her3, a zebrafish member of the *hairy-E(spl)* family, is repressed by Notch signalling

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Summary

***her3* encodes a zebrafish bHLH protein of the Hairy-E(Spl) family. During embryogenesis, the gene is transcribed exclusively in the developing central nervous system, according to a fairly simple pattern that includes territories in the mesencephalon/rhombencephalon and the spinal cord. In all territories, the *her3* transcription domain encompasses regions in which *neurogenin 1* (*neurog1*) is not transcribed, suggesting regulatory interactions between the two genes. Indeed, injection of *her3* mRNA leads to repression of *neurog1* and to a reduction in the number of primary neurones, whereas *her3* morpholino oligonucleotides cause ectopic expression of *neurog1* in the rhombencephalon. Fusions of Her3 to the transactivation domain of VP16 and to the repression domain of Engrailed show that Her3 is indeed a transcriptional repressor. Dissection of the Her3 protein reveals two possible mechanisms for transcriptional repression: one mediated by the bHLH domain and the C-terminal WRPW tetrapeptide; and the other involving the N-terminal domain and the orange domain. Gel retardation assays suggest that the repression of *neurog1* transcription occurs by binding of Her3 to specific DNA sequences in the *neurog1* promoter. We have examined interrelationships of *her3* with members of the Notch signalling pathway by the Gal4-UAS technique and mRNA injections. The results indicate that Her3 represses *neurog1* and, probably as a consequence of the *neurog1* repression, *deltaA*, *deltaD* and *her4*. Moreover, Her3 represses its own transcription as well. Surprisingly, and in sharp contrast to other members of the *E(spl)* gene family, transcription of *her3* is repressed rather than activated by Notch signalling.**

Supplemental data available online

Key words: Zebrafish, *neurog1* (*ngn1*), Her3

Introduction

Signalling mediated by Notch is a central component of a variety of developmental processes in invertebrates and vertebrates. During lateral inhibition in *Drosophila*, Delta binds to Notch on adjacent cells and determines the cleavage of its intracellular domain, which translocates into the nucleus (Hsieh et al., 1996; Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). Here, it associates with Suppressor of Hairless [Su(H)], a member of the CSL (RBP-Jκ) family (Schweisguth and Posakony, 1992), bound to its DNA recognition sequence, and forms a complex that activates the 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 (Paroush et al., 1994; Dawson et al., 1995; Fisher et al., 1996; Giebel and Campos-Ortega, 1997) suppress the expression of transcriptional activators, including the proneural genes (Oellers et al., 1994; Van Doren et al., 1994; Singson et al., 1994; Tata and Hartley, 1995; Heitzler et al., 1996; Nakao and Campos-Ortega, 1996). A regulatory feedback loop modulates *Delta* activity by regulating its transcription (Haenlin et al., 1994; Hinz et al., 1994; Kunisch et al., 1994; Heitzler et al., 1996). Proneural proteins activate the transcription of *Delta* by binding to specific sites in its promoter (Kunisch et al., 1994). Consequently, the amount of proneural protein contained in a given cell determines the amount of Delta protein produced and, ultimately, the efficacy with which that cell activates Notch in neighbouring cells. The net result of these interactions is that only single cells within cell clusters take on the neural fate, while adjacent cells become epidermoblasts (for a review, see Simpson, 1997).

The Notch signalling pathway is similarly organised in vertebrates (for a review, see Lewis, 1996). As in *Drosophila*, one of the functions of the pathway is to select individual cells for specific fates from groups of initially equivalent cells

(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, organised similar way to that described for *Drosophila*, in both *Xenopus* and zebrafish (Wettstein et al., 1997; Takke et al., 1999). Activation of Notch receptors leads to activation of *E(spl)* homologues (Jarriault et al., 1995; Tamura et al., 1995; Hsieh et al., 1996; Kopan et al., 1996; Wettstein et al., 1997; Schroeter et al., 1998; Takke and Campos-Ortega, 1999; Takke et al., 1999) mediated by CSL proteins (Wettstein et al., 1997). Thus, in mice, a loss-of-function mutation in the RBP-Jκ gene (*Rbpsuh* – Mouse Genome Informatics), the *Su(H)* homologue, leads to repression of Hes5 and, as a consequence, to upregulation of the proneural gene *Math4a* (*Neurog2* – Mouse Genome Informatics) (de la Pompa et al., 1997). In zebrafish, this feedback loop operates on the proneural gene *neurogenin 1* (*neurog1*; previously known as *ngn1*) (Blader et al., 1997), the product of which binds to E-boxes in the promoter of the *Delta* homologue *deltaD*, and activates its transcription (Hans and Campos-Ortega, 2002).

We describe here a new zebrafish *E(spl)* homologue, *her3*, the expression of which is restricted to neural territories, where it represses transcription of the proneural gene *neurog1*. Gel retardation assays show that Her3 binds specifically to N-boxes in the promoter regions of *neurog1* and of *her3*, thus contributing to its own regulation. The function of *her3*, like that of other members of the *E(spl)* family, depends on Notch signalling. However, *her3* differs from the other family members in that its transcription is repressed rather than activated by Notch1a signalling.

Materials and methods

Zebrafish embryos were obtained from spontaneous spawnings. Adult fish were kept at 28.5°C on a 14 hour light/10 hour dark cycle. The embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

Molecular cloning of *her3* and UAS plasmid construction

PCR using degenerate primers was performed on reverse-transcribed total RNA from zebrafish embryos at the 90%-epiboly to two-somite stage. PCR fragments encoding peptides with similarity to *Drosophila* bHLH proteins were used to screen a zebrafish cDNA library prepared in λZAP (Stratagene) from RNA isolated from 3-15 hour zebrafish embryos (gift from C. Fromental-Ramain and P. Chambon, Strasbourg). The GenBank Accession Number for the *her3* cDNA sequence is X97331. A genomic *her3* clone (15 kb) was obtained from a genomic DNA library (‘Easy-to-handle eukaryotic genomic library’ (zebrafish) Mo Bi Tec, Göttingen). The Accession Number for the *her3* genomic sequence is AY277702. The upstream sequence was subcloned into *pBsGAL4* (Scheer and Campos-Ortega, 1999). The plasmid *pBs2xMARher4* was generated by excision of the Notch1a:intra coding sequence from *pBs2xMAR notch1a-intra* with *Sma*I (Scheer and Campos-Ortega, 1999). The gap was filled with the *Eco*RI digested and blunt-ended coding sequence of the *her4* cDNA (Takke et al., 1999). To generate stable transgenic lines, plasmid DNA preparations and injections were carried out following the procedures described by Scheer and Campos-Ortega (Scheer and Campos-Ortega, 1999).

pCS2+her3:gfp was made by PCR using the plasmid *pCS2+her3* diluted 1:10 as template (see *her3 for* and *her3 rev* primers in Tables S1-S3 at http://dev.biologists.org/supplemental). The amplified 680 bp fragment was cut with *Eco*RI and *Bam*HI, cloned in the pCS2+EGFP vector previously cut with the same enzymes and sequenced.

RNA injections

Eight constructs encoding Her3 variants and two Her3 fusion proteins were generated by PCR using different 5′ and 3′ primers, and cloned into the *pCS2+* (Turner and Weintraub, 1994). The coding sequence was amplified from the *her3* cDNA using *her3* specific primers. The primers used for constructs with terminal deletions (see Tables S1-S3) provided an artificial ATG for the construct *her3ΔN*, an artificial WRPW motif and stop codon for construct *her3ΔC*, and an artificial stop codon for construct *her3ΔWRPW*. PCR products were digested with the appropriate restriction enzymes and cloned into the *pCS2+* vector. In order to remove the endogenous stop codon to permit the generation of the her3 fusion proteins, the coding sequence of *her3* was amplified with the primers SP6 and her3 fusion (including a *Bam*HI site), digested with *Eco*RI and *Bam*HI, and cloned into the *Eco*RI/*Bam*HI sites of the vectors *pCS2+VP16* and *pCS2+eng*, which supply the sequences encoding the VP16 transactivation and the engrailed repressor domains, respectively, in the correct reading frame. The primer combinations used for the generation of the constructs and fusion proteins are listed in Tables S1-S3 at http://dev.biologists.org/supplemental. Capped RNA was synthesised in vitro by transcription with SP6 polymerase from the constructs described above, or from a pCS2-nuclear β-Galactosidase (nβgal) template DNA, using a Message Kit from Ambion.

The RNA was injected in a volume of 5 nl into one of the first two blastomeres. In most cases, *lacZ* mRNA was co-injected at concentrations previously shown to be innocuous for the zebrafish embryo (Takke et al., 1999). β-Galactosidase was detected by antibody staining.

Morpholino injections

Morpholino-modified antisense oligonucleotides directed against *her3* (Gene Tools; MO *her3* 5′-TGCAGCCATTGTCCTTAAATGCT-CA, 2 blocker 5′-TTAAAAAATCCAGATGAATAAGGAC-3′) and the mismatch morpholino 5′-TGGAGGCATTGTGCTTAAATCCT-GA-3′ were injected at the one- to four-cell stage at a concentration of 50-200 μM in 1×Danieau (Nasevicius and Ekker, 2000). Morpholinos were injected together with 0.2% Texas Red in a total volume of 5-10 nl. As an additional control, mRNA encoding a *her3-gfp* fusion was co-injected with the morpholinos.

