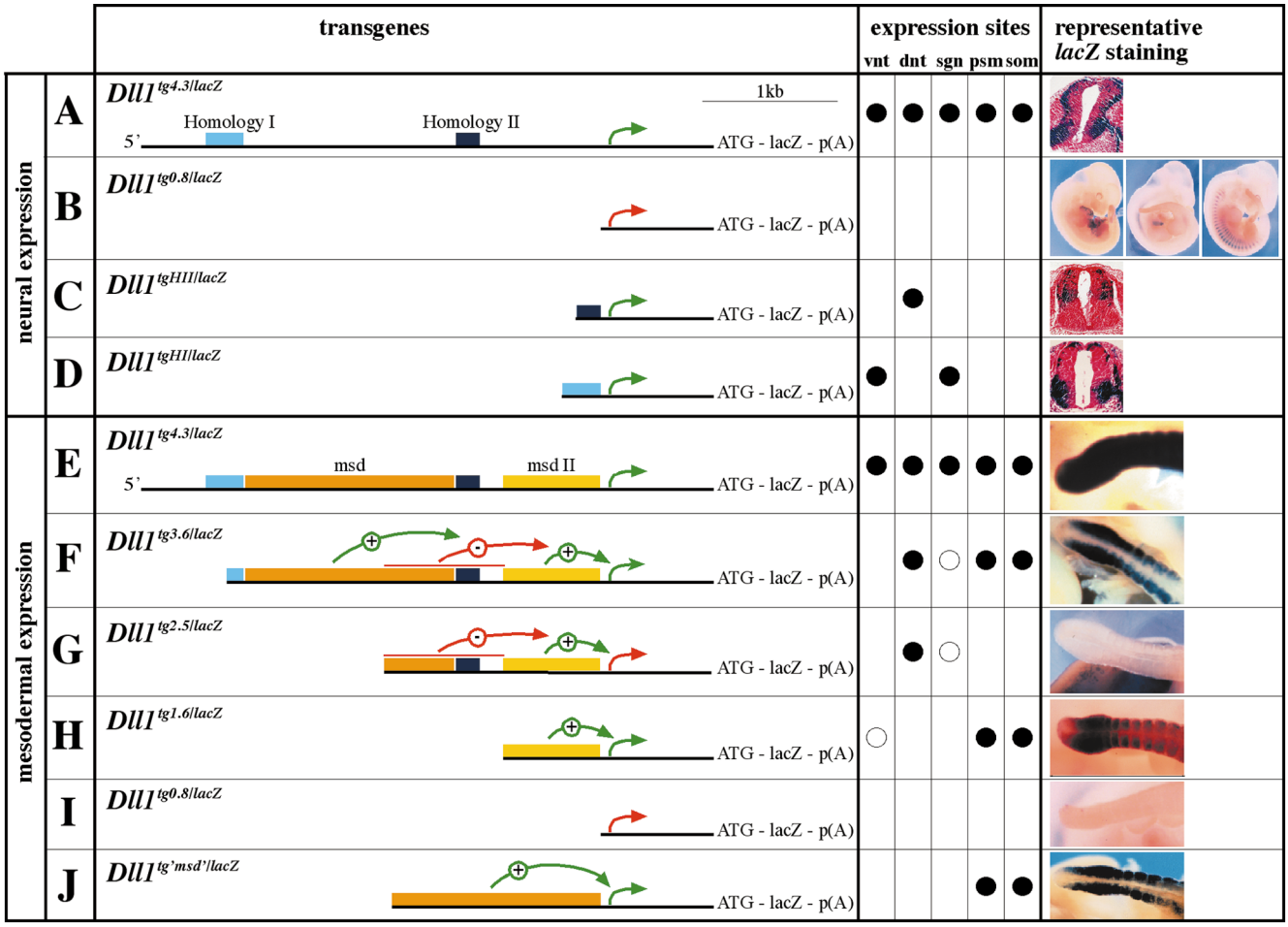

Fig. 7. Summary and interpretation of transgene expression patterns. Transgenes are grouped with respect to their significance for expression in neural tissue (A–D) and the paraxial mesoderm (E–J). Schemes of transgenes and regulatory regions defined in this study are shown in the left column (green 'promoter arrows' indicate expression, red 'promoter arrows' the lack of expression in the corresponding tissue). Major sites of transgene expression are tabulated in the middle column, filled dots indicating reproducible expression, open circles non-reproducible expression. Representative β-galactosidase stainings for each construct are shown in the right column. (A–D, neural expression) By sequence comparison to the *deltaD* gene of the zebrafish we identified two conspicuously conserved regions, Homology I (HI, light blue in A) and Homology II (HII, dark blue in A) that in the context of a minimal Delta1 promoter (*Dll1*$^{tg0.8/lacZ}$, in B) direct expression to specific domains in the central nervous system (*Dll1*$^{tgHI/lacZ}$ and *Dll1*$^{tgHII/lacZ}$; see table and representative stainings in (C,D), respectively). (E–J, mesodermal expression) The genomic region between the two homologies (msd, orange in E) functions as a specific enhancer for expression in the paraxial mesoderm when fused to the minimal Delta1 promoter (*Dll1*$^{tg'msd'/lacZ}$, in J). At least one additional mesodermal enhancer is located proximal in the Delta1 promoter (msdII, yellow; *Dll1*$^{tg1.6/lacZ}$, in H). *Dll1*$^{tg2.5/lacZ}$ is not expressed in the paraxial mesoderm (red 'promoter-arrow', see table and staining in G) suggesting that in transgene *Dll1*$^{tg2.5/lacZ}$ the function of this second mesodermal element, msdII, is suppressed by a negative regulatory element located immediately upstream. The mesodermal expression of reporter transgenes *Dll1*$^{tg3.6/lacZ}$ (F) and *Dll1*$^{tg4.3/lacZ}$ (E) apparently depends on the interaction of positive and negative regulatory influences. Abbreviations: vnt, ventral neural tube; dnt, dorsal neural tube; sgn, spinal ganglia and nerves; psm, presomitic mesoderm; som, somites.

transgene expression into specific subsets of the endogenous expression domains and can be isolated from the Delta1 promoter region.

It is important to note that the restriction of Delta1 expression to the posterior somite halves is not recapitulated by any reporter construct tested in our analysis (see, e.g. Fig. 2C). Rather, expression was found throughout the whole somites, suggesting that (a) negative regulatory element(s) is required to suppress Delta1 expression in the anterior somite halves. This element may be located either upstream or downstream the genomic region that we analyzed thus far, but not in the first intron, since deletion of this intron in the *Dll1*^{lacZ} allele preserves downregulation of transcription in anterior somite compartments. Since the restriction of Delta1 to posterior somite halves may be critical for establishment and/or maintenance of somite polarity, which, in turn, is required for the maintenance of borders between segments (Hrabé de Angelis et al., 1997), the isolation of this or these elements might lead to the identification of upstream factor(s) that are also essential for segmentation in mammals.

