**INTRODUCTION**

In *Drosophila*, the so-called neurogenic genes are involved in cell fate decisions. In the neuroectoderm and the developing epidermis their products mediate lateral inhibition to restrict neural competence to individual cells in so-called proneural cell clusters. Particularly important are Delta, Notch, Suppressor of Hairless, and the genes of the Enhancer of split complex (E(SPL)-C), which participate in sending, receiving and processing regulatory signals (Technau and Campos-Ortega, 1987; Heitzler and Simpson, 1991; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Bailey and Posakony, 1995; Lecourtois and Schweissguth, 1995). Homologues of the neurogenic genes have been cloned in many different animal species, from *Caenorhabditis elegans* to human (see reviews in Lardelli et al., 1995; Westin and Lardelli, 1997; Greenwald, 1998). In *Drosophila*, the E(SPL)-C (Lehmann et al., 1987) comprises seven genes which encode transcription factors of the basic helix-loop-helix (bHLH) family (Knust et al., 1987, 1992; Klämbt et al., 1989; Delidakis and Artavanis-Tsakonas, 1992). The products of the E(SPL)-C genes are structurally related to those of the *Drosophila* genes hairy (Rushlow et al., 1989) and deadpan (Bier et al., 1992), the zebrafish genes her1 and her5 (Müller et al., 1996a,b), the chicken hairy homologues ch-hairy-1 and ch-hairy-4 (Palmeirim et al., 1997; Henrique et al., 1997), the rat proteins HES-1, HES-3, and HES-5 (Sasai et al., 1992; Akazawa et al., 1992), and others. There is evidence that the carboxy-terminal tetrapeptide WRPW of the hairy-E(spl) proteins is required to form protein complexes with the GROUCHO protein which act as repressors in both neurogenesis and segmentation (Paroush et al., 1994; Fisher et al., 1996). However, the WRPW domain of the HAIRY protein is not necessary for the repression of SCUTE activity that occurs during the process of sex determination in *Drosophila* (Dawson et al., 1995).

We describe here a zebrafish *E(spl)* homologue, *her4*, which acts as a target for NOTCH-mediated signals during primary neurogenesis. Misexpression of *her4* following RNA injections leads to a reduction in the number of primary neurons. These results suggest that *her4* acts as a target of notch-mediated signals that regulate primary neurogenesis.

**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. (1995).
Molecular cloning of her4

PCR using degenerate primers was performed on reverse-transcribed total RNA from zebrafish embryos at the 90% epiboly stage. PCR fragments encoding peptides with similarity to *Drosophila* bHLH proteins were used to screen a zebrafish cDNA library prepared in AZAP (Stratagene) from 3-15 h (hours of development) zebrafish embryos (gift from C. Fromental-Ramain and P. Chambon, Strasbourg). her4 was isolated from these clones. The accession number for the her4 sequence is X97332.

RNA injections

The region encoding the extracellular domain and the transmembrane domain of NOTCH1 was removed from the *notch1* cDNA, and the region encoding the intracellular domain was subcloned into the myc-tag CS2\(^{+}\) expression vector, pc2SM-NIC (Turner and Weintraub, 1994). her4 and *groucho1* cDNAs were also subcloned into the CS2\(^{+}\) vector; in the case of her4 the complete coding region was amplified by PCR before subcloning. The *ngn1* cDNA, cloned in CS2\(^{+}\) and in pBS, was kindly provided by U. Strähle and P. Blader (Strasbourg).

Six constructs encoding her4\(\beta\) were made. pc2S-her4\(\Delta\)Pst was cloned by exciting the 3’ PstI-XhoI fragment from the full-length pc2S-her4 construct. For pc2SMT-her4\(\Delta\)D(Nco) the NcoI-XhoI fragment of the *her4* cDNA was cloned in frame into the pc2SMT vector. Three further constructs were cloned by PCR using the 5’ primer GAACGTAACTACAGACACAG and 3’ primer AGAGCTTACAGA ATGCAGGATT and different 3’ primers. The construct pc2SMT-her4\(\Delta\)D(5tag) encodes a peptide with a myc-tag but lacking the basic domain, and was cloned using the 3’ primer CATGAAATCTACAGGATGTCCAGAATGC; after digestion with TagI this PCR product was inserted in frame into the pc2SMT vector. The construct pc2S-her4\(\Delta\)D encoding the same HER 4 peptide as the previous construct but lacking the myc tag was cloned by PCR using the primers 5’ CAGAGGCAATGACACAGGAC and 3’ CATGAAATCTACAGGATGTCCAGAATGC. After digestion with TagI all constructs were sequenced prior to RNA synthesis.

Capped RNA was synthesized in vitro by transcription with SP6 polymerase from the constructs described above or from a pc2S2-nucleic β-galactosidase (npgal) 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.

