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# On the organisation of the regulatory region of the zebrafish *deltaD* gene

**Stefan Hans and José A. Campos-Ortega***

Institut für Entwicklungsbiologie, Universität zu Köln, 50923 Köln, Germany
*Author for correspondence (e-mail: jose.campos@uni-koeln.de)

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## SUMMARY

***deltaD* is one of the four zebrafish Delta homologues presently known. Experimental evidence indicates that *deltaD* participates in a number of important processes during embryogenesis, including early neurogenesis and somitogenesis, whereby the protein it encodes acts as a ligand for members of the Notch receptor family. In accordance with its functional role, *deltaD* is transcribed in several domains of mesodermal and ectodermal origin during embryogenesis. We have analysed the organisation of the regulatory region of the *deltaD* gene using fusions to the reporter gene *gfp* and germline transgenesis. *Cis*-regulatory sequences are dispersed over a stretch of 12.5 kb of genomic DNA, and are organised in a similar manner to those in the regulatory region of the Delta-like 1 gene of mouse. Germline transformation using a minigene comprising 10.5 kb of this genomic DNA attached to the 3′ end of a full-length cDNA clone rescues the phenotype of embryos homozygous for the amorphic *deltaD* mutation *after eight*$^{AR33}$. Several genomic regions that drive transcription in mesodermal and neuroectodermal domains have been identified. Transcription in all the neural expression domains, with one exception, is controlled by two relatively small genomic regions, which are regulated by the proneural proteins neurogenin 1 and zash1a/b acting as transcriptional activators that bind to so-called E-boxes. Transcriptional control of *deltaD* by proneural proteins therefore represents a molecular target for the regulatory feedback loop mediated by the Notch pathway in lateral inhibition.**

Key words: Zebrafish, *deltaD*, Transcriptional regulation, Proneural proteins, Neurogenin 1, Zash1a/b

## INTRODUCTION

The Notch signalling pathway mediates lateral inhibition to bring about and stabilise cell-fate decisions during development. The organisation of the pathway was initially elucidated experimentally by studies on the development of progenitor cells of the central nervous system and of the sensory organs in the neuroectoderm of *Drosophila* (for a review, see Campos-Ortega, 1993). By expressing proneural genes, such as those of the *achaete-scute* complex (AS-C), clusters of neuroectodermal cells acquire the competence to adopt a neural developmental fate. Lateral inhibition serves to ensure that the proneural clusters actually provide both epidermal and neural progenitors. The main elements of the Notch pathway are a transmembrane ligand (Delta), a transmembrane receptor (Notch) and a transcriptional repressor (Suppressor of Hairless; [Su(H)]). For *Drosophila*, there is indirect evidence that, upon binding of the ligand, the intracellular domain of Notch (Nic) is cleaved off, and translocates into the nucleus (Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998), where, analogous to the situation in vertebrates (Hsieh et al., 1996), it is assumed to associate with Su(H). The Su(H)/Nic complex then activates transcription of downstream genes (Jennings et al., 1994; Bailey and Posakony, 1995; Lecourtois and Schweissguth, 1995), most notably those of the E(SPL)-C (Knust et al., 1987). These latter genes encode transcriptional repressors of the bHLH/WRPW family (Klämbt et al., 1989; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992), which, in conjunction with Groucho, suppress expression of the proneural genes (Paroush et al., 1994; Dawson et al., 1995; Giebel and Campos-Ortega, 1997), thus giving neuroectodermal cells access to epidermal development.

A regulatory feedback loop between Notch and Delta, which modulates Delta activity by regulating its transcription (Haenlin et al., 1994; Hinz et al., 1994; Kunisch et al., 1994), is an essential element of lateral inhibition. Proneural proteins encoded by the AS-C activate transcription of Delta by binding to specific sites in the Delta promoter (Kunisch et al., 1994). Consequently, the amount of proneural protein contained in a given neuroectodermal cell determines the amount of Delta protein produced and, ultimately, the efficacy of that cell in activating the Notch receptor in neighbouring cells. Within an array of mutually interacting cells, differences in the levels of proneural proteins will tend to increase the strength of the signals emitted by one of the cells and decrease the probability that any of the surrounding cells adopt a neural fate. This in turn reduces the efficacy of the surrounding cells as sources of lateral inhibition, and thus confirms the neural progenitor cell in its developmental fate decision (Kunisch et al., 1994). However, rather than being a result of random fluctuations in proneural protein concentration in the cells of the proneural cluster, it appears that individual cells are biased towards one of the two

developmental fates prior to the separation of the two cell types (Seugnet et al., 1997).

Four different Delta homologues, *deltaA*, *deltaB*, *deltaC* and *deltaD* have been identified in the zebrafish (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). Their patterns of transcription have much in common, but they also exhibit some differences. Thus, three of the genes, *deltaA*, *deltaB* and *deltaD*, are expressed in various regions of the neural plate in similar patterns, whereas *deltaC* and *deltaD* are also expressed in various mesodermal derivatives.

One of the functions of the Notch pathway in vertebrates, as in *Drosophila*, is to select individual cells, from groups of initially equivalent ones, for specific fates (Chitnis et al., 1995; Henrique et al., 1995; Chitnis and Kintner, 1996; Dornseifer et al., 1997; Wettstein et al., 1997; Appel and Eisen, 1998; Appel et al., 2001; Haddon et al., 1998; Takke et al., 1999). Injections of mRNA encoding variants of components of the Notch pathway have provided evidence for a regulatory feedback loop, which is apparently organised similarly to that in *Drosophila*, in both *Xenopus* and zebrafish (Wettstein et al., 1997; Takke et al., 1999). However, it is not known whether, as is the case in *Drosophila* (Kunisch et al., 1994), this feedback operates on proneural proteins that can bind to the promoters of the Delta homologues.

Another major function of the Notch pathway in vertebrates is the segmentation of the mesoderm. Mouse embryos deficient for *Notch1* or for the *Delta* homologue Delta-like 1 (*Dll1*) show severe somitic defects (Conlon et al., 1995; Hrabe de Angelis et al., 1997). Similar somite defects have been observed in mice that lack RBP-$J_\kappa$ (Oka et al., 1995; de la Pompa et al., 1997), a vertebrate homologue of Suppressor of Hairless. While these observations suggest that the Notch pathway is required for segmentation of the paraxial mesoderm, it is not known how Notch signalling performs this function or at which step it affects during segmentation. However, there is evidence suggesting that Notch signalling is required to synchronise the activity of groups of presomitic mesodermal cells with respect to the activity of genes that are cyclically expressed (Jiang et al., 2000).

We address the question of how the expression of *deltaD* is regulated during embryogenesis. We attached various fragments of the *deltaD* locus to an enhanced version of the reporter gene *gfp* (Cormack et al., 1996), and transformed these into the germline in order to identify *cis*-regulatory regions. We found that regulatory sequences are dispersed over a stretch of approximately 12.5 kb of genomic DNA that includes the entire coding region. Germline transformation with a minigene comprising 10.5 kb of this genomic DNA coupled to the 3′ end of a full-length cDNA rescues the abnormal phenotype of embryos homozygous for the amorphic *deltaD* mutation *after eight*$^{AR33}$ (Holley et al., 2000). In our analysis, we concentrated on the early pattern of transcription of *deltaD* and identified genomic regions that drive expression in mesodermal and neuroectodermal domains. Two of the genomic regions relevant for expression in specific domains of the neural plate reveal a highly conserved sequence homology between the mouse *Dll1* and the zebrafish *deltaD* promoter (Beckers et al., 2000). Both regions are regulated by the proneural proteins neurogenin 1 (Blader et al., 1997) and zash1a/b (Allende et al., 1994) acting as transcriptional activators. Activation of transcription of *deltaD* by proneural proteins thus represents a molecular target for the regulatory feedback loop controlled by the Notch pathway in lateral inhibition.

## MATERIALS AND METHODS

### Isolation of deltaD genomic clones and plasmid construction

Two overlapping clones comprising a total of 21 kb were obtained from a genomic DNA library (*Easy-to-handle eukaryotic genomic library (zebrafish)*, Mo Bi Tec, Göttingen, Germany). To synthesise the reporter gene constructs, the *gfp*-coding sequence (Cormack et al., 1996) was ligated to the SV40 polyA signal (a gift from N. Scheer) and the resulting fragment was cloned either upstream or downstream of various genomic fragments in pBluescript. Different endogenous restriction sites were used for construct assembly (upstream sites: –6 *Xba*I, –3.5 *Eco*RI, –1.8 *Asp*700, –1.3 *Nsi*I; downstream sites: +2.8 *Nsi*I and +6.5 *Asp*718, the latter site lying within pBluescript). For the rescue of the *aei*$^{AR33}$ mutant phenotype, the 3′ terminal end of a full-length *deltaD* cDNA (fusion of the cDNAs Dl-1 and Dl-2) (Dornseifer et al., 1997), comprising the sequences corresponding to the tenth and eleventh exons, was linked in frame to several copies of the coding sequence for the myc epitope. The resulting fragment was then ligated to genomic DNA using a *Nru*I site located in the ninth exon. The resulting construct includes 10.5 kb of genomic DNA (6 kb of sequence upstream and 4.5 kb downstream of the transcription start site) and carries the entire coding sequence, as the fragment of the cDNA clone provides the last two exon sequences.