RT-PCR

Total RNA was extracted from 50 wild-type embryos, or 50 embryos injected with MO *her3*, using the RNA-Clean™ System (Angewandte Gentechnologie Systeme GmbH). Before precipitation, the RNA was treated with 2U of DNase (Boehringer Mannheim) for 30 minutes. Two μg of RNA was reverse transcribed using Superscript-RT (Gibco-BRL) and 100 ng of random hexamers (Boehringer Mannheim) in a 20 μl reaction, and 0.5 μl of this reaction was subjected to PCR. As an internal control, we used primers that amplified a 400 bp fragment of the gene for elongation factor e-IF4a. After 3 minutes at 95°C, amplification was carried out for 1 minute at 95°C, 1 minute at 58°C and 1 minute at 72°C (27 cycles for *her3* and 28 for *neurog1*), with a final extension step for 10 minutes at 72°C. The primers are given in Tables S1-S3 (see http://dev.biologists.org/supplemental).

Gel retardation assays

Protein preparation followed the protocol described previously (Chang et al., 1997). For each experimental determination, three lanes were loaded with increasing concentrations of protein (refer to the legends of Figs 3, 4 and 7). The specificity of binding was tested in competition assays with unlabelled oligonucleotide. Binding reactions were performed in the presence of oligonucleotides containing N boxes, one of the DNA sequences recognised by E(spl) proteins

(consensus CACNAG) (Sasai et al., 1992; Tietze et al., 1992; Oellers et al., 1994), from the promoter region of *her3* and *neurog1* (Blader et al., 2003). Band-shift assays were carried out according to Fried and Crothers (Fried and Crothers, 1984a; Fried and Crothers, 1984) and Hendrickson and Schleif (Hendrickson and Schleif, 1984). The sequences of the oligonucleotides used in binding assays were as follows (N-boxes are in bold, mutations are underlined).

neurog1 promoter

NP wt 5′ AAT TCC AAG CT**C ACA AG**C T**CA CAC G**AG CTG ATT G 3′
Mut 1+2 5′ AAT TCC AAG CTC CAT GGC TCA CCA TGG CTG ATT G3′
Mut 1 5′ AAT TCC AAG CTC CAT GGC TCA CAC GAG CTG ATT G 3′
Mut 2 5′ AAT TCC AAG CTC ACA AGC TCA CCA TGG CTG ATT G 3′

her3 promoter

N1 5′ AAT TCT GAT TGG ATG TC**C AGC AG**A AAG TAT GGA TG 3′
N2 5′ AAT TTG CAT TTT CAC CC**C ACA CG**A CCG AGG TTT CA 3′
N1 mut 5′ AAT TCT GAT TGG ATG TCA CGC ACA AAG TAT GGA TG 3
N2 mut 5′ AAT TTG CAT TTT CAC CCA CCA CCA CCG AGG TTT CA 3′

In situ hybridisation and histological methods

Hybridisation of digoxigenin-labelled RNA probes to embryo whole mounts was performed as described previously (Bierkamp and Campos-Ortega, 1993). Embryos injected with RNA were prepared for in situ hybridisation and for antibody staining, as described by Dornseifer et al. (Dornseifer et al., 1997). For sectioning, embryos were embedded in Araldite (Serva).

Results

her3 was identified among PCR fragments obtained using degenerate primers directed against the regions of the E(spl)-C genes encoding the bHLH domain and the C terminal WRPW motif. The fragments were subcloned, sequenced and used as probes to screen a zebrafish cDNA library. The *her3* cDNA *13.2.1.4* comprises a 687 bp open reading frame encoding a protein of 229 amino acids with all the features characteristic of the *Drosophila* hairy/E(spl) family. The *her3* product shows the highest degree of similarity overall to *hes3* (54%) and to the *Drosophila* mδ protein (54%). Within the bHLH domain this similarity increases to 86% for *hes3* and 69% for mδ (Bestfit, GCG programme).

her3 is expressed within neural territories

Whole-mount in situ hybridisation revealed that *her3* RNA is expressed only in neural territories during embryogenesis (Fig. 1). Expression was first detected at about 30% epiboly in a coherent patch of cells within the dorsal region of the epiblast, in a region corresponding to the prospective anlage of the neural plate (Fig. 1A) (Woo and Fraser, 1995). At 80% epiboly, transcripts disappear from the medial region, splitting the primary domain into two (Fig. 1B,C). By the tail-bud stage, each of these has evolved into two extended longitudinal expression domains, which progressively separate from each other during the formation of the first somites. One of the domains is located rostrally and will eventually split into several groups of *her3*-positive cells distributed throughout the mesencephalon and rhombencephalon (Fig. 1E-H). Double in situ hybridisation using probes for *egr2b* and *her3*, *pax2a* and *her3*, or *her5* and *her3* (Fig. 1I-L) show that the anterior margin of the *her3* domain corresponds to the anterior margin of the mesencephalic primordium (Lun and Brand, 1998) extending to rhombomere 5. At the tail-bud stage, the *her3* transcription domain ends cranially at the anterior border of the *her5* domain, which itself initially extends throughout the midbrain/hindbrain anlage (Müller et al., 1996; Bally-Cuif et al., 2000), but is later restricted to the so-called intervening zone at the midbrain/hindbrain boundary (Geling et al., 2003). Therefore, later in development, the cranial margin of the *her3* transcription domain continues to define the anterior margin of the mesencephalon, whereas the *her5* domain is located further caudal. However, the *her3* transcription pattern within the mesencephalic/rhombencephalic region is very dynamic and no attempt was made to define the different groups of *her3*-transcribing cells in this region.

The caudal expression domain occupies the intermediary regions of the neural plate, starting at the level of the presumptive cervical spinal cord, and consists of a contiguous patch of cells (Fig. 1D-H). Comparison of in situ hybridisation with *neurog1* and *her3* probes shows that, within this region, transcription of *neurog1* is restricted to those regions of the neural plate in which *her3* is not expressed (Fig. 1D′-F′). Although double in situ hybridisation with *neurog1* and *her3* failed to produce reliable results, the comparison of in situ hybridisation with each single probe strongly suggests that the two genes are expressed in mutually exclusive domains both in the developing spinal cord and in the mesencephalic/rhombencephalic territories. During later stages of neurulation, *her3* transcripts vanish from most of the cells in the anterior part of the spinal cord, becoming restricted to individual cells, while a contiguous expression domain persists in caudal regions, where neurulation is still in progress (Fig. 1E-H).

To characterise the regulatory region of the *her3* gene, a 4.7 kb segment of the 5′ upstream DNA was fused to the GAL4 coding sequence, and transformed into the germline of wild-type embryos. Three independent chromosomal insertions were recovered, of which only one was able to transactivate *UAS:notch1a-intra*. In situ hybridisation with a *gal4* cDNA probe showed that the *gal4* transcription pattern in the transgenic embryos reflects, with minor deviations, the expression of the endogenous *her3* gene (Fig. 2A-C). Thus, the expression of the *gal4* transgene in mesencephalic and rhombencephalic regions is slightly delayed relative to that of *her3*, and the density of *gal4* transcripts is higher than in the case of endogenous *her3* RNA. The transgene is also expressed in the otic placodes, unlike the endogenous *her3* gene. It is not clear whether these differences are due to position effects. For our present purposes, though, it is important to note that the 4.7-kb DNA that drives transcription in the intermediary region of the neural plate at the level of the spinal cord contains two high-affinity N boxes and a single high-affinity Su(H) binding site (TGTGAGAA) (Bailey and Posakony, 1995; Lecourtois and Schweissguth, 1995; Rebeiz et al., 2002). There are no low-affinity binding sites for E(spl)-related proteins in this DNA fragment.

The higher density of the *gal4* mRNA in the transgenic embryos might be explained by higher stability relative to