RT-PCR

Total RNA was extracted from 50 normal embryos, or 50 embryos injected with either *notch-intra* or *neurogenin1* RT, using the RNA-Clean\textsuperscript(TM) System (Angewandte Gentechnologie Systeme GmbH). Before precipitation the RNA was treated with 2 U of DNase (Boehringer Mannheim) for 30 minutes. 2 U of DNase was reverse transcribed using Superscript-RT (Gibco-BRL) plus 100 ng of random hexamers (Boehringer Mannheim) in a 20 μl reaction, and 0.5 μl of this reaction was subjected to PCR. As internal control we used primers that amplified a 400 bp fragment of the elongation factor eIF4a. After 3 minutes at 95°C 30 amplification cycles were carried out: 1 minute at 95°C, 1 minute at 58°C and 1 minute at 72°C, with a final extension step for 10 minutes at 72°C.

Primers:
- her4 upstream: CCTGCAATCAGGGATCAATCACGC;
- downstream: CGATGCTGTGATGGTCCGTCCGC;
- dlD upstream: CCCGCAATGGGACGACATATAGTACG;
- downstream: GCATTCCACTGATGGATCTGC;
- EF upstream: GCCCTGGCCAATGTA;
- downstream: GGCGCTTGCCAGGGAC.

In situ hybridization and histological methods

Hybridization of digoxigenin-labelled RNA probes to embryo whole-mounts was performed as described by Bierkamp and Campos-Ortega (1993). Digoxigenin-labelled probes were prepared using RNA labelling kits (Boehringer Mannheim). Embryos injected with RNA were prepared for in situ hybridization and for antibody staining, as described by Dornseifer et al. (1997). For sectioning, embryos were embedded in Araldite (Serva). Plastic sections (3-5 μm) were cut on a Reichert Urtrotome and observed with an Axioskop microscope (Zeiss).

RESULTS

PCR fragments obtained using degenerate primers directed against the bHLH domain and the carboxy-terminal WRPW, were subcloned, sequenced and used as probes to screen a zebrafish cDNA library. Six cDNAs encoding proteins of the HAIRY-E(SPL) family, named *her* (for hairy-Enhancer of split related) 1-6, were partially characterized (v. Weiszäcker, 1994). The *her4* cDNA 13.1a comprises a 459-nucleotide open reading frame encoding a protein of 152 amino acids with all the features characteristic of the *Drosophila* HAIRY-E(SPL) family. *her4* shows the highest degree of similarity to mouse *hes5* (64%) and slightly less (60%) to the *Drosophila* HLH-m3 gene. Within the bHLH domain this similarity increases to 84% for *hes5* and 70% for HLH-m3 (Bestfit, GCG programme).

The expression pattern of her4 during early zebrafish embryogenesis

Expression of *her4* RNA was examined by whole-mount in situ hybridization. The earliest expression was detected at about 70% epiboly in the marginal zone on either side of the dorsal midline, which is itself devoid of *her4* transcripts (Fig. 1A). As gastrulation proceeds, this domain also becomes manifest in the hypoblast, and two longitudinal rows of cells expressing *her4* appear adjacent to the notochord, extending towards the animal pole, at about 90% epiboly (Fig. 1B). After closure of the stomodeum the notochord is flanked by *her4*-expressing cells (Fig. 1J). *her4* expression in these cells persists in the 24 h embryo.

*her4* expression within the epiblast starts at about 90% epiboly. Two epiblastic transcription domains appear on either side of the dorsal midline (Fig. 1C-F). One is lateral and elongates rostrocaudally as development progresses, the other domain is oriented transversally, and somewhat obliquely, soon adopting a V-shaped arrangement, and is located in the prospective mesencephalic region (Fig. 1C-G). At the tailbud stage, two initially distinct expression stripes appear laterally, one called intermediate is slightly medial to the other, called lateral. However, during later stages of neurulation intermediate and lateral stripes widen at cranial levels of the spinal cord and meet and fuse to form apparently continuous longitudinal stripes (Fig. 1D-G). Still a third stripe of *her4*-expressing cells becomes visible in 1-somite stage embryos extending across the midline of the neural plate (Fig. 1G). The lateral stripes extend further caudally and surround the tailbud. Similar longitudinal expression domains are observed for *notch1*, *groucho1* and *id6* (Bierkamp and Campos-Ortega, 1993; Wülbeck and Campos-Ortega, 1997; Sawai and Campos-Ortega, 1997), as well as for *deltaA* (Appel and Eisen, 1998; Haddon et al., 1998), *deltaB* (Haddon et al., 1998) and *deltaD* (Dornseifer et al., 1997; Haddon et al., 1998), and for *neurogenin1* (Blader et al., 1997; see below). The longitudinal
stripes actually delineate the regions in which primary neurogenesis takes place, i.e., where the first islet-1-expressing cells arise at about 12 hpf.

In addition to the domains within myelencephalic levels of the neural plate, her4 is also transcribed within the primordia of pros-, mes- and metencephalon. The earliest expression domain in these regions is V-shaped (Fig. 1C), as mentioned above, and appears at 90% epiboly at the level of the prospective mesencephalon. In addition, several cell clusters begin to transcribe her4 in unidentified regions of the primordia of pros-, mes- and metencephalon (Fig. 1C-F).

From the tailbud stage on, and during somitogenesis, her4 is transcribed within the presomitic mesoderm, restricted to the region immediately adjacent to the last somites formed, and it is initially weak; however, during later stages, expression is much more intense and the domain forms conspicuous presomitic bands (Fig. 1H).

