The Notch targets Esr1 and Esr10 are differentially regulated in Xenopus neural precursors

Elise Lamar and Chris Kintner

Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
e-mail: lamar@salk.edu and kintner@salk.edu

Accepted 13 June 2005

Development 132, 3619-3630
Published by The Company of Biologists 2005
doi:10.1242/dev.01937

Summary

The HES family of bHLH repressors plays a key role in regulating the differentiation of neural precursors in the vertebrate embryo. Members of the HES gene family are expressed in neural precursors as targets of the Notch signaling pathway, but how this occurs in the context of neurogenesis is not known. Here, we address this issue by identifying enhancers driving Notch-dependent gene expression of two Hes5-like genes expressed in Xenopus called Esr1 and Esr10. Using frog transgenesis, we identify enhancer elements driving expression of Esr1 and Esr10 in neural precursors or in response to ectopic expression of the proneural protein, Xngnr1. Using deletion and mutation analysis, we define motifs required for enhancer activity of both genes, namely Notch-responsive elements and, in the case of Esr10, E-box motifs. We find that Esr1 and Esr10 are differentially regulated both in terms of Notch input and its interaction with heterologous factors. These studies reveal inputs required for proneural expression of genes encoding bHLH repressors in the developing vertebrate nervous system.

Key words: Notch, Esr, bHLH, E(spl)/hairy, Neurogenesis, HES, Xenopus

Introduction

The pattern of neurogenesis is regulated throughout metazoan development by repressors known as hairy/Enhancer of split [E(spl)] proteins in invertebrates or ‘HES’ proteins in mammals. These proteins are structurally related in their basic-helix-loop-helix (bHLH) DNA-binding domain and recruit corepressors through a C-terminal WRPW motif (reviewed by Davis and Turner, 2001). HES repressors block expression and activity of proneural bHLH activators such as atonal/neurogenin and achaete/scute proteins, thereby antagonizing differentiation (Van Doren et al., 1992; Sasai et al., 1992; Ishibashi et al., 1995; Cau, 2002). Identifying factors regulating bHLH repressor expression within neurogenic precursors should elucidate mechanisms controlling neural differentiation.

Paradigms for how bHLH repressors regulate neural differentiation have arisen from studies of peripheral neurogenesis in Drosophila imaginal discs (Fisher and Caudy, 1998). In one scenario, repressors such as Hairy mediate prepatterning by repressing establishment of proneural domains (Ohsako et al., 1994; Van Doren et al., 1992). By contrast, bHLH repressors encoded by genes in the E(spl) Complex (Knust et al., 1992) function within proneural domains as effectors of the Notch/LIN-12 signaling pathway, which mediates lateral inhibition in invertebrates (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991) and vertebrates (reviewed by Kintner, 2003). In Drosophila, activity of E(spl) gene enhancers during lateral inhibition is driven by direct Notch input via binding sites for the repressor Suppressor of Hairless [Su(H)] (Bailey and Posakony, 1995; Cooper et al., 2000; Nellesen et al., 1999), known as LAG-1 in worms and CBF1/RBP-Jκ in mammals, Notch signaling converts Su(H) to an activator by recruiting the Notch intracellular domain (ICD) and co-activators such as Mastermind/LAG-3 (Petcherski and Kimble, 2000; Fryer et al., 2002) (reviewed by Lamar and Kintner, 2003). Expression of several E(spl) enhancers during lateral inhibition not only requires direct input from Notch through Su(H)-binding sites but also input from the proneural bHLH proteins through E-box-binding sites (Bailey and Posakony, 1995; Nellesen et al., 1999; Cooper et al., 2000; Cave et al., 2005). This combinatorial code explains why these enhancers respond to Notch only in a proneural context (Furriols and Bray, 2001; Barolo and Posakony, 2002), and indicates that proneural proteins activate their own inhibitors not only non-cell autonomously by transactivating the gene encoding the Notch ligand Delta (Kunisch et al., 1994), but directly.

In vertebrates neural precursors also express genes encoding bHLH repressors, including proteins structurally related either to Hairy – such as mouse Hes1 (Takebayashi et al., 1994) – or to mouse Hes5 (Li et al., 2003). Numerous studies demonstrate that repressors of either family antagonize neurogenesis (Deblandre et al., 1999; Ohtsuka et al., 1999; Takke et al., 1999; Koyano-Nakagawa et al., 2000; Stancheva et al., 2003). Furthermore, many HES genes are likely direct Notch targets as many exhibit proximal Su(H)-binding sites in an ‘SPS’ motif, for Suppressor of Hairless paired sites (Bailey and Posakony, 1995). Although HES gene regulation has not been
analyzed in detail in vertebrates, their expression patterns within a species vary (Jouve et al., 2000; Hatakeyama et al., 2004; Fior and Henrique, 2005), suggesting a combinatorial mechanism.

Neural precursors in Xenopus embryos also express Hair and Hes5-like repressors. A hairless homolog, Xenopus Hair2y, is expressed during gastrulation (Tsujii et al., 2003) prior to upregulation of Delta, while a Xenopus Hes5 ortholog Esr1 is expressed at time coincident with Notch signaling (Wettstein et al., 1997). A 500 bp enhancer element regulating mesodermal Hair2y expression has been characterized (Davis et al., 2001). That element drives Hair2y expression in the brain and mesoderm (Davis et al., 2001), providing a basis for comparison with Notch effectors of lateral inhibition.