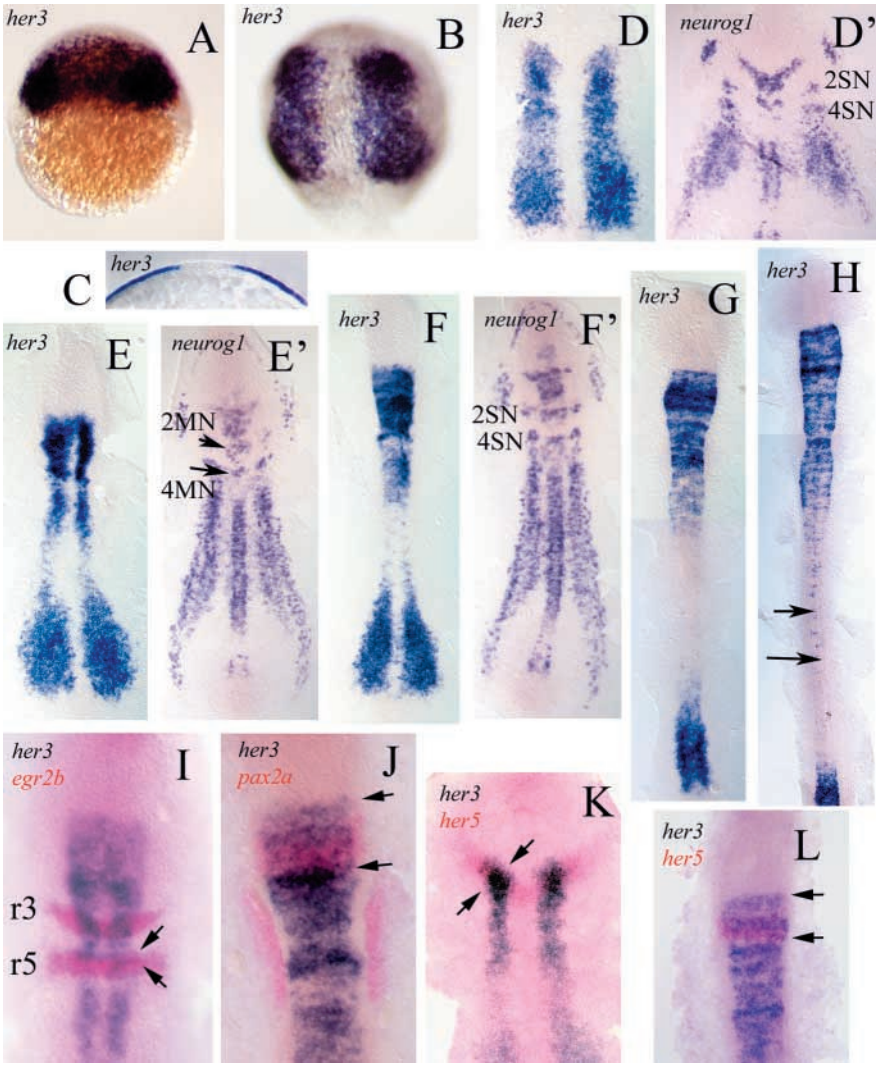

Fig. 1. Distribution of *her3* transcripts revealed by whole-mount in situ hybridisation. (A) 30% epiboly. *her3* transcription is first detected in the blastoderm. (B) 80% epiboly. The single initial transcription domain has split into two, each of which lies within the presumptive neural primordium. (C) Cross-section of B showing transcription in the epiblast. (D) Flat preparation of a tailbud-stage embryo. The *her3* expression domains form two broad longitudinal stripes in the intermediate lateral regions of the neural plate. Compare this with the embryo in D′, which was hybridised with a *neurog1* probe (see Blader et al., 1997; Geling et al., 2003). The two transcription patterns are complementary. E,E′ (two somites) and F,F′ (four somites) show that the *her3* domains occupy the regions in which *neurog1* is not transcribed. 2MN and 4MN, motoneurones of rhombomeres 2 and 4. 2SN and 4SN, sensory neurones of rhombomeres 2 and 4. (G,H) Eight-somite (G) and 16-somite stage (H) show that transcripts of *her3* later become undetectable in anterior regions but continue to be expressed in single cells within caudal regions of the developing spinal cord (arrows). (I,J) Double in situ hybridisation with *her3* (blue) and *egr2b* (red), and with *her3* (blue) and *pax2a* (red), respectively. Note that the *her3* domain progressively extends into rhombomere 5 (r5) (arrows). (K,L) In situ hybridisation with *her3* (blue) and *her5* (red), to show that the rostral margin of the *her3* domain corresponds to the anterior border of the midbrain primordium. The *her5* domain is included within the *her3* domain (between arrows). Refer to the text for further details. Anterior is towards the top in all embryos.

the endogenous *her3* mRNA. Another possible explanation, however, is that regulatory elements that suppress transcription within medial and lateral regions of the neural plate in response to Notch-dependent signalling (Haddon et al., 1998; Takke et al., 1999) are missing in the 4.7 kb genomic segment used for the transgene. To test this possibility, the *her3::gal4* transcription pattern was analysed in embryos that had been injected with mRNA encoding the constitutively active Notch receptor, Notch1a-intra. In these embryos, *gal4* transcription was suppressed (Fig. 2D,E). This result indicates that the 4.7 kb genomic fragment does indeed respond to Notch signalling, thus supporting the hypothesis that the *gal4* mRNA is more stable than *her3* transcripts.

her3 is a repressor of the proneural gene *neurog1*

Embryos injected at the two-cell stage with *her3* mRNA encoding the full-length protein were collected at the one- to three-somite stage and tested by in situ hybridisation for the expression of various other genes (*neurog1*, *deltaA*, *islet1* and *elavl3*). After injection of *her3* mRNA, a large proportion of embryos showed a pronounced asymmetry in the neural plate, which was considerably broader within the β-galactosidase-expressing territory (Fig. 3, Table 1). The same effect has been reported after misexpression of a *Xenopus* Notch variant (Coffman et al., 1993), and following misexpression of *deltaD* (Dornseifer et al., 1997) and *her4* (Takke et al., 1999) in the zebrafish. The significance of this effect on the size of the neural primordium is unclear. In most embryos that show an enlargement of the neural plate, there is a concomitant reduction in the numbers of cells expressing markers of primary neurones (*islet1* and *elavl3*, Fig. 3A-C, not shown). As *her3* is not normally expressed in the regions of the neural plate from which primary neurones originate, we asked whether this effect is due directly to the product of the injected *her3* mRNA, or is caused by the products of other genes, the activity of which may depend on Her3, and which are expressed in the territories in which primary neurones arise. The proneural gene *neurog1* elicits ectopic development of *islet1*-positive cells (Blader et al., 1997; Takke et al., 1999) and is repressed by Her4, another member of the hairy-E(spl) family (Takke et al., 1999). Thus, it is conceivable that the reduction in the number of primary neurones seen in the injected embryos is due to repression of *neurog1*. Indeed, probing with *neurog1* of embryos injected with *her3* mRNA revealed that similar proportions of embryos have an enlarged neural plate and a reduced

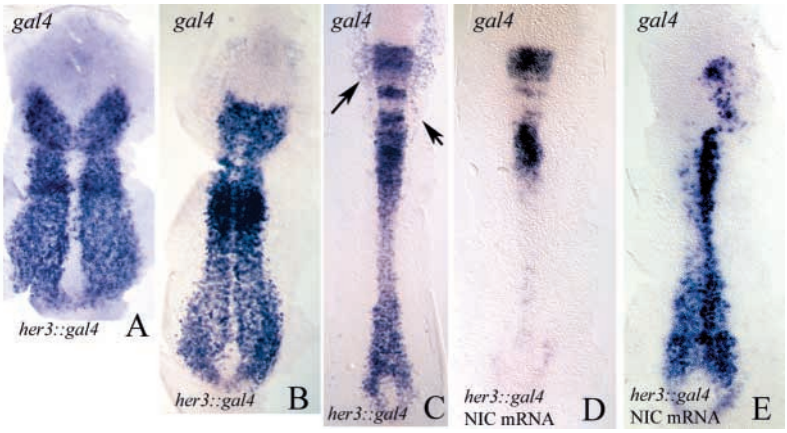

Fig. 2. (A-C) Flat preparations of *her3::gal4* embryos (tail-bud, A; two-somite stage, B; six-somite stage, C) labelled by in situ hybridisation with a *gal4* probe. The *gal4* pattern closely corresponds to the endogenous *her3* transcription pattern. Compare with Fig. 1. The arrows in C indicate areas of *gal4* expression in epidermal tissues. In cranial regions of the developing spinal cord, reporter mRNA persists for longer than do the transcripts of the endogenous *her3* gene (see Fig. 1). (D,E) Two examples of *her3::gal4* embryos injected with mRNA for notch1a-intra and probed for *gal4*. Note that transcription of *her3::gal4* is repressed. Anterior is towards the top.

density of *neurog1* transcripts (Fig. 3D,E). This observation suggests that the effect of misexpression of *her3* on primary neurones is, at least in part, due to repression of *neurog1*. Injection of *her3* mRNA also weakly suppresses transcription of *deltaA* (not shown), a target of *neurog1* (Takke et al., 1999). Gel retardation assays suggest that repression of *neurog1* transcription may be mediated by direct binding of Her3 protein to N-boxes present in the *neurog1* promoter (CT**C ACA AG**C T**CA CAC G**AG CTG) at position –129 relative to the ATG (Fig. 3F) (Blader et al.,