**Misexpression of her4 blocks primary neurogenesis**

Embryos injected at the two-cell stage with her4 RNA encoding the full-length protein were collected at the 4- to 6-somite stage and probed for islet-1 expression. No modification of the islet-1 transcription pattern was observed following injections of lacZ RNA alone, even at high concentrations (Table 1; Fig. 2A). Accordingly, in all experiments lacZ RNA was injected at low concentration together with the RNA to be tested; β-galactosidase was detected by antibody staining. Since embryos which were negative for β-galactosidase also showed no detectable effects of injection, we assumed that β-galactosidase-expressing cells also express the products of the co-injected RNAs. Therefore, the term ‘injected embryos’ refers to those which expressed β-galactosidase.

Injection of her4 RNA caused defects in the islet-1 expression pattern, ranging from reduction to complete lack of primary neurons within the territory of β-galactosidase expression (Fig. 2B,C; Table 1). Since GROUCHO is thought to function as a co-repressor together with E(SPL)-C proteins in various processes in Drosophila (Paroush et al., 1994; Fisher et al., 1996), we also injected her4 RNA together with RNA encoding the zebrafish homologue GROUCHO2 (Wülbeck and Campos-Ortega, 1997). Suppression of islet-1 expression was not significantly enhanced (t-test); misexpression of groucho2 alone did not affect the number of islet-1 cells either (Table 1). However, a high number of these embryos showed a striking asymmetry within the neural plate, which was considerably broader within the β-galactosidase-expressing territory (Fig. 2D). In most of the embryos injected with her4 RNA alone, the width of the neural plate was also enlarged, as judged from the position of neuronal markers (Fig. 2B,C). Cross sections of 24 h embryos showed that this

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**Fig. 1. Distribution of her4 transcripts revealed by whole-mount in situ hybridization.**

(A) 70% epiboly. her4 expression appears in the epibolic margin, the midline is devoid of transcript. (B) 90% epiboly. A row of hypoblastic her4-expressing cells is visible on either side of the midline (arrows). (C) 100% epiboly. her4 expression in two lateral stripes in the neural plate (is) and in a V-shaped expression domain (asterisk) in the prospective brain region; the hypoblastic expression domains elongate anteriorly. (D) Flat preparation of a tailbud-stage embryo. Expression domains in the neural plate comprise two lateral stripes, one in an intermediate position (is) and one further lateral and caudal (ls), which will fuse during later stages of development; in addition there is a medial stripe in the neural plate which overlies the hypoblastic expression domain. (E) 1-somite stage. The intermediate and lateral expression domains are still distinct. (F) 2-somite stage. Intermediate and lateral expression domains have fused and the medial stripe has elongated. her4 transcription in the primordium of the trigeminal ganglion (trg) becomes visible. (G) 5-somite stage. (H) 24 h embryo. her4 transcription is detectable in two bands in the presomitic mesoderm. (I) Transverse section through the 90% epiboly embryo shown in B, which reveals the hypoblastic expression (arrows). (J) Transverse section through a 6-somite embryo, showing the lateral and medial expression domains in the neural plate, and the hypoblastic expression in the adaxial mesoderm.
enlargement of the neural plate leads to a similar enlargement of the neural tube (Fig. 2G,H). Similar results have been reported by Coffman et al. (1993) using a Xenopus Notch variant.

A functional dissection of HER4

To gain insight into the structural requirements for her4 function, we constructed six deletion variants of the her4 cDNA and injected the corresponding RNAs into embryos (Fig. 3; Table 2). her4DWRPW, encoding a peptide which lacks the carboxy-terminal WRPW, behaved like the wild type. her4Dps encodes a terminal truncation starting at the putative helix IV. This truncation closely corresponds to the mutation found in the E(spl)D allele of Drosophila (Klämbt et al., 1989), which acts as a gain-of-function allele (Knust et al., 1987), and is identical to a variant that acts as a dominant negative in a Gal4-UAS experiment (Giebel and Campos-Ortega, 1997). However, the peptide encoded by her4Dps was apparently non-functional under the present conditions. Injection of her4Dc+WRPW RNA, whose translational product lacks the helix C. T. akke and others

Table 1. The effects of lacZ, her4 and groucho2 RNA injections on expression of islet-1, deltaA and deltaD and ngn1