### Germline transformation

DNA fragments were isolated from pBluescript with either *Asp*718/*Not*I or one of the endogenous restriction enzymes indicated in the map of the *deltaD* locus in Fig. 2. The preparation of the DNA fragments followed the previous methods (Scheer and Campos-Ortega, 1999). Injected, putative founder fish (G0) were crossed inter se and their progeny ($F_1$) were screened with a fluorescence stereomicroscope [Leica-Stereomikroskop (*MZ FLIII*)] for GFP-mediated signals. Animals scored as positive were raised to adulthood and crossed either to wild-type fish or inter se. Animals that showed no fluorescence were screened by PCR using *deltaD* (5′CAACA-GAGCATCAACCCGAGC3′)- and *gfp* (5′CGTGTCTTGTAGTTCC-CGTCATC3′)-specific primers.

### Staining procedures

In situ hybridisation was performed as described by Bierkamp and Campos-Ortega (Bierkamp and Campos-Ortega, 1993). Antibody staining (anti-myc and anti-GFP) was carried out as described by Westerfield (Westerfield, 1994) with some modifications. Embryos and larvae older than 48 hours were incubated for 30 minutes with 0.01% collagenase/PBT before bleaching in 1% $H_2O_2$/PBT overnight. EliteABC (Vectastain) was used to enhance the sensitivity of the staining. All incubation steps were performed at 4°C overnight in 5% DMSO/10% goat serum/PBT.

### CAT assays and ELISA

To determine quantitatively effects of neurogenin 1- and zash1a/b-sensitive promoter elements, CAT assays were used. Several promoter constructs and the control plasmid *pBS 6lacZ* were co-injected with mRNA for either neurogenin 1 or zash1a into wild-type zygotes; the animals were allowed to develop to the five-somite stage and the levels of CAT and β-Gal were determined (Vize, 1996) using ELISA kits (Roche). Site-directed mutagenesis of E-boxes was carried out on the CAT constructs using the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene. The following mutations were introduced into the HI and HII boxes (see Results).

For HI

Wild-type:
GTGAAAAAACGC**C**AT**T**T**G**GTTGGGAG**C**A**G**AT**G**GTTGGCTT-GGG
Mutant:
GTGAAAAAACGC**T**AT**A**T**C**GTTGGGAG**T**A**T**AT**C**GTTGGCTT-GGG

For HII

Wild-type:
GTGAGGGGAG**C**AG**G**T**G**CTGTGTGAATTACCATA**C**AG**C**T**G**A-GAGCACAGAG
Mutant:
GTGAGGGGAG**T**AG**T**T**C**CTGTGTGAATTACCATA**T**AG**T**T**C**A-GAGCACAGAG
For each reaction, 10 ng of plasmid DNA was used. Primers used for the mutagenesis experiments were:

HI 1s, GTGAAAAAACGC**T**ATA**T**C**G**TTGGGAGCAG;
HI 1a, CTGCTCCCAAC**G**ATA**T**A**G**CGTTTTTTCAC;
HI 2s, CATTTGGTTGGGAG**T**A**T**AT**C**GTTGGCTTGGG;
HI 2a, CCCAAGCCAAC**G**ATA**T**A**C**TCCCAACCAAATG;
HI 1+2s, GTGAAAAAACGC**T**ATA**T**C**G**TTGGGAG**T**A**T**AT**C**-GT;TGGCTTGGG;
HI 1+2a, CCCAAGCCAAC**G**ATA**T**A**C**TCCCAAC**G**A**T**ATA**G**C-GTTTTTTCAC;
HII 1s, GTGAGGGGAG**T**AG**T**T**C**CTGTGTGAATTACC;
HII 1a, GGTAATTCACACAG**G**AA**C**TA**C**TCCCCTCAC;
HII 2s, GTGAATTACCATA**T**AG**T**T**C**AGAGCACAGAG;
HII 2a, CTCTGTGCTCT**G**AA**C**TA**T**ATGGTAATTCAC;
HII 1+2s, GTGAGGGGAG**T**AG**T**T**C**CTGTGTGAATTACCA-TA**T**AG**T**T**C**AGAGCACAGAG; and
HII 1+2a, CTCTGTGCTCT**G**AA**C**TA**T**ATGGTAATTCACACA-G**G**AA**C**TA**C**TCCCCTCAC.

Primer orientations are: s, sense; a, antisense. Mutated bases are bold.

## RESULTS

During embryogenesis in the zebrafish, *deltaD* transcripts are distributed in a complex pattern, comprising both mesodermal and neuroectodermal expression domains. The following is a summary of the main features of the *deltaD* transcription pattern within mesodermal and neural derivatives in embryos (see also Dornseifer et al., 1997; Haddon et al., 1998).

Transcription of *deltaD* in the developing mesodermal primordia begins at 30% epiboly within the entire marginal region (Fig. 1A). At about 50% epiboly, transcription ceases in the embryonic shield, i.e. the prospective axial region of the embryo (Fig. 1B), and, at 60-70% epiboly, the expression domain extends from the marginal zone into the hypoblast. From 80-90% epiboly onwards, two transverse, band-like domains become visible – first within the hypoblast and later in the presomitic mesoderm (Fig. 1C-H). This pattern continues throughout somitogenesis. Posterior to the bands, a much lower density of transcripts can be discerned down to the tip of the growing tail, where a high concentration of transcripts is present (Fig. 1D-H). In addition to the presomitic bands, the anterior halves of the somites themselves contain *deltaD* transcripts (Fig. 1G,H).

Expression within the epiblast starts at 80-90% epiboly in the form of two bands that extend in the animal-vegetal axis to eventually become continuous stripes (Fig. 1C-F). Expression in the neural plate is characterised by a number of domains in the prosencephalic-mesencephalic primodium and in the primordium of the hindbrain. Three longitudinal expression domains, i.e. lateral, intermediate and medial, are present in the neural plate region corresponding to the hindbrain. Of these, the medial and lateral domains extend into the territory of the


**Fig. 1.** Whole-mount in situ hybridisation to show the distribution of *deltaD* transcripts. (A) 30% epiboly. *deltaD* is transcribed in the marginal zone (black arrowhead). (B) 70% epiboly. The shield region is devoid of transcripts (white arrow). (C) 95% epiboly. *deltaD* expression in a V-shaped expression domain (1) anteriorly, in two longitudinal stripes (IS) in the trunk region of the neural plate, and in one transverse, band-like domain (white arrowhead) in the hypoblast. (D) Tailbud stage embryo. Expression domains in the neural plate comprise two stripes in an intermediate position (IS) and two further lateral and caudal stripes (LS). *deltaD* is also expressed in the primordium of the trigeminal ganglion (asterisk) and in a horseshoe-like domain (2). (E) Two-somite stage embryo. Two medial stripes (MS) in the neural plate at the level of the trunk. In addition, two further expression domains (3 + 4) appear in the prospective brain region. (F) Five-somite stage embryo. Intermediate and lateral stripes are displaced due to neurulation movements. The white arrowhead indicates the presomitic expression band. (G) 12-somite stage embryo. In addition to presomitic bands (white arrowhead), the anterior halves of the somites contain *deltaD* transcripts (black arrows). (H) 20-somite stage embryo. Expression domain 2 has shifted to a new position, whereas expression domain 3 is still in place. In addition *deltaD* transcripts can be detected in the anlage of the epiphysis (E). The white arrowhead indicates the presomitic expression band.