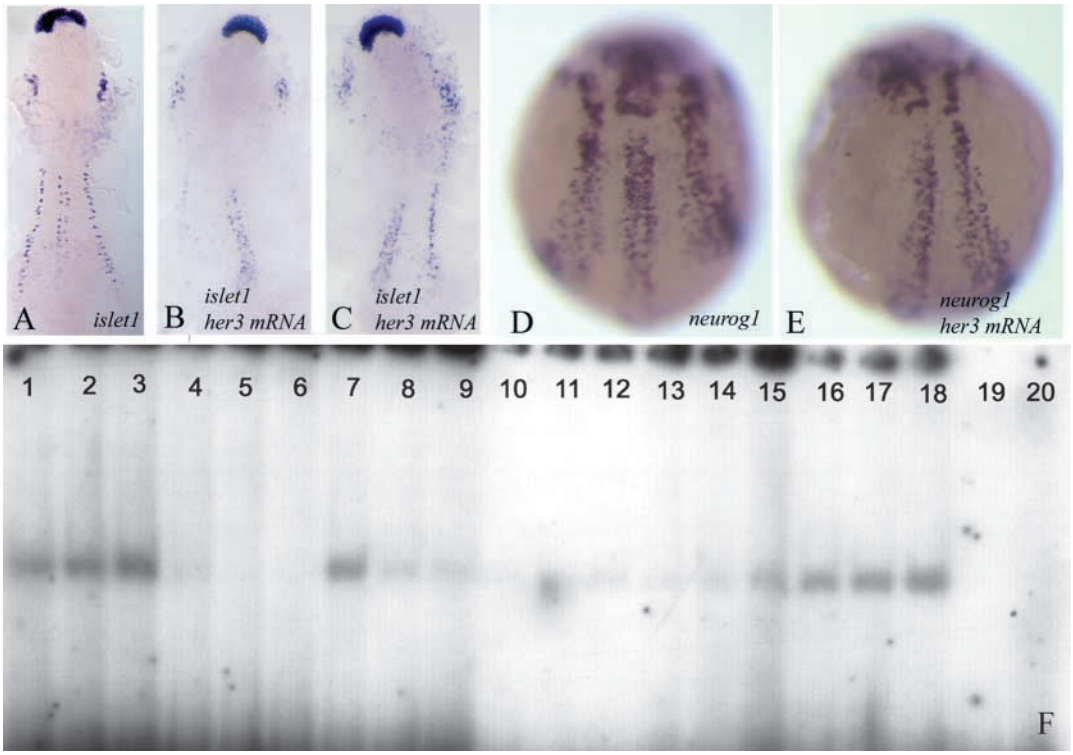

Fig. 3. (A-C) Flat preparations; (D,E) wholemounts of two-somite stage embryos. (A,D) Wild-type embryos; (B,C,E) embryos injected with *her3* mRNA encoding the full-length protein. The embryos were hybridised with *islet1* (A-C) or *neurog1* (D,E). Note that transcription of *islet1* and *neurog1* is reduced in the injected embryos. Refer to the text for further details. Anterior is towards the top. (F) Her3 protein can bind to N boxes in the *neurog1* promoter. Lanes 1-3 were loaded with increasing amounts of Her3 protein (1, 3 and 5 μg, respectively) and with a labelled (5000 cpm) oligonucleotide corresponding to part of the *neurog1* promoter and including two N-boxes (see NP oligonucleotide sequence in Materials and methods). A clear shift in the electrophoretic mobility of the oligonucleotide can be detected. Lanes 4-6 contain 3 μg aliquots of Her3 protein and 5000 cpm of the labelled oligonucleotide, together with increasing amounts of unlabelled oligonucleotide (5, 20 and 40 ng, respectively). Inclusion of non-radioactive homologous competitor prevents the band shift. Lanes 7-9 contain 3 μg of Her3 protein, 5000 cpm of labelled oligonucleotides, and 5, 20 and 40 ng of a heterologous competitor. Binding of the labelled probe is reduced but not completely blocked. Lanes 10-12, 13-15 and 16-18 contain 1, 3 and 5 μg of Her3 protein, and 5000 cpm of labelled oligonucleotides in which both N-boxes (lanes 10-12) [the N1 box (13-15) or the N2 box (16-18)] have been mutated (see Materials and methods). Mutation of both N-boxes reduces the affinity for Her3. As a control, lanes 19 and 20 were loaded with 5000 cpm of the wild-type (19) and mutated (20) oligonucleotides without any Her3 protein.

Table 1. *her3* deletion constructs and effects of injecting mRNA

Construct (300 ng)	Σ	Wild-type phenotype	Normal *neurog1* expression and enlargement of the neural plate (%)	Reduced *neurog1* expression and enlargement of the neural plate (%)
her3	59 (100%)	19%	61%	20%
her3 (600 ng)	63 (100%)	5%	44%	51%
her3 Δ *N*	62 (100%)	42%	48%	10%
her3 Δ *b*	60 (100%)	13%	80%	7%
her3 Δ *HLH*	65 (100%)	17%	71%	12%
her3 Δ *bHLH*	62 (100%)	18%	69%	13%
her3 Δ *bHLH* (600 ng)	108 (100%)	83%	14%	2%
her3 Δ *orange*	61 (100%)	11%	20%	69%
her3 Δ *C*	63 (100%)	13%	16%	71%
her3 Δ *WRPW*	64 (100%)	50%	44%	6%
her3 Δ *WRPW* (600 ng)	90 (100%)	89%	11%	–

2003). Thus, when increasing amounts of Her3 protein are incubated in the presence of oligonucleotides containing N-boxes from the *neurog1* promoter region (see Materials and methods), clear shifts in the electrophoretic mobility of the labelled probe are detectable. The shift can be selectively inhibited either by adding non-radioactive wild-type oligonucleotides, or using oligonucleotides containing mutations in both N-box sequences. In the latter case, the affinity of the nucleotide for Her3 is considerably reduced, whereas mutation of either box has a weaker effect. Furthermore, non-radioactive mutant oligonucleotides do not effectively compete with the labelled wild-type probe for binding of Her3.

Morpholino-mediated gene inactivation induces ectopic *neurog1* transcription

Two different antisense oligonucleotides (morpholinos: MO *her3* and 2 blocker) were designed to inhibit *her3* function by preventing the synthesis of Her3 protein (Nasevicius and Ekker, 2000). In addition, a mismatch morpholino was injected as a control. Whereas the mismatch morpholino did not cause any detectable deviation from the wild-type pattern, both experimental morpholinos, MO *her3* and 2 blocker, had identical effects with a penetrance of over 90%. *neurog1* transcription was activated ectopically within the primordium of the rhombencephalon (Fig. 4A,B). RT-PCR analyses confirmed the presence of additional *neurog1* transcripts in embryos after the injection of the morpholinos (Fig. 4F). In addition to the ectopic expression of *neurog1* in the rhombencephalon, other *neurog1*-positive cells were occasionally seen in other regions of the neural plate. Although it is difficult to assess the functional significance of these latter changes in the *neurog1* pattern of the morpholino injected animals, they may explain the relative abundance of *neurog1* RNA found in the RT-PCR analysis (Fig. 4F).

Double *in situ* hybridisation with *egr2b* showed that ectopic induction of *neurog1* is restricted to rhombomeres 2 and 4 (Fig. 4B), encompassing the region that connects the clusters of motoneurones and sensory neurones (Geling et al., 2003). Ectopic expression of *neurog1* is associated with ectopic activation of a number of genes involved in lateral inhibition (*deltaA*, *deltaD*, *coe2* and *her4*) in the same regions of rhombomeres 2 and 4 (Fig. 4C-E, not shown), and ectopic neurones can be subsequently detected within these same regions (Fig. 4D). The available evidence indicates that all these genes may be activated by Neurog1 (Bally-Cuif et al., 1998; Dubois et al., 1998; Takke et al., 1999; Hans and Campos-Ortega, 2002) and therefore, their ectopic activation in rhombomeres 2 and 4 might well be caused by the ectopic induction of *neurog1*, leading to the formation of ectopic neurones.

As reproducible phenotypic effects of blocking *her3* mRNA translation with morpholinos were rather weak, i.e. restricted to a fairly small region of the neural anlage, whereas the *her3* transcription domain is much broader, we suspected that the efficacy of the injected morpholinos might be compromised. To test whether the morpholinos can completely knock-down *her3* activity, a construct encoding a Her3:gfp fusion was synthesised, and 400 ng/μl mRNA transcribed from this plasmid was injected together with each of the two morpholinos. Another batch of embryos was injected with 400ng/μl *her3:gfp* mRNA without the morpholinos. After injection, embryos showing Texas Red staining were selected, and the number of embryos with GFP-mediated fluorescence was determined between 30% and at 85% epiboly. 100% of the embryos in the control series showed a fluorescent signal (Fig. 5). The simultaneous injection of either morpholino completely eliminated the fluorescent signal. This result suggests that both morpholinos completely inhibit the translation of *her3* RNA.

Autoregulation of *her3*

Injection of *her3* morpholinos results in an increase in the density of *her3* transcripts in all the *her3* expression domains (Fig. 4G). This suggests that Her3 represses transcription of its own gene within the limits of its expression domain. To test whether *her3* is indeed subject to autoregulation, gel retardation assays were carried out. Two different N-boxes are present in the promoter region of the *her3* gene (N1: TC**C AGC AG**A AAG, and N2: CC**C ACA CG**A CCG). Gel retardation assays show that labelled oligonucleotides containing either of these N boxes display a clear shift in electrophoretic mobility (Fig. 4H) following incubation with Her3. The shifts can be inhibited with increasing concentrations of the non-radioactive oligonucleotide. The presence of two or three shifted bands suggests that Her3 may bind to the target DNA in mono-, di- and trimeric forms. A similar inhibition of the DNA binding is observed if oligonucleotides with mutated N-boxes are used (see Materials and methods).