Here, we characterize two such enhancers, those of Esr1 and Esr10 (Gawantka et al., 1998). Both are expressed in neuroectodermal domains where primary neurons form, and proneural genes (Ma et al., 1996) and Notch ligands (Chitnis et al., 1995) are expressed. Esr10 is also cyclically expressed in the presomitic mesoderm, where it may function in the segmentation clock (Li et al., 2003). Using transgenic frogs (Amaya and Kroll, 1999), we show that Esr1 and Esr10 cis-elements drive reporter expression in proneural domains mirroring endogenous expression. Unlike the Hair2 regulatory element, Esr gene enhancers are upregulated by Xangur1, thereby constituting proneural enhancers. Analysis of transgenic frogs coupled with transfection assays reveals that regulation of Esr1 and Esr10 differs. Specifically, although an intact SPS motif is necessary but not sufficient for expression of either gene in a proneural context, Notch input to each intact SPS motif is necessary but not sufficient for expression of Δ-galactosidase activity using X-gal. Embryos were assayed for β-galactosidase activity using X-gal. Embryos were analyzed in detail in vertebrates, their expression patterns within a species vary (Jouve et al., 2000; Hatakeyama et al., 2004; Fior and Henrique, 2005), suggesting a combinatorial mechanism.

Neural precursors in Xenopus embryos also express Hair and Hes5-like repressors. A hairless homolog, Xenopus Hair2y, is expressed during gastrulation (Tsujii et al., 2003) prior to upregulation of Delta, while a Xenopus Hes5 ortholog Esr1 is expressed at time coincident with Notch signaling (Wettstein et al., 1997). A 500 bp enhancer element regulating mesodermal Hair2y expression has been characterized (Davis et al., 2001). That element drives Hair2y expression in the brain and mesoderm (Davis et al., 2001), providing a basis for comparison with Notch effectors of lateral inhibition.

Here, we characterize two such enhancers, those of Esr1 and Esr10 (Gawantka et al., 1998). Both are expressed in neuroectodermal domains where primary neurons form, and proneural genes (Ma et al., 1996) and Notch ligands (Chitnis et al., 1995) are expressed. Esr10 is also cyclically expressed in the presomitic mesoderm, where it may function in the segmentation clock (Li et al., 2003). Using transgenic frogs (Amaya and Kroll, 1999), we show that Esr1 and Esr10 cis-elements drive reporter expression in proneural domains mirroring endogenous expression. Unlike the Hair2 regulatory element, Esr gene enhancers are upregulated by Xangur1, thereby constituting proneural enhancers. Analysis of transgenic frogs coupled with transfection assays reveals that regulation of Esr1 and Esr10 differs. Specifically, although an intact SPS motif is necessary but not sufficient for expression of either gene in a proneural context, Notch input to each occurs through architecturally distinct sites. Furthermore, bHLH proteins probably provide both direct and indirect inputs to the Esr10 enhancer, while in the case of Esr1 that input is only indirect. These results define inputs crucial for expression of bHLH repressors within neural precursors downstream of the Notch pathway.

Materials and methods

RNA injections and in situ hybridization

Eggs were obtained from Xenopus laevis frogs using standard techniques and fertilized in vitro or by injection of sperm nuclei. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). RNA injection was performed as described previously (Chitnis et al., 1995). Before in situ hybridization, embryos were assayed for β-galactosidase activity using X-gal. Embryos were stained by whole-mount in situ hybridization with digoxigenin-labeled probes (Harland, 1991), including Esr1 (Wettstein et al., 1997), Esr7 (Deblandre et al., 1999), Esr10 (Li et al., 2003), GFP and Hair2y (Turner and Weintraub, 1994).

Identification of promoter elements and transgenic methods

Proximal elements were obtained as described (Moore and Kintner, 2004) and cloned upstream of GFP in a vector containing the 700 bp Hair2y 3′ instability element (Davis et al., 2001). Basal promoters were determined using www.fruitfly.org/seq_tools/other.html. Protein and DNA sequences were obtained from databases at www.ncbi.nlm.nih.gov (mouse, chicken, zebrafish and fugu) and http://genome.jgi-psf.org/Xentr3.home.html (Xenopus tropicalis). GenBank Accession Numbers for Esr1/RV and Esr10/Dra are DQ096795 and DQ096794, respectively.

Transgenic frogs were generated using standard (Amaya and Kroll, 1999; Sparrow et al., 2000) protocols. In addition, we delayed activation by injecting oocytes in Ca2+-free injection buffer and activating them following injection by incubation in 0.1×MMR plus Ca2+ containing 1 μM A23187 (Sigma). This protocol increased transgenic efficiency and reduced gastrulation defects.

Site-directed mutagenesis

Mutagenesis was achieved by PCR using sense and antisense oligonucleotides followed by DpnI digestion of the parent plasmid.