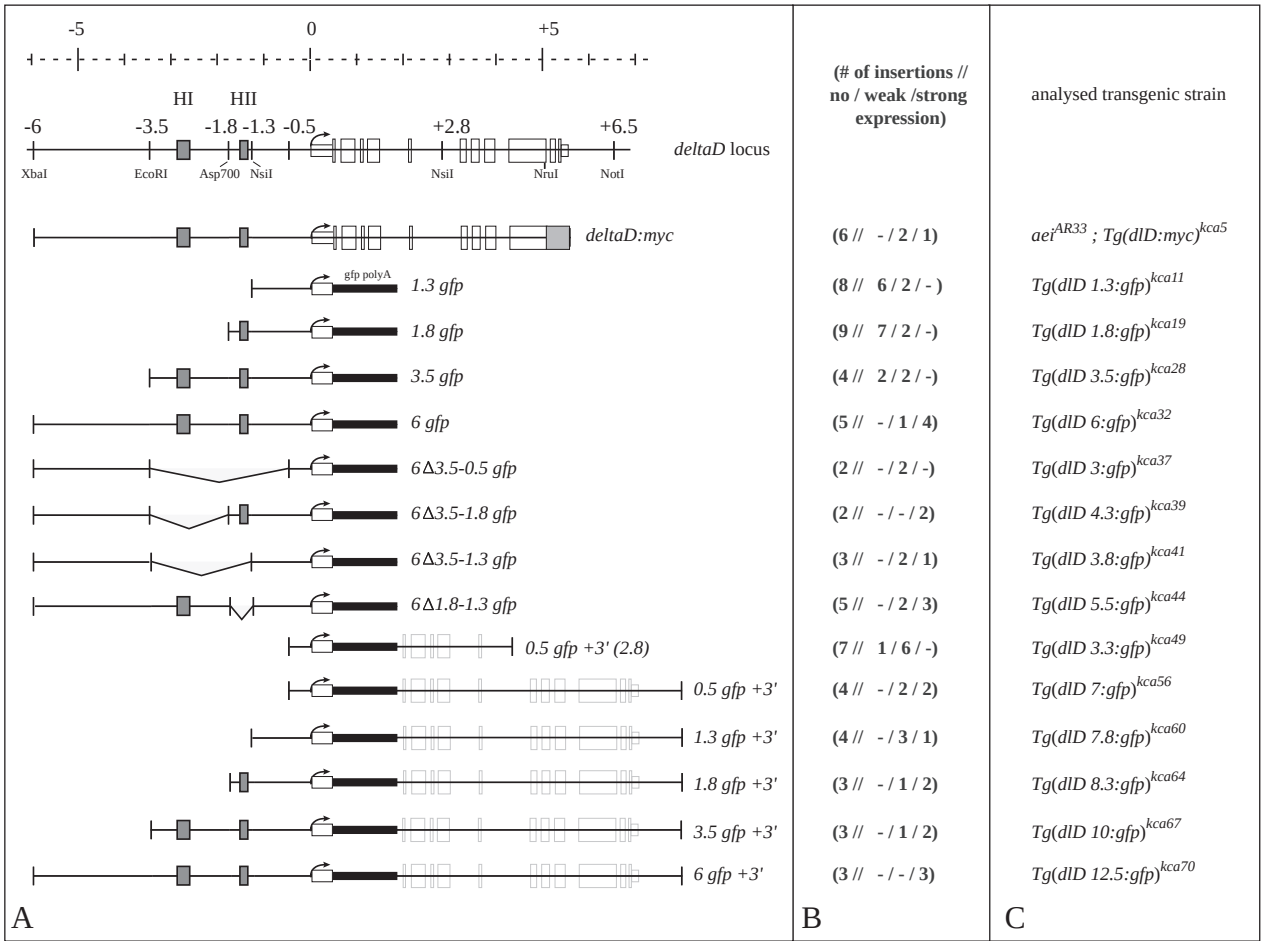


**Fig. 2.** (A) The structure of the *deltaD* locus was deduced from the sequence of 12.5 kb of genomic DNA isolated from two overlapping phage clones. The structures of the various constructs used for transformation are also shown. The transcriptional start point is indicated by an arrow. The 11 exons and the 5′- and the 3′-untranslated regions are indicated by large and small white boxes, respectively. The two conserved regions Homology I (HI) and Homology II (HII) are represented by dark-grey boxes. The light-grey box at the end of the construct *deltaD*:myc represents exons 10 and 11 derived from the deltaD cDNA and including five myc epitopes fused in frame with the genomic deltaD DNA (see Materials and Methods). (B) Number of independent insertions and their classification according to the *gfp* expression level. Strong expression indicates the detection of fluorescent GFP; weakly expressed insertions do not produce fluorescent GFP, but *gfp* transcripts are detectable by in situ hybridisation. (C) Names of the transgenic lines used in this study.

spinal cord, whereas the intermediate domain is restricted to the rhombencephalon (Fig. 1C-F). These longitudinal domains are also part of the patterns of expression of *deltaA* and *deltaB* (Haddon et al., 1998; Appel and Eisen, 1998). Experimental evidence (Dornseifer et al., 1997; Haddon et al., 1998; Appel and Eisen, 1998; Appel et al., 2001; Takke et al., 1999) indicates that primary sensory (so-called Rohon-Beard) neurones are selected from the cells that make up the lateral stripes, while primary motoneurones are generated from the medial ones, as a result of Notch-mediated signalling.

Within the primordia of forebrain and midbrain, at the tailbud and two-somite stages, four main domains of *deltaD* RNA expression can be distinguished (Fig. 1C-E), numbered 1-4 in the order of their appearance. The first one (domain 1 in Fig. 1C,D) is located at the level of the prospective mesencephalon; the second (2 in Fig. 1D-H) is prosencephalic, and is disposed in the shape of an arch, apparently delimiting the prosencephalic primordium anteriorly and laterally; the third (3 in Fig. 1E-H) is located medially in the prosencephalon; and the fourth (4 in Fig. 1E) lies in the metencephalon.

## The mutant phenotype of the *after eight*$^{AR33}$ mutation is rescued by a *deltaD* minigene

In order to determine the extents of coding and regulatory sequences in the *deltaD* gene, we chose to perform germline transformation of the *after eight* mutant *aei*$^{AR33}$, which corresponds to a loss of function of the *deltaD* gene (Holley et al., 2000). The extant *after eight* mutations (van Eeden et al., 1996) are recessive, homozygous viable mutations. The mutant phenotype is manifested in mesodermal and neuroectodermal derivatives. Fusion of the somites caudal to the seventh to ninth somite (see Fig. 3A) and mild hyperplasic defects of primary neurones are observed (Holley et al., 2000). Two overlapping genomic clones encompassing 12.5 kb of the *deltaD* locus were sequenced and the structure of the locus was defined from this sequence (Fig. 2). The *deltaD*-coding region consists of 11 exons and 10 introns (Fig. 2A). In order to add five copies of

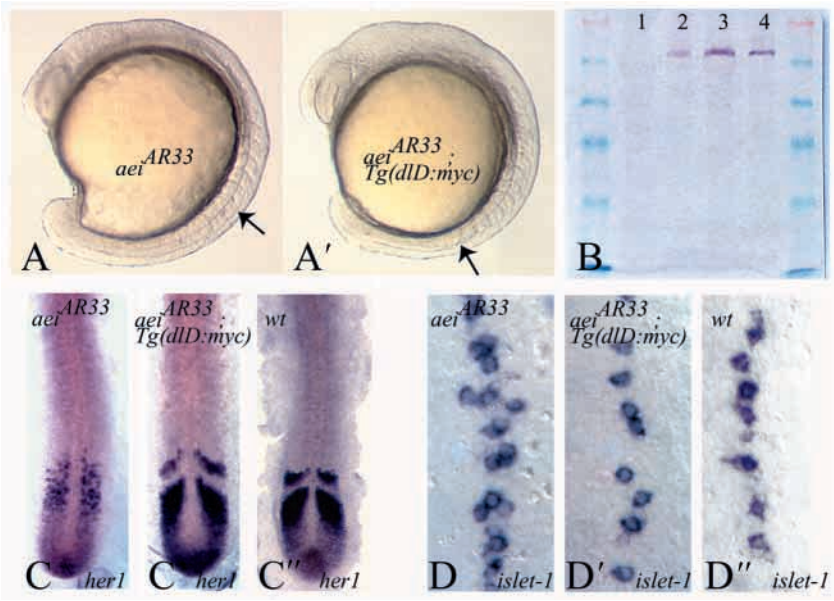


**Fig. 3.** Comparison of *after eight*$^{AR33}$ (*aei*$^{AR33}$) and *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$ embryos. (A,A′) 14-somite stage embryos, arrows indicate the last formed somites. (A) *aei*$^{AR33}$ mutant. No normal somites form beyond the eighth somite. (A′) *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$ embryos have normal somites. (B) Western blot loaded with protein extracts from 24 hpf embryos of different genotypes, probed with an anti-myc antibody. Lane 1, wild-type; lane 2, progeny of *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$ × wild type; lane 3, progeny of *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$ × *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$; lane 4, progeny of *deltaD:Gal4* × *UAS:myc:notch1a-intra* (positive control). The size of the deltaD:myc protein is approximately 91 kDa, that of notch-intra:myc 92 kDa. The left and the right lanes show the See Blue Pre-Stained Standards marker (Novex). (C,C′,C″) 14-somite stage. The pattern of expression of *her1* is aberrant in *aei*$^{AR33}$ (C) and indistinguishable from that in *aei*$^{AR33}$*; Tg(dlD:myc)kca*$^{5}$ (C′) or in wild type (C″). (D,D′,D″) Four-somite stage. The number of primary sensory neurones (*islet1* in situ hybridisation) is increased in *aei*$^{AR33}$ (D), but normal in the transformants (D′) and the wild-type embryos (D″).