Functional dissection of Her3

To gain insight into the structural requirements for Her3

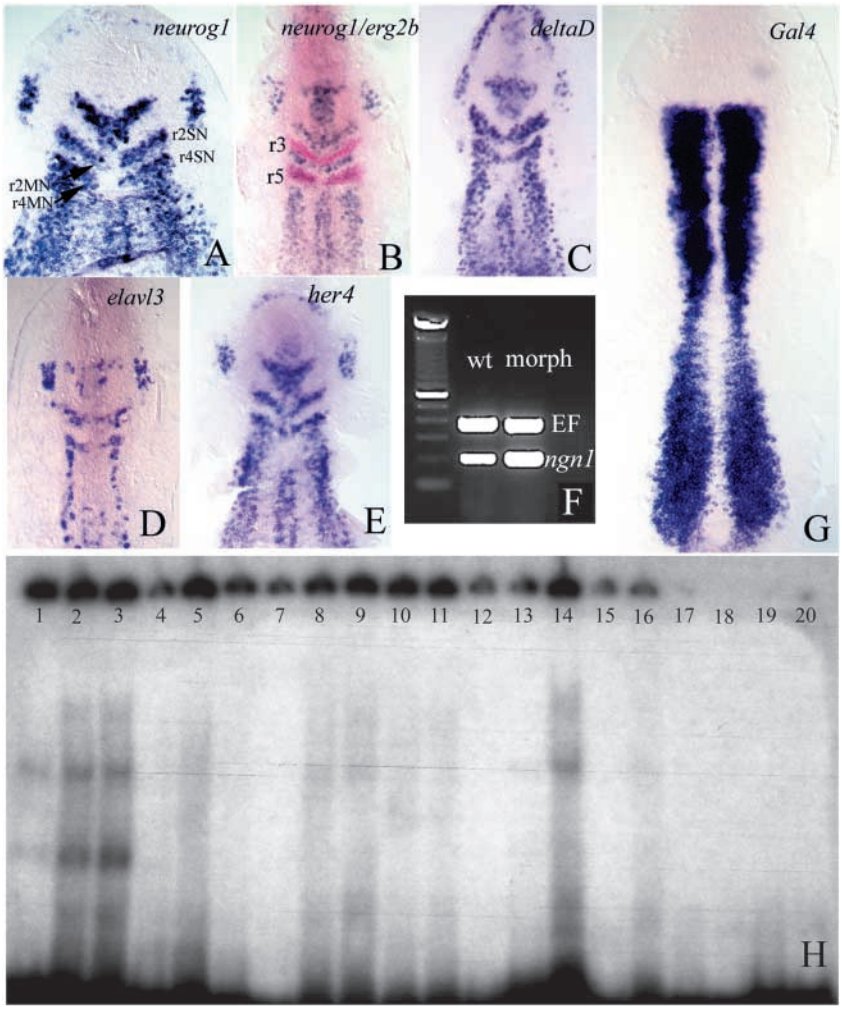

Fig. 4. (A-E) Flat preparations of embryos at the one-somite (A) and two- to three-somite (B-E) stages, which had been injected with morpholino oligonucleotides against *her3*. The embryos were hybridised with the probes indicated. Note the ectopic expression of *neurog1*, *deltaD* and *elavl3* transcripts in the region between 2SN and 2MN, and 4SN and 4MN (see Fig. 1D′). Double in situ hybridisation with *egr2b* (B) shows that ectopic expression is restricted to rhombomeres 2 and 4. (r3, r5: rhombomeres 3 and 5). (F) The density of *her3* transcripts following injection of *her3* morpholinos is higher than in the wild type (compare with Fig. 1F). (D) Wild-type embryo injected with *her3* morpholinos, showing that the density of *her3* transcripts is higher than in the controls (compare with Fig. 1E), suggesting that Her3 regulates its own transcription. (H) Her3 protein can bind to N boxes in the *her3* promoter. Lanes 1-3 contain increasing amounts of Her3 protein (1, 5 and 10 μg, respectively) and 8000 cpm of an oligonucleotide derived from the *her3* promoter that includes the N1 box (see Materials and methods). A clear shift in the electrophoretic mobility of the oligonucleotide can be detected. The presence of several bands suggests binding by Her3 oligomers. Lanes 4-6 contain 3 μg of Her3 protein, 8000 cpm of the labelled N1 oligonucleotide, and increasing amounts of unlabelled oligonucleotide (0.4, 5 and 6 pM, respectively). Binding to the labelled probe is reduced because the unlabelled oligonucleotides compete for Her3. Lanes 7-9 show the same experiment using an oligonucleotide that contains the N2 box (refer to Materials and methods). The shifted band is weaker. Lanes 10-12 show that, also in this case, the non-radioactive oligonucleotide competes for Her3. Lanes 7-9 and 10-12 contain the same amounts of protein and radioactive and non-radioactive DNA as lanes 1-3 and 4-6, respectively. Lanes 13-14 and 15-16 show the effects of mutating either the N1 or the N2 box on band shifting. Lanes contain 1 and 5 μg of Her3 protein and 8000 cpm of the labelled oligonucleotides. As a control, lanes 17-20 contain 8000 cpm of the wild-type (17-18) and mutated (19-20) oligonucleotides, but no Her3 protein.

function, we constructed seven deletion variants of the *her3* cDNA (see Fig. 6), and injected the corresponding mRNAs into embryos at the two-cell stage. Injected embryos were processed for in situ hybridisation with a *neurog1* probe. The effects of mRNA injections were comparable for five of the seven variants, $her3^{\Delta N}$, $her3^{\Delta b}$, $her3^{\Delta bHLH}$, $her3^{\Delta HLH}$ and $her3^{\Delta WRPW}$, which encode derivatives that lack the LCC (low compositional complexity) domain, the basic domain, the bHLH domain, the HLH domain and the C-terminal tetrapeptide WRPW, respectively. All these variants have partially lost the ability to suppress *neurog1* transcription (Table 1). By contrast, the last two variants tested, $her3^{\Delta orange}$ and $her3^{\Delta C}$, which encode products that lack the so-called orange domain (Dawson et al., 1995) and a C-terminal segment, respectively, behave like gain-of-function mutants. Injection of mRNA for either construct leads to a large increase in the number of embryos that show reduced *neurog1* transcription.

Her3 is a transcriptional repressor

The data described in the previous section indicate that Her3 suppresses the transcription of *neurog1*. However, they do not show whether Her3-dependent transcription repression is mediated directly or indirectly, e.g. by activating one or more other genes whose products then suppress transcription of downstream genes. To distinguish between these two alternatives, two constructs encoding C-terminal fusions of Her3 to either the transactivation domain of VP16 (*her3VP16*) or the repressor domain of engrailed (*her3eng*) were synthesised (Fig. 6). Embryos injected with the corresponding mRNAs were probed by in situ hybridisation with *neurog1* and *elavl3* cDNAs after gastrulation. Embryos expressing the Her3eng fusion had fewer primary neurones (Fig. 7B; Table 2) and showed a suppression of *neurog1* transcription (not shown). That is, injection of *her3eng* mRNA had the same effects as the injection of *her3* mRNA. By contrast, embryos expressing the Her3VP16 fusion showed strong ectopic activation of *neurog1* transcription (Fig. 7D). These results are consistent with a direct role for Her3 as a transcriptional repressor.

her3 expression is repressed by Notch signalling

Genes with homology to *E(spl)* have repeatedly been shown to

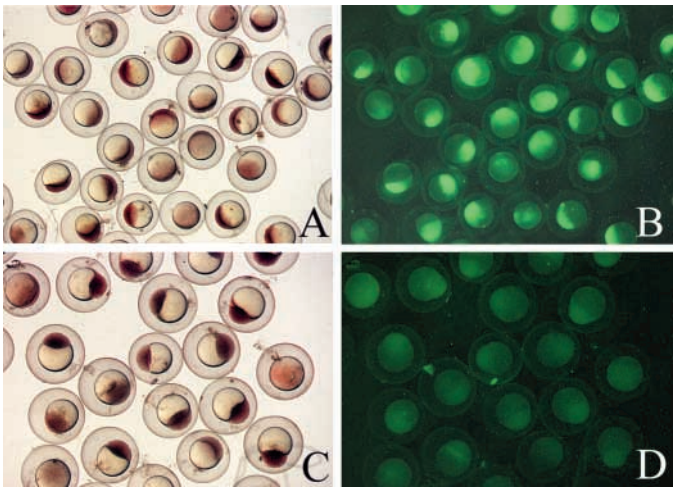

Fig. 5. (A,B) Embryos injected with mRNA encoding Her3:gfp. All embryos show GFP mediated fluorescence. (C,D) Embryos received an injection of mRNA encoding Her3:gfp and the morpholinos oligonucleotides 2 blocker. No embryo shows GFP mediated fluorescence. The same result is obtained injecting the her3 Morpholino. These results indicate that the morpholinos block completely her3 mRNA translation.

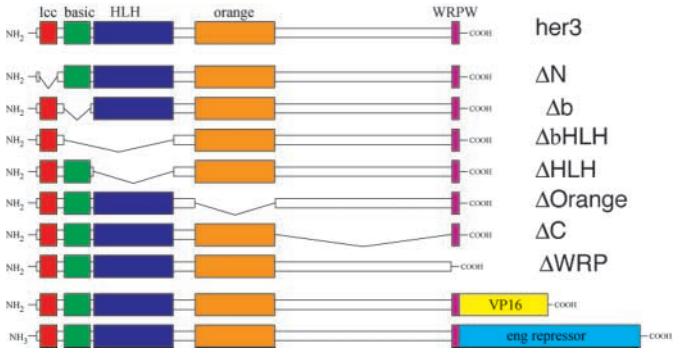

Fig. 6. Primary structures of the products encoded by the deletion derivatives of *her3*, and the *her3VP16* and *her3eng* constructs. Refer to the text for further details.