<table>
<thead>
<tr>
<th>RNA injected (concentration)</th>
<th>Hybridization probe</th>
<th>No. of embryos injected</th>
<th>No. of embryos expressing β-gal</th>
<th>No. of embryos with enhanced expression</th>
<th>No. of embryos with reduced expression</th>
<th>No. of embryos with wild-type expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacZ (1 μg/μl)</td>
<td>islet-1</td>
<td>104</td>
<td>93</td>
<td>0</td>
<td>0</td>
<td>93 (100%)*</td>
</tr>
<tr>
<td>her4 (200 ng/μl)+lacZ (40 ng/μl)</td>
<td>islet-1</td>
<td>222</td>
<td>114</td>
<td>0</td>
<td>81 (71%)*</td>
<td>33 (29%)*</td>
</tr>
<tr>
<td>deltaD</td>
<td>deltaA</td>
<td>82</td>
<td>39</td>
<td>0</td>
<td>34 (87%)*</td>
<td>5 (13%)*</td>
</tr>
<tr>
<td>ngn1</td>
<td></td>
<td>63</td>
<td>31</td>
<td>0</td>
<td>22 (71%)*</td>
<td>9 (29%)*</td>
</tr>
<tr>
<td>groucho2 (400 ng/μl)+lacZ (40 ng/μl)</td>
<td>islet-1</td>
<td>65</td>
<td>58</td>
<td>0</td>
<td>1 (2%)*</td>
<td>57 (98%)*</td>
</tr>
<tr>
<td>her4 (200 ng/μl)+groucho2 (100 ng/μl)+lacZ (40 ng/μl)</td>
<td>islet-1</td>
<td>163</td>
<td>63</td>
<td>0</td>
<td>53 (84%)*</td>
<td>10 (16%)*</td>
</tr>
</tbody>
</table>

*Percentage relative to the number of embryos that express β-gal.

Fig. 2. (A-F) Flat preparations of embryos labelled by in situ hybridization with an islet-1 probe (blue) and stained with an antibody against β-galactosidase (brown). **pmn primary motoneurons, psn primary sensory neurons. Asterisks in all panels indicate the affected side. (A) 4-somite stage control embryo injected with lacZ RNA alone. (B-C) 4-somite stage embryos injected with full-length her4 and lacZ RNA. Both embryos have been stained for islet-1 expression, the one in B, in addition, for β-galactosidase expression. A reduction in the number of islet-1-expressing cells can be observed on one side. The horizontal lines in these and the other panels indicate the extent of the enlargement of the neural plate on the affected side. (D) A 4-somite stage embryo injected with full-length her4, groucho2 and lacZ RNA, stained for islet-1 and β-galactosidase expression. Note the enlargement of the neural plate and the complete lack of islet-1-positive cells on the injected side. (E,F) Two 4-somite stage embryos which have been injected with deltaDps and lacZ RNA, and stained for her-4 and β-galactosidase expression; the anti-β-galactosidase staining was kept to a minimum in this case, to avoid brown overstaining. Notice that her-4 expression is reduced on the brown side. (G,H) Cross sections of 24 h embryos injected with groucho2 RNA and stained with two antibodies: F59, which recognises the myosin heavy chain (Miller et al., 1989) and labels the adaxial mesodermal cells and their derivatives (brown product; see Devoto et al., 1996), and Hu(C), a neuronal marker (Kim et al., 1996). Notice the asymmetry of the neural tube (not: notochord), one side of which is much larger. Hu(C) cells (blue product) are indicated with arrows on the affected side. Notice that mesodermal development, as judged by the F59 staining, is also affected on the same side.
IV-WRPW region but retains the tetrapeptide WRPW, also had no detectable effect on the development of primary neurons. Therefore, since the WRPW tetrapeptide is dispensable, the region between helix IV and the WRPW is inferred to be essential for the suppression of islet-1-positive cells in the neural plate.

Three further constructs encoding products in which the 15 amino-terminal residues and the basic domain were deleted, were also tested. The proteins encoded by MT-her4^ΔNco(taq) and MT-her4^ΔNco differ in the presence of a myc-tag in the former, which is absent in the latter; the last three amino acids of the basic domain are present in the product of MT-her4^ΔNco. Injection of RNA expressed from any of these three constructs did not affect the islet-1 expression pattern (Table 2).

**her4 expression is dependent on NOTCH-mediated signalling**

Since in *Drosophila* the E(SPL)-C is the last link in the DELTA-NOTCH signalling pathway (de la Concha et al., 1988; Lieber et al., 1993), being activated by NOTCH-mediated signals (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), we tested whether her4 is under the control of NOTCH. A variant of the zebrafish *notch1* cDNA that encodes only the intracellular domain (*notch-intra, nic*) was made. In both *Drosophila* and *Xenopus* such truncated NOTCH derivatives act as constitutively activated receptors (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Chitnis et al., 1995). A strong reduction in the number of islet-1-expressing cells and ectopic activation of her4 transcription were observed in independent samples of injected embryos (Table 3). However, her4 activation remained restricted to the territory of the neural plate (Fig. 4B,C). RT-PCR analysis carried out on cDNA reverse transcribed from total RNA of injected embryos confirmed the activation of her4 transcription (Fig. 4D). The reciprocal experiment, that is, blocking deltaD activity with deltaD^PST RNA, which encodes an antimorphic deltaD allele, led to the opposite effect: her4 expression was partially suppressed on the injected side (Fig. 2E,F).

**her4 is involved in a regulatory feedback loop**

In *Drosophila*, lateral inhibition within a proneural cluster is regulated by a sensitive feedback mechanism. This involves, on the one hand, NOTCH-mediated activation of the E(SPL)-C genes, which results in suppression of proneural activity; on the other hand, proneural activity in the prospective neural progenitor increases, and as a consequence, the strength of the DELTA signal sent also increases (Haenlin et al., 1994; Kunisch et al., 1994; Oellers et al., 1994; Heitzler et al., 1996). The experiments described in this and the following three sections indicate the existence of a similar regulatory loop in zebrafish neurogenesis.