3.2. Potential conservation of Delta regulation in the neuroectoderm

Based on the assumption that transcription factors for the regulation of Delta expression may have been conserved during vertebrate evolution and that this may be reflected in the conservation of *cis*-regulatory sequences, we have searched for sequence similarities in the promoter regions of the mouse Delta1 gene and a homologous gene in a distantly related vertebrate. In the zebrafish four genes related to the *Drosophila* gene *Delta* have been identified. Of these, *deltaA* and *deltaB* are not expressed in the paraxial mesoderm during development; *deltaC* is expressed in the paraxial mesoderm and not in the neuroectoderm (Appel and Eisen, 1998; Haddon et al., 1998). Although the evolutionary relationship between the fish and mouse Delta genes is not fully resolved, the amino acid sequence of the zebrafish *deltaD* gene (Dornseifer et al., 1997; Haddon et al., 1998) is most similar to the mouse Delta1 gene. Reminiscent of the mouse Delta1 gene, *deltaD* is expressed in the presomitic mesoderm and the neuroectoderm. In contrast to the restriction of the mouse gene to posterior somite halves, expression of the *deltaD* gene is restricted to anterior halves of somites in the developing fish (Dornseifer et al., 1997). Because of the similarity between these two genes in the amino acid sequence and their overall expression patterns, we chose the fish *deltaD* gene for our search for sequence similarities in the promoter of the mouse Delta1 gene.

The comparison of the available upstream sequences from mouse and zebrafish identified two regions of homology (Homology I and II; Figs. 3 and 7A). Both conserved regions directed transgene expression reproducibly in distinct regions of the neural tube and, when they were absent from reporter gene constructs, these aspects of Delta1 expression were lost. This suggests that these regions contain element(s) that are necessary and sufficient for Delta1 transcription in subsets of neuroectoderm cells. The sequence conservation of both neuronal elements suggests that in the neuroectoderm at least some interactions of transcription factors with Delta promoters are conserved between mammals and teleosts. Both conserved sequence blocks contain multiple binding sites for known transcription factors. For example, E-boxes thought to be involved in bHLH-factor dependent transcriptional activation (Blackwell and Weintraub, 1990), are located in Homology I as well as in Homology II. Interestingly, the truncation of the distal part of HI in *Dll1*$^{tg3.6/lacZ}$ removes the two E boxes present in Homology I suggesting that these sequences are functionally important. However, considering the low specificity of such recognition sequences (in this case CANNTG) and their high abundance outside the two conserved regions, the significance of such potential binding sites for Delta1 expression remains to be tested in further studies.