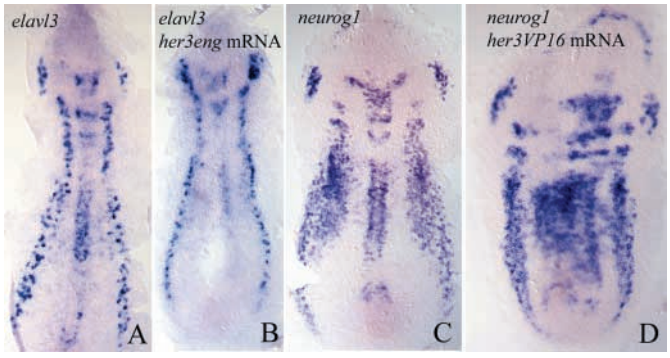

Fig. 7. Expression of *elavl3* (A, three-somite stage) and *neurog1* (C, one-somite stage) in wild-type embryos is compared with the expression of the same genes in embryos that had been injected with *her3eng* (B) or *her3VP16* mRNA (D). Whereas the injection of *her3eng* mRNA leads to loss of primary neurones, *her3VP16* causes ectopic activation of *neurog1*.

be activated by Notch signalling, both in *Drosophila* (Lieber et al., 1993; Jennings et al., 1994; Bailey and Posakony, 1995; Lecourtois and Schweissguth, 1995) and vertebrates (Furukawa et al., 1995; Jarriault et al., 1995; Hsieh et al., 1996; Kopan et al., 1996; Wettstein et al., 1997; Takke and Campos-Ortega, 1999; Takke et al., 1999). Therefore, we tested whether *her3* is also under the control of Notch signalling. We used Gal4-mediated gene misexpression to activate, in the first instance, a constitutively active variant of the zebrafish notch1a receptor (*UAS:myc-notch1a-intra*) (Scheer and Campos-Ortega, 1999; Scheer et al., 2001; Lawson et al., 2001). Crosses were made between heterozygous *hsp70::Gal4* and *UAS:myc-notch1a-intra* individuals, and embryos derived from these crosses were heat-shocked, at the 50% epiboly stage, for 30 minutes at 40°C and allowed to develop until the 1- to 2- to the 5- to 6-somite stage. Fixed embryos were either stained with antibodies against Myc and probed by in situ hybridisation with *her3* cDNA, or processed directly for *her3* in situ hybridisation without anti-Myc staining. *her3* transcription was found to be downregulated, particularly in the mesencephalic/rhombencephalic domain in embryos expressing Myc (Fig. 8A). To test whether the observed repression of *her3* is indirect, i.e. caused by Notch1a-mediated activation of transcriptional repressors, we misexpressed *her4*, another member of the *E(spl)* family that is activated by Notch1a:intra (Takke et al., 1999), by the Gal4-UAS technique and by mRNA injection. First, we crossed *hsp70::Gal4* heterozygotes with *UAS:her4* homozygotes, heat-shocked the progeny embryos at 50% epiboly and analysed them by *her3* in situ hybridisation. In all cases, individual embryos were genotyped by PCR after in situ hybridisation (Fig. 8G). Second, wild-type zygotes were injected with *her4* mRNA and the embryos were then analysed in the same manner. The results were the same in both cases: *her3* transcription was strongly repressed (Fig. 8B,C). It should be noted that stronger repression of *her3* transcription was obtained upon misexpression of *her4* than after misexpression of *notch1a-intra*, suggesting that activation of *her4* is a prerequisite for transcriptional repression of *her3*. *her3* transcription was also repressed after the injection of *neurog1* mRNA (not shown), most probably owing to transcriptional activation of genes that encode some of the links in the feedback loop that regulates lateral inhibition, e.g. *deltaA*, *deltaD* and *her4* (Takke et al., 1999).

To probe further the functional significance of these observations, we analysed *her3* transcription in embryos in which Notch signalling had been perturbed by injecting *deltaDPst* mRNA, which encodes a dominant-negative deltaD variant (Takke et al., 1999) [see Haddon et al. (Haddon et al., 1998), for the effects of the corresponding variant of *Xenopus* Delta, deltastu, in zebrafish]. Embryos injected with *deltaPst* mRNA showed ectopic *her3* transcription in various regions, both within and outside the neural plate (Fig. 8D).

Gel retardation assays suggest that Her4-mediated repression of *her3* occurs by direct binding of Her4 to N-boxes (Tietze et al., 1992; Sasai et al., 1992; Oellers et al., 1994) present in the *her3* promoter (see Materials and methods). Thus, incubation of increasing concentrations of Her4 protein in the presence of oligonucleotides containing N-boxes from the *her3* promoter region results in clear shifts in the electrophoretic mobility of the labelled probes. The shifts

Table 2. Her3 is a transcriptional repressor

Construct (300 ng)	Σ	Wild type	Wild type expression with enlargement of the neural plate	Reduced expression with enlargement of the neural plate	Ectopic expression
her3 VP16	50 (100%)	8%	46%	6%	40%
her3 eng	45 (100%)	2%	65%	33%	0

suggest binding of oligomeric forms of Her4 to the target DNA (Fig. 8H).

We tested to what extent transcription of other zebrafish genes of the *hairy/E(spl)* family, such as *her5* and *her6*, is also dependent on Notch signalling. To do this, we probed embryos from both of the crosses mentioned above (*hsp70::Gal4* to *UAS:myc-notch1a-intra* and *hsp70::Gal4* to *UAS:her4*) with *her5* and *her6* cDNAs. Whereas transcription of *her5* is strongly repressed in embryos from both crosses (Fig. 8E,F), transcription of *her6* seems not to be affected at all (not shown). Therefore, the fact that is a member of the *hairy/E(spl)* family does not necessarily imply that it is activated as a result of Notch signalling.

Discussion

Two main conclusions can be drawn from the work presented above. First, *her3* encodes a zebrafish homologue of the *Drosophila* E(spl) proteins, which represses the transcription of *neurog1* and is a target of Notch1a signalling. Second, in contrast to other members of the *E(spl)* family, the *her3* gene itself is repressed rather than activated by Notch signalling.

Her3 is an E(spl) homologue

The first conclusion is based on structural and functional considerations. Structurally Her3 shows considerable sequence identity to proteins of the E(spl) family in its bHLH domain, the region that binds DNA and is involved in target recognition (Akazawa et al., 1992; Tietze et al., 1992; Oellers et al., 1994). Furthermore, Her3 also exhibits the other characteristics of members of this family, such as the C-terminal tetrapeptide WRPW and the orange domain (Dawson et al., 1995), which corresponds to helix III/IV defined by Knust et al. (Knust et al., 1992). Davis and Turner (Davis and Turner, 2001) classify the hairy-E(spl) proteins, on structural grounds, into four different groups. In their phylogenetic tree, Her3 belongs to the group of E(spl) proteins. With respect to functional criteria, the effects of fusions to the transactivation domain of VP16 and