Misexpression of nic represses transcription of the zebrafish genes deltaA and deltaD (Fig. 5B,E; Table 3), although only within neural primordia. To test whether her4 is involved in this process, we injected her4 RNA and probed for deltaA and deltaD expression (Table 1). A reduction in the amount of deltaA and deltaD transcripts was detected (Fig. 5C,F). However, these effects were weaker than those seen after injection of nic RNA. Since the amount of her4 RNA is lower following activation by nic than after her4 RNA injection (Fig. 4F-G), we assume that nic RNA injection leads to activation of additional bHLH genes besides her4.

### Misexpression of her4 suppresses transcription of neurogenin1

In *Drosophila*, misexpression of genes of the E(SPL)-C suppresses neural development; this suppression is associated with suppression of proneural gene activity (Tata and Hartley, 1995; Nakao and Campos-Ortega, 1996). Blader et al. (1997) have cloned a homologue of the *Drosophila* proneural gene atonal, called neurogenin1 (*ngn1*), whose expression is modulated by lateral inhibition: whereas misexpression of the *Xenopus* Delta homologue X-delta-1 in zebrafish embryos strongly reduces or eliminates *ngn1* expression, misexpression of the antimorphic X-delta^mut* causes an increase in *ngn1* expression (Table 3).

#### Table 2. The effects of her4 variants on islet-1 expression

<table>
<thead>
<tr>
<th>RNA injected (concentration)</th>
<th>No. of embryos injected</th>
<th>No. of embryos expressing β-gal</th>
<th>No. of embryos with increased islet-1 expression</th>
<th>No. of embryos with reduced islet-1 expression</th>
<th>No. of embryos with wild-type islet-1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-her4^ΔD(Nco) (400 ng/μl)+lacZ (40 ng/μl)</td>
<td>70</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>40 (100%)*</td>
</tr>
<tr>
<td>MT-her4^ΔD(taq) (400 ng/μl)+lacZ (40 ng/μl)</td>
<td>71</td>
<td>45</td>
<td>0</td>
<td>2 (4%)*</td>
<td>43 (96%)*</td>
</tr>
<tr>
<td>her4^ΔD (800 ng/μl)+lacZ (40 ng/μl)</td>
<td>84</td>
<td>44</td>
<td>0</td>
<td>1 (2%)*</td>
<td>43 (98%)*</td>
</tr>
<tr>
<td>her4^ΔPst (1 ng/μl)+lacZ (40 ng/μl)</td>
<td>32</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>28 (100%)*</td>
</tr>
<tr>
<td>her4^ΔWRPW (400 ng/μl)+lacZ (40 ng/μl)</td>
<td>88</td>
<td>60</td>
<td>0</td>
<td>52 (87%)*</td>
<td>8 (13%)*</td>
</tr>
<tr>
<td>her4^AC+WRPW (400 ng/ml)+lacZ (40 ng/ml)</td>
<td>99</td>
<td>61</td>
<td>0</td>
<td>1 (2%)*</td>
<td>60 (98%)*</td>
</tr>
</tbody>
</table>

*Percentage relative to the number of embryos that express β-gal.
expression (Blader et al., 1997). We used the zebrafish \(\delta^{\text{A/PST}}\) variant. Indeed, \(\delta^{\text{A/PST}}\) also behaves as a dominant negative, eliciting development of supernumerary islet-1-positive cells restricted to the territory of the lateral and medial expression domains (Fig. 6A-C). Injection of \(\delta^{\text{A/PST}}\) RNA led to activation of \(ngn1\) transcription; activation of \(ngn1\) was intense but restricted to the expression stripes in the neural plate (Fig. 6D-G). Furthermore, we tested whether suppression of \(ngn1\) is due to activation of the zebrafish \(\text{NOTCH}\) pathway, and probed for \(ngn1\) expression after injection of either \(nic\) (Table 3) or \(her4\) RNA (Table 1). In the first case, local suppression of \(ngn1\) transcription was found. The same effect, albeit in fewer embryos, was found after \(her4\) misexpression (Fig. 5H-I).

**NEUROGENIN1 activates transcription of \(\delta\text{A}, \delta\text{D} and her4\)**

In *Drosophila*, proneural proteins activate transcription of the neurogenic genes \(\delta\text{A} and E(spl)\) directly, i.e., by binding to specific sites in their promoters (Haenlin et al., 1994; Hinz et al., 1994; Kramatschek and Campos-Ortega, 1994; Kunisch et al., 1994; Oellers et al., 1994; Singson et al., 1994; Heitzler et al., 1996). \(\delta\text{A}, \delta\text{D} and her4\) transcription was activated in \(ngn1\)-injected zebrafish embryos (Fig. 7D-F; Table 4). Ectopic activation of \(\delta\text{A}, \delta\text{D} and her4\) by \(ngn1\) was in all cases restricted to the neural plate, suggesting that \(NEUROGENIN1\) may activate the lateral inhibition network only within the neural plate.