the myc epitope to the rescue construct, part of the genomic DNA was deleted and replaced by the 3′ end of a full-length cDNA (see Materials and Methods). The rescue clone consisted of 6 kb of genomic sequence upstream and 4.5 kb downstream of the putative transcription start site (as defined by the TATA box and the maximal extent of cDNAs), including the first nine exons of the protein coding region. The tenth and eleventh exons and the 3′UTR were provided by the 3′ terminal region of a full-length cDNA with the myc tags. Hence the entire coding sequence is present in this construct, which was injected into *aei*$^{AR33}$ homozygous zygotes. Upon reaching adulthood, the injected animals were backcrossed to *aei*$^{AR33}$ homozygotes and embryonic progeny of these crosses were screened for insertions of the construct by PCR using *myc*-specific primers. Progeny of a total of 102 injected animals were screened and six independent insertions were recovered, of which three *[Tg(dlD:myc)*$^{kca5–7}$*]* were further analysed. Mutants carrying each of the three *deltaD:myc* insertions expressed a deltaD-myc fusion protein of the expected size of 91 kDa**,** as shown on western blots probed with the anti-myc antibody (Fig. 3B). These animals showed none of the abnormal phenotypic traits characteristic of the *aei* mutants and were indistinguishable from the wild type.

The most obvious difference between insertion-bearing embryos and mutants was the appearance of normal somitic boundaries caudal to the ninth somite in the *aei*$^{AR33}$*;Tg(dlD:myc)*$^{kca5-7}$ transformants (Fig. 3A′), as this trait is completely penetrant in the *aei* mutants (van Eeden et al., 1996). Several genes, e.g. *her1*, *myoD*, *mesp-a*,

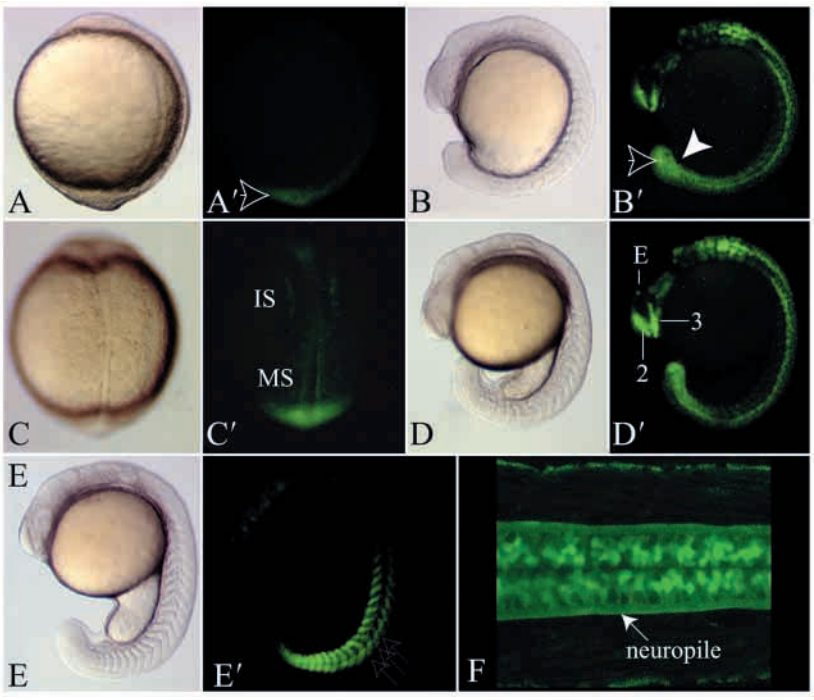


**Fig. 4.** GFP expression driven by the *deltaD* promoter in transgenic embryos. Living embryos carrying the *3.5gfp+3′* construct at tailbud stage (A,A′), 14-somite (B,B′), four-somite (C,C′) and 22-somite (D,D′) stages show GFP expression in neural and mesodermal domains. This pattern is nearly identical to that of the endogenous gene with the exception of the somitic expression, which is much weaker in the transgenic embryos. (E,E′) Strong somitic GFP expression at the 20-somite stage in an embryo carrying the *6Δ1.8-1.3gfp* construct. (F) Subcellular distribution of GFP within the trunk neural tube of an embryo carrying the construct *3.5gfp+3′*. GFP fluorescence was detected by stereomicroscopy in A-E′; (F) GFP fluorescence analysed by laser confocal microscopy. Tailbud, black arrowhead; presomitic expression, white arrowhead; somitic expression, black arrows; intermediary and medial expression stripes, IS and MS; epiphysis, E; telencephalic and diencephalic expression domains, 2 and 3.

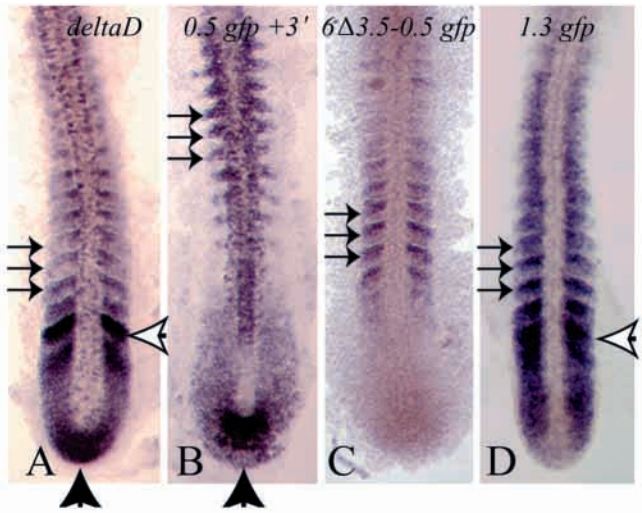


**Fig. 5.** Distribution of *gfp* transcripts in mesodermal derivatives of embryos carrying different *gfp* constructs (B-D) in comparison with the expression of the endogenous gene (A). 14-somite stage embryos. (A) *deltaD* transcripts are visible in the tailbud (black arrowhead), in one to two transverse bands in the presomitic mesoderm (white arrowhead) and in the anterior halves of the somites (black arrows) decreasing in intensity from caudal to rostral. (B) Embryos carrying the *0.5gfp+3′* construct reveal strong expression in the tailbud and in the posterior half of the somites from the eighth somite onwards. (C) Embryos carrying the *6Δ3.5-0.5gfp* construct show *gfp* transcripts only in newly formed somites. (D) Embryos carrying the *1.3gfp* construct also reveal *gfp* transcription in newly formed somites and in the presomitic mesoderm in a stripe pattern, whereas the tailbud is transcript free.

*papc* and *deltaC*, exhibit a conspicuous pattern of transcript expression in stripes made up of presomitic and/or somitic, paraxial mesodermal cells in the wild-type. This pattern is perturbed in the *aei* mutants (Fig. 3C) (van Eeden et al., 1996; Holley et al., 2000; Durbin et al., 2000; Jiang et al., 2000). However, the expression patterns of these genes were normal in the transformants (Fig. 3C′) and indistinguishable from wild type (Fig. 3C″). The number of primary neurones (detected with an *islet1* probe), which is increased in the *aei* mutants (Holley et al., 2000), was lowered in the transformants to a wild-type level (Fig. 3D-D‴). We therefore conclude that the artificial *deltaD* construct is functionally equivalent to the wild-type *deltaD* gene.