Oligonucleotides to mutate Esr1 were (mutant nucleotides underlined): mS1, GCTAAACGAGTGTGGC; mS2, GTAGCAGGTGGATGAATGAGGTTTGG; mS4, GATGGGAATCCTTTGCCAGGTTCCACACCTT; mE1, GCCCTAGTCACACTTCTTTCCTGTTATACCAATTACGTC; mE2a, TGAAACACTCTGACCAGTTGTTTCCACTGGGAGCC; mE3, GCCTCCACAGCTCATACTCCCTCAGCACACTAGC. Oligonucleotides to mutate Esr10 were: mE1, GTATCTCA-GGTTGATTCCCCACACCTT; mE2, GTGTCAGGCTCTCGCTTCCACCACCTTAATATTGAC; mS1, GCTACTGAGTGGCGAAC-CTCTGTTCCAGCC; mS2, CTACGCTGATCGTACCATATTGTCA; mCAAT, CTGCAGCCGCTGAGCTGACTAGTGGTGT.

Animal cap assay

RNA injection, preparation of neutralized caps, RNAse protection assay, and probes for Esr1 and EFair were as described were as previously described (Koyano-Nakagawa et al., 1999). The Esr10 probe was a 276 bp fragment of the 3′UTR of clone 11A10 (Gawantka et al., 1998), cloned into Bluescript (Stratagene), linearized with BamI, and transcribed using T7. Caps were cut at stage 10 and harvested when embryo controls reached stage 12. Quantification was carried out using a Phosphor Imager (Molecular Dynamics).

Transfections and EMSA

HeLa cells were transfected with Lipofectamine2000 (Invitrogen) as described (Lamar et al., 2001). Effectors were Xenopus ICD (Wettstein et al., 1997), Xangur1 (Ma et al., 1996) and E47 (Lee and Pfaff, 2003). In addition to those described in the text, reporters included Xenopus Hairy2y (Davis et al., 2001), Hes1 (Jarriault et al., 1995) and multimerized Su(H)-binding sites (Ling et al., 1994). Transfection efficiency was assessed using either co-transfected IacZ expression vectors and ONPG substrate (Sigma) or tk-Renilla reporters.

For EMSAs proteins were synthesized using a TnT reticulocyte lysate kit (Promega). Oligos were end-labeled with [32P]dUTP using Klenow to a specific activity of 2×106 CPM/pmol. Heterodimers were preincubated 30 minutes at room temperature prior to binding. Binding reactions included 1-5×105 CPM of probe, 2 μg poly(dI-dC) (Roche), 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 3% glycerol. After incubating 45 minutes at room temperature, DNA/protein complexes were loaded onto a 5% (30:1) nondenaturing polyacrylamide gel and run for 3 hours at 200 V at 4°C.

Results

Embryonic expression of neural E(spl) homologs

Xenopus embryos express several bHLH repressors related to the two main subfamilies of mammalian HES proteins (Fig. 1). One of these repressors, Hairy2A/B (Turner and Weintraub, 1994), belongs to the Hes1-like subfamily (closely related in sequence to Drosophila Hairy). By contrast, Xenopus Esr1,
Esr7, Esr9 and Esr10 (Wettstein et al., 1997; Deblandre et al., 1999; Li et al., 2003) belong to the Hes5-like subfamily, which is more distantly related to either Drosophila Hairy or the bHLH repressors encoded in the E(spl) complex (Fig. 1).

The expression patterns of Hairy/Hes1-like and Hes5-like repressors in Xenopus suggest distinct functions in regulating differentiation of neural precursors. Esr1 (Fig. 2A,B), Esr10 (Fig. 2D,E), Esr9 (Li et al., 2003) and Esr7 (Deblandre et al., 1999) are expressed in neural tissue in a pattern consistent with a role in lateral inhibition. Their expression corresponds with sites of neurogenesis as marked by the expression of the Notch ligand Delta1 (Fig. 2G,H) and the proneural gene Xngnr1 (Ma et al., 1996). At early tailbud stages, when primary neurogenesis is completed posteriorly, Esr1 and 10 expression is accordingly downregulated in the spinal cord and upregulated in eye and in brain (data not shown), coincident with the onset of neurogenesis anteriorly (Papalopulu and Kintner, 1996). Neural Delta1 expression occurs in a broader pattern, e.g. in the pronephros and presomitic mesoderm, than that of individual Esr genes, such as Esr1. Thus, Esr1 and 10 expression coincides with Notch activity in neural precursors but is not seen in all tissues where Notch signaling occurs. Finally, at neurula (Fig. 2D) and tailbud (data not shown) stages, Esr10 is also expressed in the presomitic mesoderm, where its expression oscillates in a manner similar to that of the closely related Esr9 (Li et al., 2003). Esr1 is not expressed in the presomitic mesoderm (Fig. 2A).

By contrast Hairy2 is expressed predominantly in neural crest cells arising at the border of the neural plate and later migrating into the branchial arches (Fig. 2J,K). At early neurula stages, Hairy2 is also expressed in the neural

---

**Fig. 1.** Xenopus Esr proteins are orthologs of HES proteins. Tree compares primary sequence of bHLH domain. Esr1 and Esr7 are orthologs of mouse (Mm) Hes5, zebrafish (Dr) Her4 and a chick (Gg) Hes5-like protein. Esr9 and Esr10 form a subgroup within this family. MmHes1 is homologous to Drosophila (Dm) Hairy. Xenopus laevis (Xi) Hairy2A, Gg hairy1 and DrHer6. Drosophila E(spl) proteins are shown for comparison.