**NEUROGENIN1 affects development of primary neurons**

Injection of \(ngn1\) RNA leads to ectopic development of islet-1-positive cells (Blader et al., 1997; our observations, Fig. 7A). This effect is dose-dependent since its frequency increases with the concentration of \(ngn1\) RNA injected (Table 5). \(ZfE12\) encodes a zebrafish homologue of the human E12 protein (Wülbeck et al., 1994), a class A bHLH protein which forms heterodimers with class B bHLH proteins (Murre et al., 1989). When low doses are used, ectopic islet-1 cells develop in 89% of all embryos co-injected with \(ngn1\) and \(ZfE12\) RNA, as compared to only 17% following injection of \(ngn1\) RNA alone at the same dose (Table 5). These results suggest that \(ZfE12\) interacts with \(ngn1\). Ectopic islet-1-positive cells follow the differentiation pathway characteristic of Rohon-Beard or trigeminal neurons (Blader et al., 1997, our observations). Therefore, zebrafish \(ngn1\) behaves like a proneural gene in that it can elicit neural development. Surprisingly, though, development of ectopic neurons was restricted to ectodermal regions outside the neural plate (Blader et al., 1997). We found, however, that the number of islet-1-expressing cells was reduced within the neural plate in some of the embryos (Fig. 7B-C). This effect is also dose dependent, being particularly strong after injection of \(ngn1\) RNA at a concentration of 4 ng/ml or higher (Table 5).

The differences between the effects of \(ngn1\) within and outside the neural plate can be explained by taking into account that \(NGN1\) apparently activates lateral inhibition only within the neural plate. To test this hypothesis we co-injected \(ngn1\) and \(her4\) RNA. Indeed, the number of islet-1 cells outside the neural plate is in this case considerably reduced. In the region of highest density of ectopic islet-1 cells this number is reduced from 13.9±3.4/0.01 mm² after \(ngn1\) RNA injection, to 6.8±2.1/0.01 mm² after injection of equal amounts (200 ng/μl) of \(ngn1\) and \(her4\) RNA. The number of ectopic islet-1 cells is further reduced increasing the concentration of \(her4\) RNA threefold (4.9±2.2/0.01 mm²).
**Table 3. The effects of notch-intra RNA injection* on expression of islet-1, her4, deltaA and deltaD, and ngn1**

<table>
<thead>
<tr>
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<th>No. of embryos with reduced expression</th>
<th>No. of embryos with wild-type expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>islet-1</td>
<td>63</td>
<td>56</td>
<td>0</td>
<td>54 (96%) ‡</td>
<td>2 (4%) ‡</td>
</tr>
<tr>
<td>her4</td>
<td>136</td>
<td>101</td>
<td>99 (98%) ‡</td>
<td>0</td>
<td>2 (2%) ‡</td>
</tr>
<tr>
<td>deltaD</td>
<td>163</td>
<td>135</td>
<td>130 (96%) ‡</td>
<td>0</td>
<td>5 (4%) ‡</td>
</tr>
<tr>
<td>deltaA</td>
<td>132</td>
<td>91</td>
<td>87 (96%) ‡</td>
<td>0</td>
<td>4 (4%) ‡</td>
</tr>
<tr>
<td>ngn1</td>
<td>81</td>
<td>53</td>
<td>47 (88%) ‡</td>
<td>0</td>
<td>6 (12%) ‡</td>
</tr>
</tbody>
</table>

*nic (200 ng/μg)+lacZ (40 ng/μl)
‡Percentage refers to the number of embryos that express β-gal.

**DISCUSSION**

**HER4 is an E(spl) homologue**

The main conclusion of our work is that her4 encodes a zebrafish homologue of the Drosophila E(spl)-C proteins, which acts as a target of NOTCH to suppress primary neurogenesis. This conclusion is based on structural and functional considerations. HER4 shows considerable sequence identity in the bHLH domain, the region that binds DNA and is involved in target recognition (Tietze et al., 1992). Furthermore it also exhibits the other characteristics of the Hairy-E(spl) protein family, such as the carboxy-terminal tetrapeptide WRPW. her4 is expressed in the neural plate region in which the primary neurons are formed. In Drosophila the E(spl) proteins suppress the activity of the proneural genes (Oellers et al., 1994; Tata and Hartley, 1995; Heitzer et al., 1996; Nakao and Campos-Ortega, 1996). Misexpression of her4 suppresses ngn1 and the development of primary neurons.