Similar to the two 'msd' regions in the Delta1 promoter, the zebrafish *deltaD* promoter contains a distal and a proximal mesodermal enhancer region directing reporter gene expression in the paraxial mesoderm of transgenic zebrafish (S. Hans and J. Campos-Ortega, unpublished observation). However, in contrast to the elements for neural expression (HI and HII), the mesodermal elements of the mouse Delta1 promoter do not share significant sequence homologies with the zebrafish *deltaD* promoter. This suggests that the regulatory sequences (and the corresponding transcription factors) that direct mesodermal expression have considerably diverged during evolution. Alternatively, since the Delta1 promoter appears to consist of modules that can interact with the minimal promoter in different distances, it is possible that the mesodermal elements in the zebrafish corresponding to the mouse 'msd' regions reside in a portion of the zebrafish promoter outside the analyzed region. In the somitic mesoderm the expression pattern of the zebrafish *deltaC* gene resembles that of the mouse Delta1 gene, as its expression is also restricted to posterior somite compartments. Sequence comparisons between the mouse Delta1 and the zebrafish *deltaC* gene could be helpful to identify (a) potential element(s) that may repress expression in anterior somite compartments – if the transcription factors for this regulation have been conserved during vertebrate evolution.

4. Materials and methods

4.1. Constructs and transgenic mice

The transgene *Dll1*$^{tg4.3/lacZ}$ has its 5′ end 4265 bp upstream the first codon (ATG) of the *Dll1* coding sequence (see Fig. 1 for an overview of all transgenes). In all transgenes tested in this study, the *E. coli lacZ* gene was fused in frame into the first codon of the *Dll1* sequence followed by the SV40

and PGK polyadenylation signals in the 3′ end. The XhoI site within Homology I (HI; Fig. 3), lying 3660 bp upstream the first codon, is the 5′ end of *Dll1*$^{tg3.6/lacZ}$. Transgene *Dll1*$^{tg2.5/lacZ}$ has its 5′ end at an *SphI* site 2465 bp upstream the reporter gene. In transgene *Dll1*$^{tg1.6/lacZ}$ the 5′ end lies at a *HindIII* site 1615 bp upstream the first codon. The 5′ end in the minimal promoter construct, *Dll1*$^{tg0.8/lacZ}$, is at an *EagI* site 775 bp upstream the coding sequence and 40 bp upstream the putative major transcription start site of the Delta1 gene (Bettenhausen et al., 1995). In construct *Dll1*$^{tgHI/lacZ}$ the conserved sequence Homology I of the mouse Delta1 gene was introduced upstream the minimal promoter transgene as a PCR product of 304 bp starting 10 bp upstream and ending 15 bp downstream the sequence shown in Fig. 3. Similarly, in transgene *Dll1*$^{tgHII/lacZ}$ the conserved mouse sequence of Homology II was introduced as a 207 bp long PCR product that had its 5′ end 21 bp upstream and its 3′ end 27 bp downstream the sequence shown in Fig. 3. The cloning of these PCR products and the insertion of the *lacZ* gene were controlled by sequencing. The sequence between Homology I and Homology II that contains the mesodermal ('msd') element was isolated as 1495 bp *FokI* fragment and introduced upstream the minimal promoter transgene to generate reporter gene *Dll1*$^{tg'msd'/lacZ}$. The *FokI* fragment has its 5′ end 70 bp downstream of the HI sequence and its 3′ 100 bp upstream the HII sequence shown in Fig. 3. Transgenic mice were generated by injecting linearized constructs without vector sequences into the pronuclei of hybrid CByB6 fertilized eggs according to standard procedures. Mice were analyzed for transgene expression either in transient assays or transgenic lines as described in the results. The *Dll1*^{lacZ} allele is described in (Hrabé de Angelis et al., 1997).

4.2. Staining for β-galactosidase activity, histology, and whole-mount in situ-hybridization

Whole-mount detection of β-galactosidase activity was done as described in (Zachgo and Gossler, 1994). In brief, embryos were collected from timed pregnancies and fixed for 15–60 min in 0.2% glutaraldehyde in 100 mM potassium phosphate buffer, pH 7.4, containing 5 mM EGTA and 2 mM $MgCl_2$. Embryos were then washed three times in 0.01% Na-desoxycholate and 0.02% Nonidet P-40 in 100 mM potassium phosphate buffer with 5 mM EGTA and 2 mM $MgCl_2$. For detection of β-galactosidase activity embryos were incubated in washing solution containing 0.5 mg/ml X-gal, 10 mM $K_3Fe(CN)_6$, and 10 mM $K_4Fe(CN)_6$ at 37°C. After staining embryos were washed again in washing solution and finally in PBS. For histology embryos were dehydrated in isopropanol, cleared in xylene and transferred to paraffin. Embedded embryos were sectioned at 7–10 μm and stained with eosin according to standard procedures. For the detection of *lacZ* mRNA we used whole-mount in situ-hybridizations with a Digoxygenin-labelled probe following standard procedures.

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