---

**Fig. 2.** Esr1 and Esr10 are expressed in proneural domains. Esr1, Esr10 and Delta are expressed in the neural tube (A,D,G, white arrowhead), cranial ganglia (A,D,G, arrow) and anterior neural tube (B,E, arrowhead). Esr10 is also expressed in the tailbud (D, black asterisk) and somitomeres (D, white asterisk). Delta1 is also seen in somitomeres (G, white asterisk), although the Notch ligand predominantly expressed in the tailbud is Delta2 (Jen et al., 1997). At neurula stages, Hairy2 is barely detectable in the neural tube (J) but expressed in presumptive neural crest (K, arrowhead). Hairy2 expression in the eye (K, arrow) precedes that of Esr1 and Esr10. Misexpression of mRNA encoding Xngnr1 induces Esr1 (C), Esr10 (F) and Delta1 (I); C’, F’ and I’ show un.injected sides. Hairy2 is not upregulated by misexpressed Xngnr1 (L; L’, uninjected side). Turquoise stain in C,F,I,L reflects activity of the lacZ tracer gene. (M, left) RNase protection assay showing that expression of Xngnr1 in neuralized animal caps analyzed at stage 12 induces Esr1 and Esr10 that can be inhibited by expression of SuH, and in the case of Esr1, further increased by co-injection of ICD. EF1a expression serves as a loading control; ‘Embryos’ indicates staged-matched controls. Quantification (right) shows fold increases in Esr1 and Esr10 relative to their respective ‘noggin only’ control, which is set arbitrarily to 1.
管在一个狭窄的祖细胞群体中沿着背腹轴（数据未显示）。

Differing expression patterns in neural precursors of Esr1 and Esr10 compared with Hairy2 suggest that these two structural classes of genes respond to different transcriptional inputs. To distinguish these inputs, we exploited the fact that, when misexpressed, Xngnr1 induces ectopic or premature neurogenesis, marked by expression of the Notch ligand XDelta1 (Ma et al., 1996) (Fig. 2I, I') and of neuronal differentiation genes, such as N-tubulin (Ma et al., 1996). Indeed, when embryos were injected with Xngnr1 at the two-cell stage and assayed for Esr1 and Esr10 expression at neurula stages, both were induced in the neural and non-neural ectoderm (Fig. 2C, C', F, F'). By contrast, embryos injected with Xngnr1 and assayed for Hairy2 expression showed no such increases (Fig. 2L, L'). Thus, based on this criterion, expression of Esr1 and Esr10 responds to proneural activity whereas Hairy2 does not.

Proneural expression of the Xenopus bHLH repressors was also examined in an animal cap assay in which premature neuronal differentiation is induced in neutralized ectoderm by misexpression of Xngnr1. In this assay, expression levels of both Esr1 and Esr10 (Fig. 2M), but not of Hairy2A (data not shown) are markedly upregulated in response to Xngnr1. Significantly, the response of Esr1 and Esr10 to Xngnr1 in this more quantitative assay differs by several criteria. Although Esr1 RNA levels increased 17-fold in response of Xngnr1, the levels of Esr1 RNA increased only 7.6 fold. Moreover, the response of Esr1 and 10 to Xngnr1 differed when assayed in the presence of either excess ICD or Su(H). Whereas the levels of Esr1 RNA induced by Xngnr1 increased twofold with excess ICD and halved with excess Su(H), the levels of Esr10 remained relatively unchanged (Fig. 2M, left; quantified on the right). In this assay, therefore, the response of Esr1 and Esr10 to proneural input was similar but not equivalent.

Identification of genomic elements flanking Esr1 and Esr10
To identify elements required for proneural expression of the Esr genes, we isolated genomic sequences lying upstream of Esr1, Esr7, and Esr10 (Davis et al., 2001) show high and moderate homology of S1 and S2, respectively, in the SPS (green). All exhibit a conserved CCAAT motif (blue). GFP expressed by deletion mutants of Esr1 (B,C-E) and Esr10 (B,F-H) in transgenic frogs, followed by whole-mount in situ hybridization, indicates that short elements drive Esr gene expression in the neural tube (D,G, arrowheads). Esr10/Dra also drives somitomic (G, arrow) and tailbud (G, asterisk) GFP expression. Deletion to a Hin3 (E) site attenuates Esr1 GFP, although expression remains restricted to neural tissue (E, arrowhead). Deletion to a Pst site (H) abrogates Esr10 neural expression, although diffuse somitomic expression (H, arrow) remains. Activities using the neural tube (NT) as a reference are summarized in (B, right; see Table 1 for details).

Sections through the neural tube of stage 20 Esr1/RV transgenic embryos (J) show GFP-positive cells in the ventricular zone in a pattern similar to the endogenous gene (I). Also summarized (B, right) are data reported in Figs 4, 6 and 8 and Table 2 that are relevant to responses to ectopic Xngnr1 (NA; not assayed).
Esr1, Esr7, Esr10 and Hairy2 is high in the proximal 100 base pairs, with Esr1 exhibiting comparable identity with Esr7 (56%), Esr10 (56%) and Hairy2 (51%). However, the degree of homology between -100 and -200 reflects the degree of identity of the proteins (see Fig. 1), with the Esr1 promoter exhibiting 64%, 41% and 27% identity with Esr7, Esr10 and Hairy2, respectively.