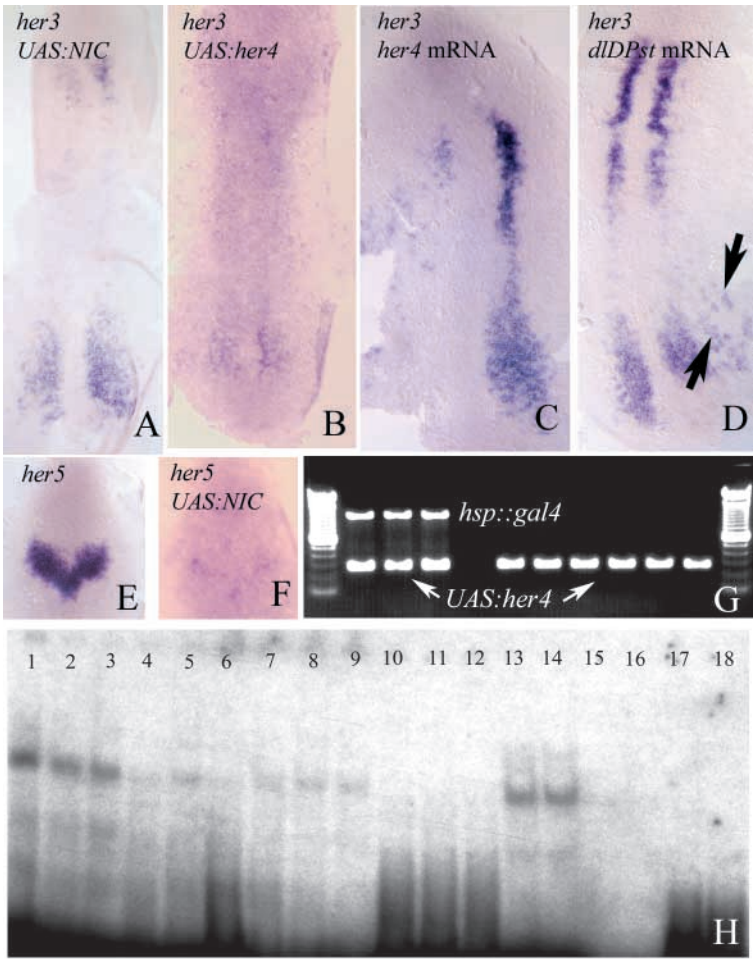

Fig. 8. (A-D) *her3* in situ hybridisation in two-somite embryos. Transcription of *her3* is repressed by Gal4 mediated misexpression of *notch1a-intra* (A) or *her4* (B), and by injection of the *her4* mRNA (C). *her3* expression is also repressed by injection of *neurog1* mRNA (D), probably as an indirect consequence of the activation of *her4* (Takke et al., 1999). (E,F) *her5* in situ hybridisation to two-somite embryos. Transcription is repressed following misexpression of *notch1a-intra* by the Gal4-UAS technique. Misexpression of *her4* represses the transcription of *her5* to the same extent. (G) Genotyping of individual embryos by PCR. Embryos that exhibit transcriptional repression of *her3* (or of *her5*) carry both transgenes (*hsp::gal4* and *UAS:her4*, three first lanes). Phenotypically wild-type embryos carry one transgene. (H) Her4 protein can bind to N boxes in the *her3* promoter. Lanes 1-3 contained increasing amounts of Her3 protein (2, 3 and 4 μg, respectively) and 8000 cpm of an oligonucleotide derived from the *her3* promoter and including the N1 box (Materials and methods). A clear shift in the electrophoretic mobility of the oligonucleotide can be detected. Lanes 4-6 contain 3 μg of Her4 protein, 8000 cpm of the labelled N1 oligonucleotide, and increasing amounts of unlabelled oligonucleotide (1, 2 and 4 pM, respectively). The band shift is competed out. Lanes 7-9 show the same experiment using an oligonucleotide that contains the N2 box (Materials and methods). Binding to the N2 oligonucleotide is weaker. Lanes 10-12 show that, also in this case, binding can be competed with non-radioactive oligonucleotide. Lanes 7-9 and 10-12 contain same amounts of protein and radioactive and non-radioactive DNA as lanes 1-3 and 4-6, respectively. Lanes 13-14 and 15-16 show the effects of mutating either the N1 or the N2 box on the mobility of the labelled probe. Binding to the N1 mutant is still strong. Lanes contain 1 and 5 μg of Her3 protein and 8000 cpm of the labelled oligonucleotides. As a control, lanes 17-20 contain 8000 cpm of the wild-type (17-18) and mutated (19-20) oligonucleotides, without Her4 protein.

the repression domain of Engrailed indicate that Her3 is a transcriptional repressor. Gel retardation assays and deletion analyses support the contention that Her3 represses transcription by binding directly to so-called N-boxes, a major DNA target for the E(spl) proteins (Sasai et al., 1992; Tietze et al., 1992; Oellers et al., 1994) (for a review, see Davis and Turner, 2001).

The variant *her3*$^{\Delta WRPW}$, which encodes a Her3 derivative that lacks the C-terminal tetrapeptide WRPW, reveals an additional element of functional similarity to E(spl). In *Drosophila*, the WRPW motif is essential for the association of hairy-E(spl) proteins with the co-repressor groucho (Wainwright and Ish-Horowicz, 1992; Paroush et al., 1994; Dawson et al., 1995; Fisher et al., 1996; Giebel and Campos-Ortega, 1997), and its removal results in a non-functional polypeptide. Similarly, injections of *her3*$^{\Delta WRPW}$ mRNA show that this variant has partially lost the ability to suppress target gene expression. Similar results have been reported for *her5*, another member of the same protein family (Geling et al., 2003). However, the WRPW tetrapeptide appears to be functionally dispensable in the case of other members of the family. Thus, a Her4 variant lacking the WRPW domain was found to behave like the wild-type protein (Takke et al., 1999).

Finally, we find that *her3*$^{\Delta orange}$ and *her3*$^{\Delta C}$, which encode products that are devoid of the orange domain and of the region between the orange domain and the WRPW motif, respectively, behave like gain-of-function mutants. This conclusion is based on the fact that their expression leads to a more pronounced reduction in *neurog1* transcription than does the wild-type Her3. The deletion derivative encoded by *her3*$^{\Delta C}$ is similar to the product of the *E(spl)*^{D} allele of *Drosophila* (Knust et al., 1987; Klämbt et al., 1989), with the exception of the WRPW-coding region, which is still present in the former and absent in the latter. The Her3$^{\Delta C}$ deletion behaves like the product of *E(spl)*^{D} when the expression of this gene is driven by Gal4 in a Gal4-UAS experiment (Giebel and Campos-Ortega, 1997). When expressed under the control of Gal4, E(spl)^{D} behaves like a dominant-negative variant. Dominant-negative effects were interpreted as being due to inhibition of the function of the endogenous E(spl) proteins by competitive or neutralising interactions with the truncated proteins (Giebel and Campos-Ortega, 1997). As the gel shift analyses suggest that Her3 may bind to DNA as dimers or trimers (Fig. 4F), association of the endogenous proteins with those supplied exogenously might also explain the gain-of-function and dominant-negative effects seen with the Her3 variants. In *Drosophila*, deletion of either the orange domain or the WRPW leads to strong impairment of the E(spl) function (Wainwright and Ish-Horowitz, 1992; Schrons et al., 1992; Dawson et al., 1995; Fisher et al., 1996). It is assumed that the region between the orange domain and the WRPW motif may be required as a spacer to accommodate Groucho, so that its removal prevents the association of the WRPW with Groucho (Dawson et al., 1995; Giebel and Campos-Ortega, 1997).

Taken together, the results described above suggest that Her3 binds directly to DNA and acts as a transcriptional repressor. However, mechanisms of transcriptional repression other than direct DNA binding can not be excluded. Thus, in addition to the bHLH domain required for DNA binding, both the orange domain and the WRPW tetrapeptide appear to play a prominent functional role. In fact, despite the abundance of data available (see Davis and Turner, 2001), it remains difficult to make generalisations with regard to how E(spl) proteins function.

neurog1 is repressed by Her3

Our present results point to the proneural gene *neurog1* as one of the targets of Her3 function. Indeed, *neurog1* transcription, as well as that of several target genes of Neurog1, is repressed following injection of *her3* mRNA. Gel retardation assays show that *neurog1* repression might be due to direct binding of Her3 to N-boxes in the *neurog1* promoter. This function is clearly compatible with the known function of members of the E(spl) family as strong suppressors of proneural gene function. Our data do not allow us to decide whether Her3 acts on *deltaA*, *islet1* and *elavl3* directly or via *neurog1*. Injection of morpholinos, either MO *her3* or 2 blocker, leads to ectopic expression of *neurog1* and subsequent induction of a number of targets of Neurog1, as for example *deltaA*, *deltaD*, *coe2* and *her4*, and the ectopic induction of primary neurone development.

However, ectopic induction of *neurog1* following morpholino injections is restricted to rhombomeres 2 and 4, whereas the remaining domains of *neurog1* expression remain unaffected. As injection of *her3* mRNA affects *neurog1* transcription in all its expression domains, the relatively mild effect of morpholino injection is a striking result. Although we do not yet have a satisfactory explanation for this observation, two possible hypotheses can be considered. First, it is conceivable that under normal conditions regulatory interactions between Her3 and *neurog1* are restricted to the regions of rhombomeres 2 and 4 that connects r2MN and r2SN, and r4MN and r4SN, respectively. In this case, the remaining expression domains of *her3* would not manifest regulatory interactions with *neurog1*. However, in view of the complementary nature of the transcription patterns of *her3* and *neurog1*, this seems rather improbable. The second hypothesis is based on functional redundancy of the genes of the *E(spl)* family. There are several examples of members of this family being expressed in overlapping domains, both in *Drosophila* and in vertebrates. In *Drosophila*, six out of the seven E(spl)-C genes show identical expression pattern in the neuroectoderm (Knust et al., 1987; Knust et al., 1992; Klämbt et al., 1989); in the zebrafish, *her4* and *her2* exhibit virtually identical expression patterns in the neural plate (Takke et al., 1999; Takke and Campos-Ortega, unpublished), *her1* and *her7* within the presomitic mesoderm (Oates and Ho, 2002; Gajewski et al., 2003), and at least one other gene of the *her* family display the same expression pattern as *her5* (L. Bally-Cuif, personal communication) (Müller et al., 1996; Bally-Cuif et al., 2000). The overlap of their expression domains and their common function explains why the genes of the *Drosophila* E(spl)-C show marked functional redundancy in early neurogenesis (reviewed by Campos-Ortega, 1993) (see also Delidakis et al., 1991; Schrons et al., 1992). A similar redundancy can be invoked to explain why *neurog1* transcription outside the region delimited by rhombomeres 2 and 4 is not affected, at least to levels detectable by in situ hybridisation, by misexpression of *her3*. However, as RT-PCR shows a considerable increase in the amount of *neurog1* RNA (Fig. 4G), transcription of *neurog1* might in fact be affected in all its domains, albeit either below levels detectable by conventional in situ techniques, or with low penetrance. We

have mentioned that, in addition to the rhombencephalon, *neurog1*-positive cells are occasionally seen in other regions of the neural plate. This would require the expression of other *her* genes in the *her3* domains under discussion, for which there is as yet no evidence. Therefore, for the time being our results do not allow us to decide between the two possibilities.

her3 is repressed by Notch signalling

Despite all the similarities between Her3 and the *Drosophila* E(spl) proteins, there is one important difference, which concerns the response to signalling through Notch1a. We find that *her3* transcription is repressed by Notch signalling, in clear contrast to the behaviour of several other members of the E(spl) family, both in *Drosophila* and in vertebrates, which are activated (Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Tamura et al., 1995; Hsieh et al., 1996; Kopan et al., 1996; Wettstein et al., 1997; Takke et al., 1999; Takke and Campos-Ortega, 1999) (for a review, see Lewis, 1996). This conclusion is based on the results of three types of experiment: (1) the downregulation of *her3* transcription observed following misexpression, both by mRNA injection and by the Gal4:UAS technique, of *notch1a-intra* and *her4*; (2) the upregulation of *her3* transcription in embryos expressing a dominant-negative variant of DeltaD; and (3) the downregulation of *gal4* transcription in *her3::gal4* embryos, in which *gal4* is driven by the regulatory region of *her3*. Moreover, our results suggest that transcriptional repression of *her3* is mediated by direct binding of Her4 to the N-boxes present in the promoter of *her3*. The functional significance of the Notch1a-mediated repression of *her3* remains unclear. As Her3 represses *neurog1* expression, being thus required to block neurogenesis, one possibility is that Notch signalling promotes, rather than blocks, neurogenesis. However, this possibility requires experimental support, which, for the time being, is still missing.