## Spatial and temporal regulatory elements of *deltaD* are distributed over 12.5 kb of genomic DNA

In order to identify genomic regions containing *cis*-acting elements that regulate transcription of *deltaD*, the coding region of the reporter gene *gfp*, beginning immediately after the 5′UTR, was fused to various fragments of the 12.5 kb genomic sequence that rescues the $aei^{AR33}$ phenotype. The resulting constructs were injected into wild-type zygotes and, after reaching adulthood, the injected animals (putative G0 transgenics) were crossed inter se and the progeny embryos were screened for GFP-mediated fluorescence and by PCR analysis of DNA prepared from pools of embryos, using a forward primer located in the *deltaD* promoter and a reverse primer in the *gfp*-coding sequences (see Materials and Methods). Transgenic animals were used to establish lines carrying the different transgenes. Although transcription of the endogenous *deltaD* gene begins at 30-40% epiboly (Dornseifer et al., 1997; Haddon et al., 1998) (Fig. 1), GFP-mediated fluorescence was first detected as late as the tailbud stage in embryos carrying some of the constructs used here (Fig. 4A). Thus, to allow comparison with the early endogenous expression pattern, we assayed for *deltaD-gfp* transcription by in situ hybridisation using a digoxigenin-labelled *gfp* probe. Fig. 2 shows the transgenes analysed in this study. All strongly expressed transgenes were associated with GFP-mediated fluorescence. The remaining transgenes were either weakly expressed, and could be analysed only by *gfp* in situ hybridisation, or were not expressed at all.

The pattern of *gfp* transcripts in transformants carrying construct *6gfp+3′*, in which the *gfp* gene is sandwiched between the 5′ and 3′ halves of the entire 12.5 kb genomic DNA, displays all temporal and spatial elements of the *deltaD* expression pattern. Therefore, together with the rescue of the *aei* mutant, this observation strongly suggests that the entire *deltaD* locus is contained within a 12.5 kb segment of genomic DNA (Fig. 4A-F).

## Regulatory regions for mesodermal expression

Three different regions of the *deltaD* promoter contain all regulatory elements required for transcription in the tailbud, in the presomitic paraxial mesoderm and in the somites (Fig. 5A). One element required for expression in the marginal zone and, at later stages, in the tailbud, is located in the 3′ region, as all constructs carrying the fragment 0 to +2.8 showed, albeit weakly, expression in the marginal zone and the tailbud (Fig. 5B). The strength of 3′-mediated expression was considerably increased when the +2.8 to +6.5 fragment was added. The 3′ region also contributes weakly to the control of diffusely distributed transcripts within the presomitic mesoderm and, from the eighth somite on, in the somites. Double in situ staining with *myoD* and *gfp* revealed that this somitic *gfp* expression is in fact an ectopic expression, as it is restricted to the posterior half of the somites (not shown). An additional regulatory element that controls transcription within the newly formed somites is located in the upstream DNA, between –6 and –3.5 (construct *6Δ3.5-0.5gfp*) (Fig. 5C). The third mesodermal element is located proximal to the basal promoter, and is also responsible for control of transcription in the somites (construct *1.3gfp*) (Fig. 5D). More importantly, this region drives expression in the stripe domains in the presomitic mesoderm. On its own, this element drives transcription weakly. However, transcription is quite intense when the element is combined with the –6 to –3.5 region. Besides the timing of the mesodermal expression, we also observed a change in the amount of transcripts depending on the upstream DNA fragment driving *gfp*. Embryos carrying the construct *6gfp* gave rise to a normal level of transcripts in the presomitic mesoderm, i.e., comparable with that of the endogenous gene; embryos transgenic for *1.3gfp* exhibited the same expression in the presomitic mesoderm. Strikingly, embryos transgenic for *6Δ1.8-1.3gfp* showed a higher density of transcripts in the presomitic mesoderm (see Fig. 7A,F), whereas embryos transgenic for *6Δ3.5-1.8gfp* did not show any expression in the presomitic mesoderm (see Fig. 7C,H). This suggests the existence of an element reducing

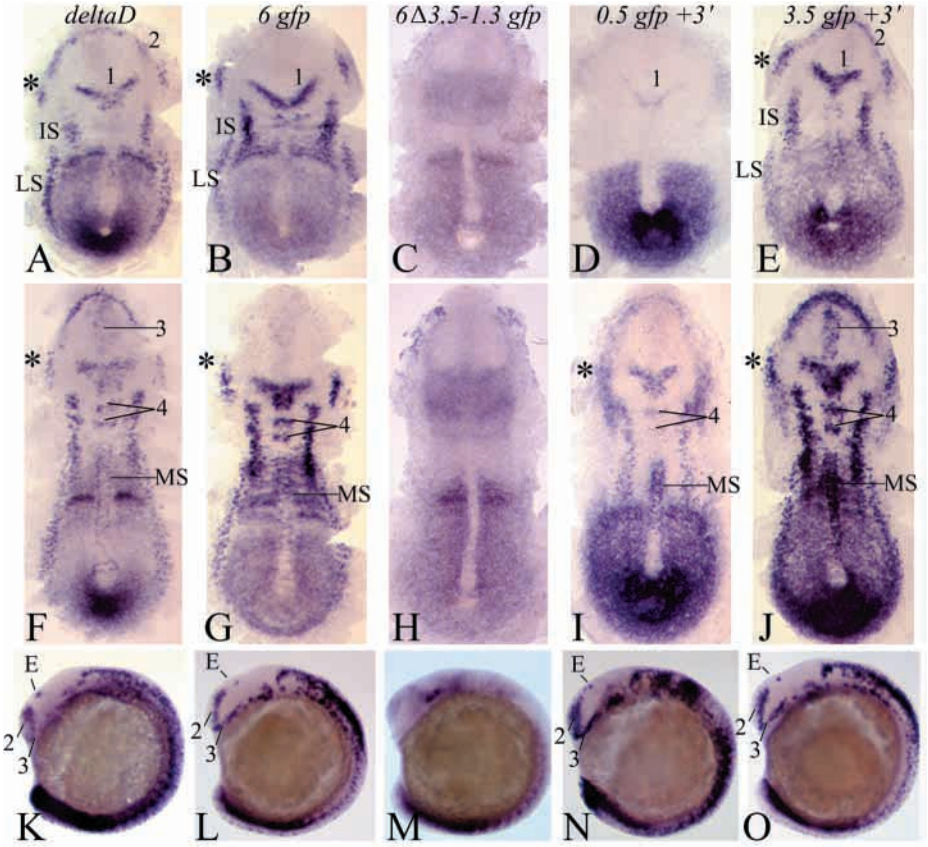


**Fig. 6.** Neuroectodermal distribution of *gfp* transcripts in embryos carrying different constructs (B-E,G-J,L-O) in comparison with transcripts of the endogenous gene (A,F,K). Tailbud stage (A-E), two-somite stage (F-J) and 12-somite stage embryos (K-O). Embryos carrying the *6gfp* construct (B,G,L) show all the neural expression domains of *deltaD* with the exception of the early expression of domain 2 and anterior region of domain 3 (A,F,K). Embryos carrying the *6Δ3.5-1.3gfp* construct show no neural *gfp* expression at all (C,H,M). *gfp* expression in neural domains in embryos carrying the *0.5gfp+3′* construct (D,I,N) shows a striking delay in comparison with the endogenous gene. Expression domain 2 requires sequence elements located both in the upstream and the downstream regions, as only progeny of transformants carrying the *3.5gfp+3′* (E,J,O) and *6gfp+3′* construct show *gfp* transcripts here. Prosencephalic, mesencephalic and rhombencephalic expression domains, 1-4; developing trigeminal ganglion, asterisk; epiphysis, E; medial, intermediary and lateral expression stripes, MS, IS and LS.

transcription in the presomitic mesoderm located between –1.8 and –1.3.

### Regulatory elements for neural expression

Three different regions were found to drive transcription within the neural primordia (Fig. 6A,F,K). Particularly striking are two well-defined regions located between –3.5 and –1.8 (HI) and –1.8 and –1.3 (HII) (Fig. 2A), which show substantial sequence similarity to two regions in the promoter of the mouse *Dll1* gene (Beckers et al., 2000). The 6 kb of genomic DNA upstream of the putative transcription start site (*6gfp*), which includes HI and HII, contains regulatory sequences necessary for the expression of all features of the transcription pattern in the neuroectoderm, with the exception of the early expression (tailbud- to five-somite-stage) of the horseshoe-like domain at the boundary of the prosencephalon (domain 2) and the anterior expression of domain 3 (Fig. 6B,G,L). This latter domain requires additional sequences in the downstream region, which could not be mapped to a defined segment (Fig. 6E,J,O). From the five-somite-stage on, *gfp* expression corresponds to the endogenous expression of *deltaD*. Absence of HI, as in the constructs *6Δ3.5-1.8gfp* and *1.8gfp*, is associated with the loss of reporter gene expression in the trigeminal ganglion and in the three stripes in the neural plate at the level of the prospective rhombencephalon and spinal cord (Fig. 7C,H). Absence of HII, as in construct *6Δ1.8-1.3gfp*, leads to loss of various expression domains in forebrain, midbrain and hindbrain (Fig. 7A,F). Finally, absence of both HI and HII, as in constructs *6Δ3.5-1.3gfp* and *1.3gfp*, determines the loss of essentially all elements of the neural pattern (Fig. 6C,H,M).