**Esr gene proximal sequences drive neural reporter expression**

To determine if the isolated genomic fragments contained proneural enhancers, they were assayed in transgenic frogs using vectors containing proneural enhancers, they were assayed in transgenic frogs carrying its own basal promoter and the vector contained the 3' Hairy2 UTR, which mediates RNA instability and is required for the striped pattern of mesodermal Hairy2 expression (Davis et al., 2001). Although GFP expression was apparent at neural plate stages (data not shown), we analyzed embryos at neurula stages (18-20) owing to the robust response. The neural expression of GFP RNA in frogs transgenic with the longest (FL) fragments of Esr1 and Esr10 (Fig. 3C,F) was indistinguishable from that of the endogenous genes (compare Fig. 3C,F with Fig. 2A,B,D,E). FL-Esr10 drove reporter expression in the neural tube, cranial ganglia and brain (Fig. 3C). FL-Esr10 also recapitulated neural expression of endogenous Esr10 (Fig. 3F), including tailbud expression, indicating that these sequences contain some elements required for mesodermal expression. FL-Esr10 also drove mesodermal GFP expression in somitomeric stripes, a pattern similar to that observed with endogenous Hairy2 and Esr10. Finally, a 516 bp Esr7 element drove robust GFP expression in a pattern similar to the endogenous gene but was not further analyzed (Table 1).

Analysis of GFP expression in Esr1 and Esr10 deletion mutants (summarized in Fig. 3B and Table 1) showed that deletions to -446 in Esr1 (Esr1/RV) and to -354 in Esr10 (Esr10/Dra) drove neural (and in Esr10, mesodermal) GFP expression indistinguishable in pattern and intensity from FL constructs (Fig. 2A,D; Fig. 3C,D,F; data not shown). Transverse sections through the neural tube of Esr1/RV (Fig. 3J) transgenic embryos showed GFP expression in cells of the ventricular zone as was seen with the endogenous gene (Fig. 3I). Similar results were obtained with Esr10/Dra (data not shown). Further deletion of 216 bp in Esr1 (Esr1/Hin3) (Fig. 3E) greatly attenuated GFP expression in the spinal cord relative to controls, although residual expression was restricted to neural tissue. Significantly, deletion of 123 bp of Esr10 (Esr10/Pst) (Fig. 3H) abrogated GFP expression in the neurectoderm and presomitic mesoderm, with only traces of possibly somitomeric expression remaining (see below). Overall, these observations show that short regions proximal to the TATA box are sufficient for neural Esr1 and Esr10 expression, and that – with the caveat that cyclic Esr10 expression is not addressed – it is likely that Esr10/Dra can activate transcription in the mesoderm.

**Esr1 and 10 enhancer elements are appropriately responsive to Xngnr1**

Endogenous Esr1 and Esr10 can be induced ectopically by misexpression of the proneural gene Xngnr1 (Koyano-Nakagawa et al., 1999) (Fig. 2C,F). Therefore, we injected mRNA encoding Xngnr1 and β-galactosidase tracer into one blastomere of two-cell embryos that were transgenic for Esr1/RV or Esr10/Dra, and asked whether embryos showed ectopic GFP expression. In both cases, GFP expression was expanded, although, in general, Esr10/Dra showed broader expression on the injected side than did Esr1/RV (Fig. 4A,C). We then asked whether Xngnr1 upregulated GFP in Esr1/Hin3 and Esr10/Pst transgenic embryos, which show attenuated GFP expression (Fig. 4F). Neither Esr1/Hin3 (Fig. 4B) nor Esr10/Pst (Fig. 4D) exhibited ectopic GFP expression in response to Xngnr1, indicating that sequences required for such a response are upstream of Hin3 and Pst in Esr1 and Esr10, respectively. These observations confirm that both elements contain neural enhancers responsive to Xngnr1, and that elements responsive to Xngnr1 lie upstream of the SPS.

Data presented here (Fig. 2L) and by others (Glavic et al., 2003; Tsuji et al., 2003) strongly suggests that Xenopus Hairy2 inhibits neurogenesis primarily through a prepattern function and is not responsive to proneural genes. Therefore, we asked if the 500 bp Hairy2 proximal genomic element, which drives Hairy2 expression in the anterior neurectoderm and in the mesoderm (Davis et al., 2001), was upregulated by Xngnr1. Transgenic frog embryos harboring the Hairy2-GFP construct