The results obtained using the Gal4-UAS technique for targeted gene misexpression, i.e. using both *UAS:notch1a-intra* and *UAS:her4*, suggest that Notch1a signalling suppresses transcription of the *her5* gene as well, whereas transcription of *her6*, yet another zebrafish *hairy-E(spl)*-related gene, remains unchanged under the same experimental conditions. Therefore, Notch1a signalling allows us to classify the Her genes into three different groups, depending on whether transcription is activated (*her1* and *her4*) (Takke and Campos-Ortega, 1999; Takke et al., 1999), repressed (*her3* and *her5*; present results) or remains unaffected (*her6*; present results). Whereas members of the first and the second group repress neurogenesis in zebrafish as well as rodents, members of the third group promote neurogenesis, at least in mice (Hes6) (Bae et al., 2000; Koyano-Nakagawa et al., 2000).

her5 might well be a special case among the Her genes. Thus, Geling et al. (Geling et al., 2003) refer to unpublished data indicating that *her5* within the neural plate is independent of Notch signalling in vivo. However, we find that *her5*, like *her3*, is downregulated by Notch1a-intra, thus suggesting that *her5* can be a target of Notch signalling under conditions of ectopic Notch expression. Altogether, these results suggest that Notch1a-intra can repress both *her3* and *her5* expression, but only *her3* expression is activated by inhibition of Notch1a-intra, supporting the contention that *her3* is a target of Notch signalling in the embryo in vivo, and hence a new player in the regulation of neurogenesis in zebrafish.

A case of Notch-mediated repression of Hes genes has previously been described in the mouse. Using an in vitro assay, Beatus et al. (Beatus et al., 1999) found that misexpression of the intracellular domain (IC) of Notch 3 represses transcriptional activation of Hes genes mediated by the intracellular domain of Notch1. If cells from any of a number of different lines are transfected with Notch1 IC together with a much larger amount of Notch3 IC, Notch1 IC-mediated transcriptional activation of Hes1 and Hes5 is repressed. Moreover, Hes5 transcription is repressed in vivo by Notch3 IC when expression is driven by the nestin promoter.

Formally, the effect of Notch3 IC signalling on Hes5 is the same as that of Notch1a on *her3*. However, the mechanism of transcriptional repression may not be the same in each case. The promoters of both Hes1 and Hes5 contain RBP-Jκ [Su(H)] binding sites and can be activated by Notch1 IC. Beatus et al. (Beatus et al., 1999) suggest that Notch3 IC may either compete with Notch1 IC for access to RBP-Jκ; or, alternatively, as Notch3 IC cannot activate Hes genes, it may compete with Notch 1 IC for a co-factor present in limited amounts. In the case of *her3*, sequence comparisons uncover a single high-affinity Su(H)-binding site in the 4.7 kb genomic fragment that was used here to drive spatially regulated transcription of *her3*, and has been shown to contain Notch responsive elements. Because our results suggest that Notch1a cannot activate *her3*, it is not clear what role this binding site might play. Given that we have not tested whether other Notch receptors can activate *her3*, we cannot exclude the possibility that a mechanism like the one proposed by Beatus et al. (Beatus et al., 1999) is involved in the repression of *her3* transcription. However, the available data suggest that *her3* repression is caused by the activation of *her4* by Notch1a signals, and by binding of the Her4 protein to the N-boxes present in the *her3* promoter. This hypothesis is supported by the observation, mentioned above, that *her4* transcription is activated by Notch1a signalling probably via Su(H) binding to several sites in the promoter. Consequently, the mechanism would be different from that in mouse.

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Table S1. Sequence of the primers

SP6	5′-CTTGATTTAGGTGACACTATAG-3′
her3f1	5′-ATTT**GAATTC**ATGGCTGCAGCATCCAAC-3′
her3f2	5′-AGTGCG**GAATTC**ATGAAACCCCAAAACGTTAAA-3′
her3f3	5′-AAAAGA**GGATCC**CGTATAAATAAATGTTTG-3′
her3f4	5′-GCTTGT**GGATCC**GCTGAATATCACGCTGGT-3′
her3f5	5′-CACAAT**GGATCC**CCTGATTTCAGTACCGTG-3′
her3r1	5′-GCAGTC**TCTAGA**CTACCAAGGTCGCCAATA-3′
her3r2	5′-CTTTTT**GGATCC**TTGGGGTTTTGCCGTCGC-3′
her3r3	5′-ATTTAT**GGATCC**CCTTCTTTTTTTCTCCATC-3′
her3r4	5′-GTGATA**GGATCC**AGAGTCACAAGCTTTGGAC-3′
her3r5	5′-ATCGCT**TCTAGA**CTACCAAGGTCGCCAAACTCTATTGT-GGTTAAGACC-3′
her3r6	5′-CTACCA**TCTAGA**CTAATAATTCTGTTTGAAAATG-3′
her3fusion	5′-CATT**GGATCC**CCAAGGTCGCCAATAATT-3′
her3for	5′-CATTTAGGATCCATGGCTGCAGCATCCAAC-3′
her3rev	5′-GTCATGAATTCGCCAAGGTCGCCAATAATT-3′

Table S2. Primer combinations

Construct	5′-primer/3′-primer	5′-primer/3′-primer
her3	*her3f1/her3r1*	-
her3ΔN	*her3f2/her3r1*	-
her3Δb	*SP6/her3r2*	*her3f3/her3r1*
her3ΔHLH	*SP6/her3r3*	*her3f4/her3r1*
her3ΔbHLH	*SP6/her3r2*	*her3f4/her3r1*
her3Δorange	*SP6/her3r4*	*her3f5/her3r1*
her3ΔC	*SP6/her3r5*	-
her3ΔWRPW	*SP6/her3r6*	-
her3:VP16 and *her3:eng*	*SP6/her3* fusion	-
pCS2+her3:gfp	*her3for/her3rev*	-
Primers for RT-PCR		
her3 upstream	5′-CGCAAGCGAAAACTGGAAAAGGC;	
her3 downstream	5′-GTCAGCATGATGGAGCGGGAATC;	
neurog1 upstream	5′-ACGTCGTGAAGAAGAACCGCAGG;	
neurog1 downstream	5′-CCCTGCTTCTGGTCTGCGATCCG;	
EF upstream	5′-GCCCCTGCCAATGTA;	
EF downstream	5′-GGGCTTGCCAGGGAC	

Table S3. Sequence of *pCS2her3-5′EGFP* and location of the morpholino sequences

CGAATTCCGTTGCTGTCGCCGTTGCTGNCCCCTTTGCTGTCGGTCCTTATTCATCT
GGATTTTTTAACACACTGAGCATTTAAGGACAATGGCTGCAGCATCCAACAGTG
CGGCGACGGCAAAACCCCAAAACGTTAAAAAGGTTTCAAAACCACTGATGGAGA
AAAAAAGAAGGGCGCGTATAAATAAATGTTTGAATCAATTAAAGAGCCTTCTGG
AAAGCGCCTGTTCTAACAATATCCGCAAGCGAAAACTGGAAAAGGCTGACATTCT
GGAGCTCACTGTGAAACATCTGAGGCATCTGCAAAATACGAAAAGAGGACTGTC
CAAAGCTTGTGACTCTGCTGAATATCACGCTGGTTACAGAAGTTGTCTCAACACT
GTAAGTCATTATCTACGAGCGTCGGACACAGACAGAGATTCCCGCTCCATCATGC
TGACAAATCTTACGAGCGGTCTTAACCACAATAGAGTTCCTGATTTCAGTACCGT
GGAGAGCGATCCCGCATTGATCTTTACACTACCAAGTACACTTCGACGACCACAC
AAAGTTCCCATTAGAACAGACGTCTCTTATTCCAGCTTTCAACAAACTGCAGAGA
GAAAAGTTTGCTTAATGCCCAAGAGGACTGAGATTGGTGATAGCGACAGAATGT
CATTGGACGCAGCTCTTAGGAGCCAGGAATCCAAAAAGGCAGAAACCACACACT
TCAGACCTAAAGATTTAAAGGTCATTGAGTGCTGCATTTTCAAACAGAATTATTG
GCGACCTTGGCGAATTCAAGNGGGGGCCGGTCGCCACCATGGTGAGCAAGGGCG
AGGAGCTGTTCACC

A, h3MO
A, 2T-Blocker
The yellow ATG is the start for EGFP