Constructs containing the most proximal 2.8 kb of genomic DNA in the 3′ region, or even the complete 3′ fragment down to +6.5 which encompasses all the coding sequences of *deltaD*, are also associated with *gfp* expression in all neural expression domains (Fig. 6D,I,N). However, expression mediated by this 3′ DNA in these domains is significantly delayed and much weaker than when the 3′ region is accompanied by 5′ DNA. Therefore, we assume that control of neural expression by the 3′ region is subordinate to control by the 5′ region. It should be noted that there is a putative MyT1 binding site at +271. We will come back to this point later.

### HI and HII contain neurogenin 1- and zash1a/b-sensitive E-boxes

*neurogenin 1* (Blader et al., 1997; Korzh et al., 1998) and *zash1a/1b* (Allende et al., 1994) encode putative proneural proteins. Injection of *neurogenin 1* mRNA has been shown to ectopically activate transcription of the endogenous *deltaD* gene (Takke et al., 1999), and neurogenein 1 is therefore very likely to regulate *deltaD* directly. Similarly, injection of mRNA encoding neurogenin 1 into zygotes carrying the *3.5gfp+3′* transgene leads to ectopic activation of *gfp* transcription (data not shown) as in the case of the endogenous *deltaD* gene (Takke et al., 1999). In addition, it is worth noting that constructs containing HI and HII drive reporter gene expression in a pattern that reflects the distribution of *neurogenin 1* and *zash1a/1b* transcripts. Thus, in the two-somite stage embryo, *neurogenin 1* is transcribed in domain 1, in domain 4, and in the medial, intermediary and lateral stripes of *deltaD* expression in the prospective rhombencephalon and

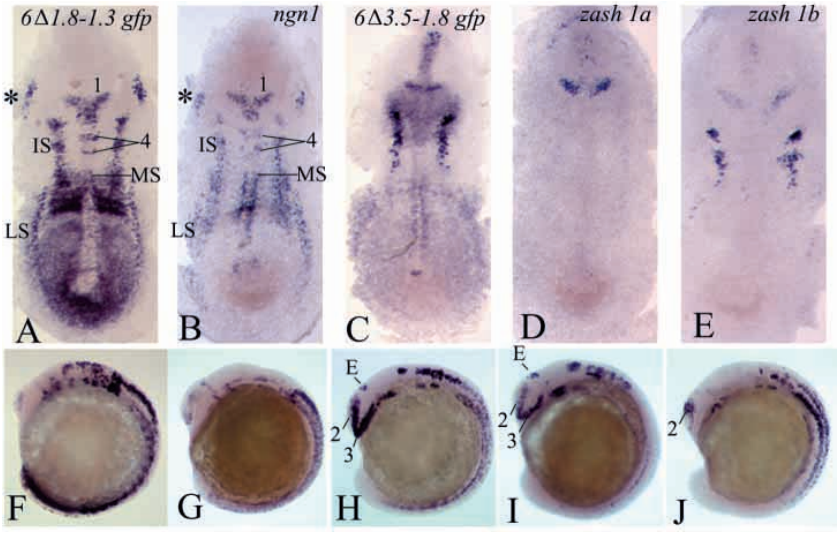


**Fig. 7.** Comparison of the distribution of *gfp* transcripts driven by *6Δ1.8-1.3gfp* and *6Δ3.5-1.8gfp* with that of *neurogenin 1* (*ngn1*), *zash1a* and *zash1b*. (A-E) Two-somite stage and (F-J) 12-somite stage embryos. Neural *gfp* expression driven by the *6Δ1.8-1.3gfp* construct (A,F) comprises *gfp* transcripts in the primordium of the trigeminal ganglion (asterisk), in domains 1 and 4 and in the lateral, intermediate and medial stripes (LS, IS, MS) of the neural plate, but lack expression in domains 2 and 3 and the epiphysis anlage (E). This expression is similar to that of *neurogenin 1* (B,G). Embryos carrying the *6Δ3.5-1.8gfp* construct (C,H) reveal a complementary expression pattern in the distribution of *gfp* transcripts, which is highly reminiscent of the expression of *zash1a* (D,I) and *zash1b* (E,J).

spinal cord (Fig. 7A,B,F,G) (Blader et al., 1997; Korzh et al., 1998; Takke et al., 1999). However, in the 12-somite stage embryo *zash1a* is transcribed in the epiphysis, ventrally in the diencephalon and dorsally in the hindbrain, whereas *zash1b* is transcribed dorsally in the telencephalon, in rhombomeres 2 and 4, and in cells of the spinal cord (Fig. 7C,D,E,H-J) (Allende et al., 1994). HI and HII may thus represent direct targets for the proneural proteins neurogenin 1 and zash1a/1b.

We used CAT assays to look for possible activation of *deltaD* by neurogenin 1 and/or zash1a/1b. For this, plasmids carrying specific regions of the *deltaD* promoter fused to the CAT-coding sequence were injected, together with mRNA for either neurogenin 1 or zash1a and zash1b, into wild-type zygotes (Fig. 8A). The amount of chloramphenicol acetyl transferase (CAT) was determined by ELISA in extracts of the injected animals when they had reached the five-somite stage. When co-injected with mRNA for neurogenin 1, constructs including HI (*6CAT* and *3.5CAT*) expressed high levels of CAT; constructs lacking HI (*1.3CAT*, *1.8CAT* and *6Δ3.5-1.8CAT*) generated much lower amounts of the protein (Fig. 8B). Deletion constructs, particularly one comprising HI and the basal promoter (*HI 0.5CAT*), which still express significant amounts of CAT, show that the neurogenin 1-mediated CAT activation requires HI (Fig. 8D).

Pilot experiments with either zash1a or zash1b gave similar results for both proteins (results not shown). Therefore, as the DNA-binding regions of both proteins are identical (Allende et al., 1994), we assume that the conclusions drawn for zash1a can be extended to zash1b. After co-injection with mRNA for zash1a, constructs carrying HII (*6CAT*, *3.5CAT* and *1.8CAT*) express high levels of CAT, whereas those without HII (*1.3CAT* and *6Δ1.8-1.3CAT*) gave rise to much lower amounts of the reporter (Fig. 8C).

Neurogenin 1 and zash1a/b are bHLH proteins, and bHLH proteins are known to bind to E-boxes. HI contains two E-boxes, which are conserved in position and orientation in mouse and zebrafish (Beckers et al., 2000). Three nucleotides were replaced in each E-box in HI (see Materials and Methods). Mutation of either of the E-boxes brings about a marked reduction in the amount of CAT expressed. Mutation of both E-boxes in HI causes a further reduction to the basal level (Fig. 8D). Two E-boxes are also located within HII, which, like those in HI, are conserved in position and orientation in the HII region of the mouse gene. Mutation of the distal E-box does not have any apparent effect on the amount of CAT, whereas mutation of the proximal E-box causes a strong reduction in the level expressed (Fig. 8E).

We have seen that the 0 to +2.8 fragment located in the 3′ region mediates reporter gene expression in all neural domains, although with considerable delay relative to the full-length gene, and that this control is temporally subordinate to that by the –3.5 to –1.3 region. It has also been mentioned that there is a putative MyT1-binding site at +271 (Park et al., 2000), as well as 13 E-boxes distributed throughout the 0 to +2.8 region. MyT1 has been described as a direct target of neurogenin 1 (Bellefroid et al., 1996; Koyano-Nakagawa et al., 1999). Therefore, to further characterise the regulatory region and the possible roles played by MyT1, the 13 E-boxes and neurogenin 1, two additional constructs were synthesised (Fig. 8A). One construct comprised the 0 to +2.8 region placed downstream of CAT; the control region in the other reporter construct carried a mutation in the putative MyT1-binding site. mRNA encoding either neurogenin 1 or MyT1 was injected into zygotes together with each one of the plasmids, and the level of CAT was determined as described above. However, none of these constructs could be activated by either MyT1, neurogenin 1 or both. Therefore, the putative MyT1 binding site and the 13 E-boxes contained in this region, appear to be irrelevant in the context studied here.

## DISCUSSION

### Regulatory sequences of *deltaD* are dispersed through 12.5 kb of genomic DNA

Two main conclusions bearing on the organisation of the *deltaD* promoter can be drawn from our observations.