### Table 1. Enhancer activity of Esr gene deletions and point mutants

<table>
<thead>
<tr>
<th>Construct</th>
<th>Detectable GFP</th>
<th>Total embryos</th>
<th>Relative GFP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esr1-FL</td>
<td>57 (44%)</td>
<td>128</td>
<td>++</td>
</tr>
<tr>
<td>Esr1 (RV)</td>
<td>123 (56%)</td>
<td>219</td>
<td>++</td>
</tr>
<tr>
<td>Esr1 (Hin3)</td>
<td>78 (28%)</td>
<td>277</td>
<td>+</td>
</tr>
<tr>
<td>Esr1 (RV) mS1S2</td>
<td>34 (31%)</td>
<td>109</td>
<td>–</td>
</tr>
<tr>
<td>Esr1 (RV) mS1</td>
<td>7 (5%)</td>
<td>142</td>
<td>+</td>
</tr>
<tr>
<td>Esr1 (RV) mS2</td>
<td>39 (53%)</td>
<td>73</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) mE1E2</td>
<td>19 (32%)</td>
<td>59</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) 3xMSuH</td>
<td>10 (5%)</td>
<td>210</td>
<td>+</td>
</tr>
<tr>
<td>Esr1 (RV) mE2a</td>
<td>79 (55%)</td>
<td>142</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) mE2b</td>
<td>120 (68%)</td>
<td>176</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) mE3</td>
<td>49 (90%)</td>
<td>54</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) mE123</td>
<td>110 (72%)</td>
<td>152</td>
<td>+++</td>
</tr>
<tr>
<td>Esr1 (RV) mS4</td>
<td>22 (18%)</td>
<td>121</td>
<td>+</td>
</tr>
<tr>
<td>Esr10-FL</td>
<td>140 (45%)</td>
<td>310</td>
<td>+++</td>
</tr>
<tr>
<td>Esr10 (Dra)</td>
<td>146 (45%)</td>
<td>325</td>
<td>++</td>
</tr>
<tr>
<td>Esr10 (Pst)</td>
<td>138 (40%)</td>
<td>344</td>
<td>–</td>
</tr>
<tr>
<td>Esr10-FL mE1E2</td>
<td>15 (12%)</td>
<td>122</td>
<td>–</td>
</tr>
<tr>
<td>Esr10 (Dra) mE1E2</td>
<td>83 (40%)</td>
<td>208</td>
<td>–</td>
</tr>
<tr>
<td>Esr10 (Dra) mE2E2</td>
<td>12 (5%)</td>
<td>226</td>
<td>–</td>
</tr>
<tr>
<td>Esr10 (Dra) mS1</td>
<td>7 (5%)</td>
<td>214</td>
<td>–</td>
</tr>
<tr>
<td>Esr10 (Dra) mS2</td>
<td>13 (11%)</td>
<td>120</td>
<td>–</td>
</tr>
<tr>
<td>Esr10 (Dra) mCAAT</td>
<td>72 (52%)</td>
<td>140</td>
<td>+++</td>
</tr>
<tr>
<td>Esr7</td>
<td>28 (40%)</td>
<td>70</td>
<td>GFP+</td>
</tr>
</tbody>
</table>

Constructs are described in the text and point mutant sequences are provided in Materials and methods. Total embryos are embryos completing gastrulation following sperm injection; embryos showing skin staining indicative of non-integrated DNA or severe morphological defects were not counted.

*Any embryo showing GFP staining and therefore known to be transgenic but is not necessarily a measure of absolute transgenic efficiency.

†Level of GFP staining in neural tube of stage 19-20 frog embryos relative to non-mutant controls included in every assay.

Scores do not reflect levels of tailbud, cranial ganglia and forebrain staining. Esr10 (Dra) mE1E12 and mE2E12 are different mutations in Esr10 E-boxes (see Materials and methods); mE1E12 showed loss of neural staining but gave ectopic GFP in the heart field.
and injected with Xngnr1 mRNA showed no GFP upregulation (Fig. 4E), in support of results seen with the endogenous Hairy2 gene (Fig. 2L). Thus, we propose that in contrast to the element flanking Hairy2, Esr1 and Esr10 constitute proneural enhancers upregulated by bHLH proteins during lateral inhibition.

An intact SPS is required for Esr1 and Esr10 expression

We next asked whether Su(H)-binding sites within the SPS were required for Esr1 and Esr10 expression. S1 is absolutely conserved among Esr genes and their homologs (Fig. 5I, left) and exactly matches RTGRGAR, the optimal in vitro Su(H) site (Tun et al., 1994). Mutating the S1 G5 to C, which abrogates DNA binding in vitro (Tun et al., 1994), in either element blocked enhancer activity in transgenic frogs (Fig. 5A,B,E,F) in agreement with reports demonstrating an absolute requirement for S1 for Hes1 promoter activity in transfected cells (Jarriault et al., 1995) and Hairy2 mesodermal enhancer activity in vivo (Davis et al., 2001).

By contrast, S2 diverges among Hes5-like genes and

<table>
<thead>
<tr>
<th>Construct</th>
<th>Induced by Xngnr1</th>
<th>Total embryos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esr1 (RV)</td>
<td>75 (78%)</td>
<td>95</td>
</tr>
<tr>
<td>Esr1 (Hin3)</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>Esr1 (RV) mS1</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Esr1 (RV) mE1E2</td>
<td>35 (83%)</td>
<td>42</td>
</tr>
<tr>
<td>Esr1 (RV) 3xSuH</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>Esr10 (Dra)</td>
<td>31 (88%)</td>
<td>35</td>
</tr>
<tr>
<td>Esr10 (Pst)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Esr10 (Dra) m1E1E2</td>
<td>3 (6%)</td>
<td>47</td>
</tr>
<tr>
<td>Esr10 (Dra) m2E1E2</td>
<td>4 (7%)</td>
<td>54</td>
</tr>
<tr>
<td>Esr10 (Dra) mS1</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Hairy2 (500 bp)</td>
<td>0</td>
<td>250</td>
</tr>
</tbody>
</table>

Constructs are described in the text. Hairy2 is identical to 'H2pm' (Davis et al., 2001). *Total surviving embryos scored transgenic based on GFP signals and presence of β-gal (i.e. injected with Xngnr1 RNA). Except for wild-type Esr1 (RV), Esr10 (Dra) and H2A, this number probably greatly underestimates the number of transgenic embryos because (with the exception of the Esr1/RV construct mE1E2) deletion and point mutants show minimal GFP expression.