However, there are two major differences between HER4 and the E(spl) proteins. In Drosophila the latter require GROUCHO for their function in neurogenesis whereas our data do not provide evidence for interactions between HER4 and zebrafish GROUCHO2. Injections of grouch2 RNA do not have any apparent effect on the development of islet-1 cells, and coinjection of her4 and grouch2 RNA does not enhance the neural suppression mediated by her4 alone. In the association between GROUCHO and E(spl)-C proteins in Drosophila, the WRPW domain plays an essential role in segmentation and neurogenesis; thus removal or mutation of this domain renders the protein non-functional (Wainwright and Ish-Horowicz, 1992; Paroush et al., 1994; Fisher et al., 1996; Giebel and Campos-Ortega 1997). In contrast, the WRPW of HER4 is apparently not needed to suppress primary neurogenesis in zebrafish. It is important to note in this context that there is at least one precedent for the present result: Dawson et al. (1995) have reported that the WRPW of HAIRY is not required to suppress SCUTE in the sex determination pathway in Drosophila. The other important difference between the Drosophila E(spl)-C proteins and HER4 concerns their amino-terminal regions, including the basic domain, which has been shown to bind a specific DNA sequence called the N-box (Tietze et al., 1992). In misexpression experiments using the Gal4-UBAS system in Drosophila, deletion of either the basic domain (Nakao and Campos-Ortega, 1996) or both the basic and the HLH domains (Giebel and Campos-Ortega, 1997) did not seriously affect the ability of E(spl) proteins to suppress neural development. However, the amino-terminal domain of HER4 seems to be essential for activity, as its deletion results in non-functional proteins in our present conditions. Therefore, DNA binding of the HER4 protein might play a more important role during neurogenesis in the zebrafish than in the case of the E(spl) proteins in Drosophila.

**her4 is a target of NOTCH signalling**

In both Xenopus and zebrafish, differentiation of primary neurons is perturbed following misexpression of Notch and Delta homologues (Chitnis et al., 1995; Chitnis and Kintner 1996; Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). The results of these experiments, as well as those presented here, strongly support the idea that primary neurons are selected from equivalence groups within the neural plate, and that this process is mediated by lateral inhibition. Our data indicate that her4 is one of the target genes of the NOTCH signalling cascade in the zebrafish. Transcription of her4 is activated by the constitutively active NOTCH1 variant encoded by nic, and misexpression of her4 leads to a reduction in the number of islet-1 positive cells. Similar observations were made by Wettstein et al. (1997) concerning ESR-1, a Xenopus homologue of the E(spl) proteins. However, the effects of misexpression of her4 on the islet-1 cells are less severe than those caused by nic. We suggest that, in analogy to Drosophila

**Table 4. The effect of misexpression of ngn1* on ectopic transcriptional activation of her4, deltaA and deltaD**

<table>
<thead>
<tr>
<th>Hybridization probe</th>
<th>No. of embryos injected</th>
<th>No. of embryos expressing β-gal</th>
<th>No. of embryos with ectopic expression</th>
<th>No. of embryos with reduced expression</th>
<th>No. of embryos with wild-type expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>her4</td>
<td>69</td>
<td>42</td>
<td>42 (100%) ‡</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>deltaD</td>
<td>64</td>
<td>37</td>
<td>36 (97%) ‡</td>
<td>0</td>
<td>1 (3%) ‡</td>
</tr>
<tr>
<td>deltaA</td>
<td>50</td>
<td>30</td>
<td>26 (87%) ‡</td>
<td>0</td>
<td>4 (13%) ‡</td>
</tr>
</tbody>
</table>

*ngn1 (400 ng/μl)+lacZ (40 ng/μl) injected.
‡% refers to embryos with β-gal expression.
(Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), this difference is due to the concomitant activation by NOTCH of additional bHLH genes involved in regulation of primary neurogenesis.

The reduction in the number of islet-1 positive cells following misexpression of her4 might be due either to direct inhibition of proneural gene transcription, or to an effect on the target genes of the proneural proteins. Transcription of zebrafish ngn1 is modulated by misexpression of Delta variants that are assumed to activate or repress NOTCH (see Blader et al., 1997). We find that inhibition of ngn1 transcription correlates with transcriptional activation of her4, thus suggesting a causal relationship between the two events.

A feedback loop between NOTCH and DELTA in zebrafish that is organised similarly to that in Drosophila is strongly supported by our data. We find that activation of NOTCH has two detectable effects. First, it leads to an increase in transcription of her4, resulting in repression of proneural activity and primary neural fate. Whether proneural activity is directly repressed by transcriptional suppression, and/or indirectly by posttranslational modifications, has to be analysed in further detail. In any case, the second effect, a feedback loop, is established that leads to a reduction in the concentration of delta RNA in the cells in which NOTCH has been activated. This in turn reduces the intensity of NOTCH activation in the neighbouring cells, and allows them to differentiate as primary neurons.

**Table 5. The effects of ngn1 on islet-1 expression**

<table>
<thead>
<tr>
<th>RNA injected (concentration)</th>
<th>No. of embryos injected</th>
<th>No. of embryos expressing β-gal</th>
<th>No. of embryos with ectopic islet-1 cells</th>
<th>No. of embryos with reduced islet-1 expression</th>
<th>No. of embryos with wild-type islet-1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ngn1 (100 ng/μl) + lacZ (40 ng/μl)</td>
<td>32</td>
<td>29</td>
<td>6 (21%)*</td>
<td>1 (17%)‡</td>
<td>23 (79%)*</td>
</tr>
<tr>
<td>ngn1 (200 ng/μl) + lacZ (40 ng/μl)</td>
<td>64</td>
<td>35</td>
<td>22 (63%)*</td>
<td>9 (41%)‡</td>
<td>13 (37%)*</td>
</tr>
<tr>
<td>ngn1 (400 ng/μl) + lacZ (40 ng/μl)</td>
<td>75</td>
<td>62</td>
<td>60 (97%)*</td>
<td>51 (85%)‡</td>
<td>3 (3%)*</td>
</tr>
<tr>
<td>ngn1 (800 ng/μl) + lacZ (40 ng/μl)</td>
<td>62</td>
<td>41</td>
<td>38 (93%)*</td>
<td>38 (100%)‡</td>
<td>2 (7%)*</td>
</tr>
<tr>
<td>ngn1 (1000 ng/μl) + ZfE12 (100 ng/μl) + lacZ (40 ng/μl)</td>
<td>115</td>
<td>59</td>
<td>37 (63%)*</td>
<td>33 (89%)‡</td>
<td>22 (37%)*</td>
</tr>
</tbody>
</table>