(1) *Cis*-regulatory sequences are distributed over 12.5 kb of genomic DNA encompassing 6 kb upstream and 6.5 kb downstream of the transcription start site. This DNA can correct all abnormal phenotypic traits associated with the mutation $aei^{AR33}$ (van Eeden et al., 1996; Holley et al., 2000),

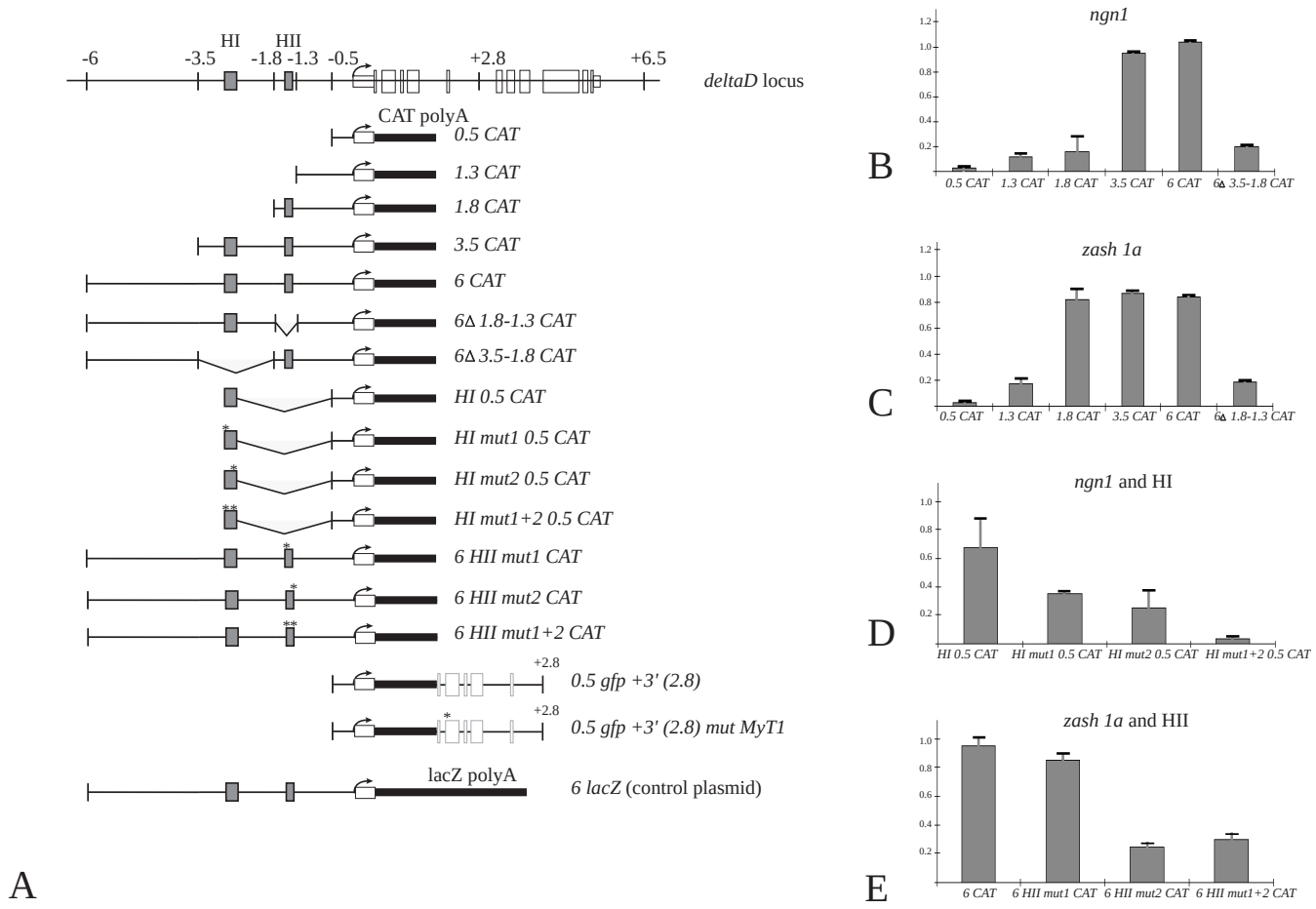


**Fig. 8.** (A) CAT reporter gene constructs. Structures are as in Fig. 2. (B-E) Assays of transient CAT expression in zebrafish embryos. (B) In the presence of neurogenin 1, constructs with HI (*3.5CAT* and *6CAT*) express a high level of CAT, whereas constructs without HI (*0.5CAT, 1.3CAT, 1.8CAT* and *6Δ3.5-1.8 CAT*) show a much weaker response. (C) In the presence of zash1a, constructs without HII (*0.5CAT, 1.3CAT* and *6Δ1.8-1.3 CAT*) express little CAT protein and constructs with HII (*1.8CAT, 3.5CAT* and *6CAT*) generate larger amounts. (D) HI is sufficient for a strong CAT response in the presence of neurogenin 1 (*HI 0.5 CAT*). Mutation of either the distal (*HI mut1 0.5 CAT*) or the proximal E-box (*HI mut2 0.5 CAT*) leads to a decrease in the amount of CAT produced. When both E-boxes are mutated (*HI mut1+2 0.5 CAT*) CAT production drops to the basal level. (E) In the presence of zash1a, mutation of the distal E-box in HII (asterisk in *6 HII mut1 CAT*) has no apparent effect on the levels of CAT, whereas mutation of the proximal E-box (asterisk in *6 HII mut2 CAT*) results in a decrease in the amount of CAT. Mutation of both E-boxes simultaneously (asterisks in *6 HII mut1+2 CAT*) leads to no further reduction.

and is thus sufficient to provide the normal function of the *deltaD* gene. No additional control sequences are expected to occur outside this region, as the *6gfp+3′* construct, which contains the entire 12.5 kb genomic region, displayed all the temporal and spatial features of *deltaD* transcription that we were able to assess. Genomic regions required for the regulation of expression in mesodermal and neuroectodermal derivatives can be distinguished within this DNA segment. In addition, two putative quantitative enhancers were identified, one between –6 kb and –3.5 kb in the 5′ region, and the other between +2.8 kb and +6.5 kb in the 3′ region. However, this proposal is based solely on the observation that their presence substantially increased the intensity of expression driven by other DNA fragments.

(2) Sequences required for the regulation of specific traits were found to be concentrated in discrete zones, whereas others are dispersed over a wider area. Thus, mesodermal elements were found to be dispersed in relatively large pieces of genomic DNA, whereas two discrete boxes, HI and HII, were found to be necessary and sufficient for almost all neural expression domains, as the comparison of reporter gene expression directed by several constructs clearly shows. Moreover, E-boxes within HI and HII were found to be targets for neurogenin 1 and zash1a, and these elements are therefore responsible for the expression directed by HI and HII in neural domains (see below; Fig. 9).

Besides HI and HII, genomic sequences in the interval 0 to +2.8 in the 3′ region appear to regulate transcription within the same regions of the neural plate, although with a striking delay in comparison with reporter expression driven by the upstream sequence, or relative to the endogenous *deltaD* gene. As the pattern elements controlled by this genomic interval suggest the participation of proneural proteins and/or their downstream targets, we used CAT assays to probe the role played by 13 E-boxes and a putative MyT1 binding site located there in responding either to neurogenin 1 or to MyT1. On the one