Table 2. Activity of deletion and point mutants following Xngnr1 injection

<table>
<thead>
<tr>
<th>Construct</th>
<th>Induced by Xngnr1</th>
<th>Total embryos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esr1 (RV)</td>
<td>75 (78%)</td>
<td>95</td>
</tr>
<tr>
<td>Esr1 (Hin3)</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>Esr1 (RV) mS1</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Esr1 (RV) mE1E2</td>
<td>35 (83%)</td>
<td>42</td>
</tr>
<tr>
<td>Esr1 (RV) 3xSuH</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>Esr10 (Dra)</td>
<td>31 (88%)</td>
<td>35</td>
</tr>
<tr>
<td>Esr10 (Pst)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Esr10 (Dra) m1E1E2</td>
<td>3 (6%)</td>
<td>47</td>
</tr>
<tr>
<td>Esr10 (Dra) m2E1E2</td>
<td>4 (7%)</td>
<td>54</td>
</tr>
<tr>
<td>Esr10 (Dra) mS1</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Hairy2 (500 bp)</td>
<td>0</td>
<td>250</td>
</tr>
</tbody>
</table>

Fig. 5. An intact SPS is required for Esr1 and Esr10 expression. S1 (I, left) is highly conserved in Esr1, Esr10 and homologous genes, and matches the optimal RTGRGAR consensus determined by Tun et al. (Tun et al., 1994). S2 of Esr1, Esr10 and several E(spl) homologs is less conserved (mismatches in red). S2 is reported as the bottom strand. Su(H) sites within the SPS of Esr1 (B,C) and Esr10 (F,G) were mutated individually (mS1 or mS2) by changing G5 to a C, and GFP expression in transgenics was monitored by in situ hybridization and compared with wild-type controls (A,E). Neural and somitomeric Esr10 expression required two intact Su(H) sites (F,G), while neural Esr1 expression required only S1 (B,C). Injection of Xngnr1 (ngn; injected side down) mRNA could not rescue GFP expression in embryos carrying S1 mutations of Esr1 (Esr1/RvmS1) (D) or Esr10 (Esr10/DramS1) (H). (J) Luciferase activity of HeLa cells transfected with Esr1/RV SPS mutants showed that whereas mS1 abrogated transcription, mS2 had no effect.

Fig. 4. Proximal elements constitute Esr1 and Esr10 proneural enhancers. Sequences driving neural GFP expression of Esr1 and Esr10 constructs are shown schematically in F. (A-E) Xngnr1 mRNA (ngn) with a lacZ tracer mRNA was injected into embryos made transgenic with sequences flanking Esr1, Esr10 and Hairy2. Embryos were stained for GFP by in situ hybridization. GFP expression driven by Esr1/RV (A) and Esr10/Dra (C) is induced by Xngnr1. Esr1/Hin3 (B), Esr10/Pst (D) and the 500 bp H2A flanking sequence (E) are not, indicating that they lack elements responsive to proneural input.

Table 2. Activity of deletion and point mutants following Xngnr1 injection

<table>
<thead>
<tr>
<th>Construct</th>
<th>Induced by Xngnr1</th>
<th>Total embryos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esr1 (RV)</td>
<td>75 (78%)</td>
<td>95</td>
</tr>
<tr>
<td>Esr1 (Hin3)</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>Esr1 (RV) mS1</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Esr1 (RV) mE1E2</td>
<td>35 (83%)</td>
<td>42</td>
</tr>
<tr>
<td>Esr1 (RV) 3xSuH</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>Esr10 (Dra)</td>
<td>31 (88%)</td>
<td>35</td>
</tr>
<tr>
<td>Esr10 (Pst)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Esr10 (Dra) m1E1E2</td>
<td>3 (6%)</td>
<td>47</td>
</tr>
<tr>
<td>Esr10 (Dra) m2E1E2</td>
<td>4 (7%)</td>
<td>54</td>
</tr>
<tr>
<td>Esr10 (Dra) mS1</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Hairy2 (500 bp)</td>
<td>0</td>
<td>250</td>
</tr>
</tbody>
</table>

Constructs are described in the text. Hairy2 is identical to ‘H2pm’ (Davis et al., 2001). *Total surviving embryos scored transgenic based on GFP signals and presence of β-gal (i.e. injected with Xngnr1 RNA). Except for wild-type Esr1 (RV), Esr10 (Dra) and H2A, this number probably greatly underestimates the number of transgenic embryos because (with the exception of the Esr1/RV construct mE1E2) deletion and point mutants show minimal GFP expression.
between Esr1 and 10, and S2 sites of several HES homologs constitute potentially suboptimal binding sites (Fig. 5I, right). Mutating the S2 G5 to C in Esr1/RV or Esr10/Dra revealed a significant difference between the two: mutating the Esr1 S2 (Fig. 5A,C) had no effect on GFP expression, while mutating the Esr10 S2 (Fig. 5E,G) strongly blocked GFP staining in neural tissue. These observations suggest that the Esr1 S2 is not a Su(H)-binding site in vivo and were supported by transfection analysis of Esr1/RV showing that mutant S2 had little effect on ICD-mediated transcription, while mutating S1 blocked activation (Fig. 5J). These findings indicate that S1 and S2 of Esr10 probably constitute a bona fide SPS, while analogous sequences of Esr1 resemble the SPS but contain only a single functional Su(H) site (S1). For the sake of simplicity, however, we refer to this motif in Esr1 as an ‘SPS’ although it is technically a misnomer.