*Percentage relative to the number of embryos that express β-gal.
‡Percentage relative to the number of embryos with ectopic islet-1 cells.

Proneural proteins activate the genes that mediate lateral inhibition only in the neural plate: lateral inhibition predominates over proneural gene function

Within the neural plate, the number of islet-1-positive cells is reduced following

![Fig. 5. Wild-type expression patterns of deltaA (A), deltaD (D) and ngn1 (G) in 5-somite stage embryos as revealed by in situ hybridization. (B,E,H) Embryos of the same age injected with nic RNA and scored for expression of deltaA (B), deltaD (E) and ngn1 (H). Transcription of these genes is reduced on the injected side of the embryo (asterisks). (C,F,I) Embryos injected with full-length her4 RNA and scored for expression of deltaA (C), deltaD (F) and ngn1 (I). Transcription of these genes is reduced (asterisks).](image)
A zebrafish E(spl) homologue

ngn1 RNA injection, while cells of this type develop ectopically outside the neural plate of the same animals. A similar behaviour has been observed with XASH-3 in Xenopus (Chitnis and Kintner, 1996). However, misexpression of Xenopus neurogenin leads in Xenopus to ectopic development of primary neurons in the neural plate (Ma et al., 1996).

These apparently paradoxical effects of ngn1 can be understood when one considers that it activates transcription of deltaA, deltaD and her4, i.e. of the effectors responsible for lateral inhibition, only within the limits of the neural plate. This may explain the reduction in the number of islet-1-positive cells observed within the neural plate following injection of ngn1 RNA. ngn1 cannot activate deltaA, deltaD and her4 and, consequently, lateral inhibition outside the neural plate, thus permitting ectopic development of islet-1-positive cells in the non-neural ectoderm. The observation that the co-injection of her4 suppresses ngn1-mediated development of ectopic islet-1 cells supports this hypothesis.

In a Drosophila embryo that overexpresses proneural genes the situation is remarkably similar to that of ngn1 misexpression in the zebrafish. Gal4-mediated overexpression of lethal of scute leads to ectopic development of neurons only within the amnioserosa; this effect is suppressed by the concomitant activation of NOTCH in the amnioserosa. However, selection of neural and epidermal progenitor cells takes place normally in the neuroectoderm – in spite of the presence of large amounts of proneural gene products. The proneural gene products seem to activate lateral inhibition strongly, since a reduction in the complement of copies of the Notch+ gene from 2 to 1, leads to strong neurogenic phenotypes (Giebel et al., 1997).

Based on the findings of this study, we suggest that, like the E(spl)-C genes in Drosophila, her4 constitutes the last link in

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Fig. 6. Flat preparations of 6-somite stage embryos injected with deltaD(Pst) RNA and stained for islet-1 expression by in situ hybridization (A-C). Arrows and asterisks label the affected side. (D,E) 1-somite stage embryos injected with deltaD(Pst) RNA and scored for ngn1 transcription, detected by in situ hybridization with a ngn1 probe (D) and by double staining for β-galactosidase (E). (F,G) 6- and 8-somite stage embryos, treated as in D and E. Inhibition of DELTA signalling leads to an activation of ngn1 transcription within the limits of its normal expression stripes.

Fig. 7. (A-C) 4-somite stage embryos injected with ngn1 RNA and stained for islet-1 expression by in situ hybridization. (A) Lateral view of an injected embryo showing ectopic islet-1-positive cells in the ectoderm adjacent to the neural plate. (B,C) Dorsal views reveal a reduction in the number of islet-1-positive cells within the neural plate following misexpression of ngn1 (asterisks). (D,E,F) 3-somite stage embryos injected with neurogenin1 RNA and stained for the expression of deltaA (D), deltaD (E) and her4 (F). Transcription is activated within the neural plate (asterisks).
the NOTCH signalling cascade in zebrafish neurogenesis. There are several other her genes in the zebrafish (v. Weizsäcker 1994). Their complex patterns of expression during embryogenesis suggest their involvement in other processes besides differentiation of islet-1-positive cells. Since other genes encoding bHLH proteins of the same family are known to be expressed in other regions of the body, e.g. her1 and her5 in the presomitic mesoderm and in the midbrain anlage, respectively (Müller et al. 1996a,b), they may represent different endpoints of this signalling pathway and regulate cell fate decisions other than that which results in the appearance of the primary neurons.

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