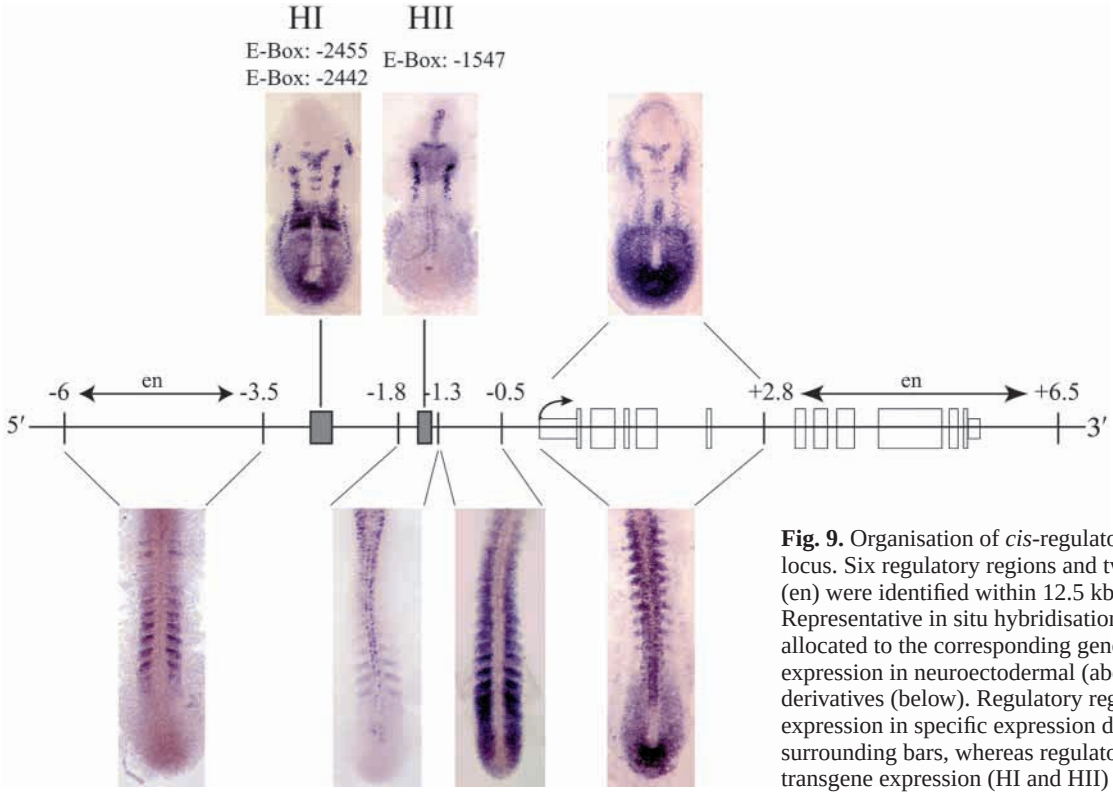


**Fig. 9.** Organisation of *cis*-regulatory regions of the *deltaD* locus. Six regulatory regions and two quantitative enhancers (en) were identified within 12.5 kb of the *deltaD* locus. Representative in situ hybridisation experiments have been allocated to the corresponding genomic regions with respect to expression in neuroectodermal (above) or mesodermal derivatives (below). Regulatory regions sufficient to direct expression in specific expression domains are indicated by the surrounding bars, whereas regulatory regions necessary for transgene expression (HI and HII) are indicated by boxes.

hand, the pattern elements controlled by this interval include those which, as discussed above, are neurogenin 1-responsive. On the other hand, the MyT1 protein has been shown in *Xenopus* to be part of the neurogenic cascade, appearing following the activity of proneural bHLH proteins and rendering cells expressing these proteins insensitive to lateral inhibition (Bellefroid et al., 1996). However, surprisingly enough, the results of CAT assays suggest that these 13 E-boxes do not respond to neurogenin 1, nor does the putative MyT1 site react to MyT1. Therefore, the question as to how expression in neural primordia is driven by the 0 to +2.8 segment remains unanswered.

### Proneural genes regulate transcription of *deltaD* in the neural plate

The similarity of the pattern of reporter gene expression directed by constructs including HI and HII to that of the proneural genes *neurogenin 1* and *zash1a/b* (Blader et al., 1997; Allende et al., 1994) was the first hint that transcription of *deltaD* in these domains might be activated by the proneural proteins encoded by these genes. Indeed, the CAT assays have provided convincing evidence that both types of proneural proteins activate transcription of *deltaD* through the E-boxes present in them, either by directly binding, or by activating other bHLH proteins which themselves bind, to the E-boxes. Previous work had shown that injection of mRNA encoding neurogenin 1 activates *deltaD* transcription ectopically in embryos (Takke et al., 1999). Our present findings indicate that activation of transcription occurs by direct binding of either neurogenin 1, or some other bHLH protein induced by neurogenin 1, to specific E-boxes. It seems highly probable that neurogenin 1 binds directly to the *deltaD* promoter, as the timing of its expression and of that driven by the neurogenin 1-responsive reporter gene constructs are virtually coincident. In addition, *Xenopus Delta1* can be activated by low doses of *X-ngnr-1* mRNA, which do not elicit any response from several other downstream targets of X-ngnr-1 (Koyano-Nakagawa et al., 1999).

Transcription of *Drosophila Delta* is directly activated by the binding of proneural proteins to several E-boxes distributed through the promoter, and this provides the target for the regulatory feedback loop responsible for lateral inhibition (Haenlin et al., 1994; Kunisch et al., 1994). Therefore, in this respect, the promoters of *Drosophila Delta* and zebrafish *deltaD* are similarly organised, as transcription of both genes in the neuroectoderm can be activated by proneural proteins. In both cases, the amount of proneural protein determines the strength with which the Delta ligand signals to the Notch receptor and, therefore, the intensity of lateral inhibition (Kunisch et al., 1994; Takke et al., 1999). However, although in *Drosophila* a relatively large number of active binding sites for proneural proteins are distributed over a large segment of genomic DNA (Kunisch et al., 1994), far fewer binding sites are found in the zebrafish *deltaD* gene, and these are concentrated in two discrete regions. In fact, in *Drosophila* 12 E-boxes in a stretch of 4.3 kb of genomic DNA were found to serve as binding sites for homodimers of daughterless, achaete and lethal of scute, and their heterodimeric combinations, and all were required for the neuroectodermal pattern of transcription of *Delta* (Kunisch et al., 1994). In the zebrafish, however, only three E-boxes that respond to proneural proteins are sufficient to drive transcription in all neuroectodermal domains but one, namely domain 2, which is regulated by sequences contained within the up- and downstream regions.

As there is only one *Delta* gene in *Drosophila*, whereas at least three *delta* genes in zebrafish exhibit largely overlapping, but not identical, expression patterns in neural primordia, it is conceivable that this is the reason why a larger number of E-boxes is required to modulate the transcriptional activity and thus the function of *Delta* in the fruitfly. This modulation may be achieved in the zebrafish by the subtle differences that can be observed between the transcriptional patterns of the three Delta genes expressed in neural primordia.

### The regulatory regions of zebrafish and mouse *Delta* genes are similarly organised

Our transgenic analysis of the *deltaD* locus has revealed six distinct *cis*-regulatory regions, five upstream and one downstream of the transcription start site, that direct gene expression in neuroectodermal and mesodermal subdomains of the embryo. The upstream region of the mouse *Dll1* locus shows a similar organisation (Beckers et al., 2000). We propose that both promoters are organised in five modules, of which at least three are phylogenetically conserved. Two of these modules correspond to the regions HI and HII, identified on the basis of their high sequence similarity; in addition, both regions are located in the same relative positions and in the same orientation in both species. However, there is a difference in the pattern of expression driven by these elements in zebrafish and mouse. In stably transformed mouse embryos, HI coupled to the minimal *Dll1* promoter is able to direct reporter gene expression primarily to the ventral tube and some derivatives of the neural crest, such as dorsal root and spinal ganglia. By contrast, transformants bearing HII fused to the minimal *Dll1* promoter direct expression in the marginal zone of the dorsal region of the neural tube (Beckers et al., 2000). In zebrafish, the expression patterns of HI and HII do not exhibit a restriction to dorsal or ventral regions of the neural tube. It seems probable that this apparent difference is due to the different expression of neurogenin 1 and Mash1 (Ascl1 – Mouse Genome Informatics), and neurogenin 1 and zash1a/b, in mouse and zebrafish, respectively. Indeed, in the mouse embryo the expression patterns of neurogenin 1 and Mash1 show a similar restriction in the neural tube, and it appears that there is a complete overlap in the expression patterns of HI and HII with neurogenin 1 and Mash1 (Ma et al., 1996). Thus, the regulatory network appears to be conserved in zebrafish and mouse, although the expression domains of the corresponding proneural genes have changed during evolution.

With respect to the three mesodermal modules, two appear to be conserved whereas the other has diverged. In both species, one mesodermal element in the region immediately proximal to the minimal promoter [‘msd II’ according to Beckers et al. (Beckers et al., 2000)] is able to direct reporter gene expression in the presomitic mesoderm and nascent somites. However, sequence comparison of these two regions in zebrafish and mouse revealed only minor stretches of similarity, the significance of which still remains to be tested. A second module is represented by the putative silencer of transcription in the presomitic mesoderm at –1.8 to –1.3, flanked by enhancer elements. In mouse, Beckers et al. (Beckers et al., 2000) describe negative regulators flanked by positive regulators of expression in the presomitic mesoderm, i.e. a similar organisation to that in zebrafish. Unfortunately, again in this case the comparison of both DNA sequences has failed to show any similarity in this region. The third mesodermal module identified in the mouse *Dll1* promoter, called ‘msd’, is located within the two elements HI and HII. This module is not present in the zebrafish *deltaD* and might be responsible for the difference in the expression in mature somites: zebrafish *deltaD* is expressed in the anterior halves of the mature somites, whereas mouse *Dll1* is expressed in the posterior halves. Therefore, all these considerations reveal a great deal of phylogenetic conservation in the organisation of the regulatory regions of *deltaD* and *Dll1* expression in zebrafish and mouse.

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