Loss of Esr1 and Esr10 enhancer activity following S1 mutation indicates that proneural expression of both requires direct Notch input through this site. Therefore, we asked whether enhancer activity of S1 mutants could be induced by ectopic Xngnr1. Xngnr1 injection into embryos transgenic with S1 mutants of Esr1/RV or Esr10/Dra did not drive GFP expression in either case (Fig. 5D,H), indicating that bHLH input and/or high levels of Notch signaling driven by Xngnr1 cannot rescue enhancer activity in the absence of S1 function.

The Esr10 neural enhancer requires intact E-boxes

The Esr1 or 10 SPS is necessary but not sufficient for enhancer activity. To identify potential heterologous inputs, we searched for motifs conserved between both enhancers or for candidate transcription factor binding sites (using Matinspector from www.genomatix.com). Among the latter, we found E-boxes (CANNTG) (binding sites for bHLH proteins) and several consensus sites for Sox and NF-Y factors. Mutating the latter produced little effect (Table 1; data not shown). Therefore, we focused on E-boxes, as they are required for proneural expression of several Drosophila E(spl) genes (Kramatschek and Campos-Ortega, 1994; Nellesen et al., 1999; Cooper et al., 2000; Cave et al., 2005; Reeves and Posakony, 2005).

Esr10/Dra contains two E-boxes (Fig. 6A, upper), both of which are conserved in the Xenopus tropicalis (Xt) Esr10 gene, although the Xt E1 is CAAATG. To determine if E-boxes responded to proneural input, both were mutated and assayed in transfection assays (Fig. 6A, lower). Luciferase reporters driven by wild-type Esr10/Dra were inactive when induced by exogenous Xngnr1 plus E47 alone and were activated approximately threefold by exogenous ICD. Co-transfection of both factors synergistically activated transcription approximately threefold over ICD alone (Fig. 6A, left). Synergy was lost when mE1E2 constructs were assayed (Fig. 6A, right), demonstrating that E-boxes are required for this activity.

Next, we asked if intact E-boxes were required for expression in transgenics. E-box mutants of Esr10/Dra drove markedly reduced GFP expression relative to controls in neural tissue in vivo (Fig. 6B,C). Mutation of both sites also abrogated mesodermal GFP expression (Fig. 6C; data not shown). To confirm that E-boxes are required for enhancer activity, we misexpressed Xngnr1 in transgenic embryos and evaluated GFP expression in embryos harboring wild-type or mutant enhancers. Following Xngnr1 misexpression, mutant enhancer activity was greatly attenuated relative to controls (Fig. 6D,E), almost as severely as that of Esr10/Pst (see Fig. 3D), which lacks both E1 and E2. These results indicate that the insufficiency of Esr10/Pst is due in part to lack of E-box input and that high levels of proneural activity cannot compensate for that loss.

Finally, we asked whether proneural proteins bind in vitro to E-box sequences present in Esr10/Dra. The sequence of Esr10 E2 (cCAGATGc) resembles the reported ‘high affinity’ bHLH site (cCAGSTG) targeted by Drosophila proneural proteins (Nellesen et al., 1999) and exactly matches the required NeuroM/E47 binding site in the HB9 enhancer (Lee and Pfaff, 2003). EMSA analysis showed a robust shift of an E2 oligonucleotide against ICD alone (A, left). Synergy was lost when E-boxes were mutant (A, right). E-box motifs were also required for GFP expression driven by Esr10/Dra in transgenic frogs (compare C with B). (D,E) Injection of Xngnr1 mRNA with a lacZ tracer into Esr10/Dra transgenic embryos (E) could not activate enhancer activity as was seen with controls. (F) EMSA showing that Xngnr1 (N) and E47 (E) proteins shift an E2 oligo; shifts were competed by 10× and 100× cold competitor (WT) but not by similar increases mutant E2 oligos (Mut) or oligos corresponding to a binding site of a heterologous activator (Vax) (Mui et al., 2005). O, oligo; R, reticulocyte lysate; N/E, Xngnr1 plus E47. Complexes formed by E47 homodimers (Ex2) are of higher mobility than those formed by Xngnr1/E47 (N/E) heterodimers. ns, nonspecific complexes attributable to reticulocyte proteins.

Fig. 6. Esr10 proneural enhancer activity requires intact E-boxes. HeLa cells were transfected with expression vectors for ICD, Xngnr1 and E47 (N/E), or both, and with luciferase vectors driven by wild-type (Esr10/Dra) or mutated (Esr10/DraE1E2) elements, shown schematically above (A). In the case of Esr10/Dra, ICD and N/E synergistically activate transcription approximately three times more over
Development 132 (16) 3626

Research article

Fig. 7. The Esr1 enhancer does not require E-boxes and responds to Notch through two loci. (A) Luciferase activity of Esr1/RV and Hin3 fragments co-transfected with activated Notch (ICD) plus or minus Xngnr1 (ngn). (B) Luciferase activity of Esr1/RV and Esr1/Hin3 vectors co-transfected with ICD compared with proximal elements from mouse Hes1 (Jarriault et al., 1995) and Xenopus Hairy2 (Davis et al., 2001), Esr10/Dra and a vector containing eight multimerized Su(H) sites (Ling et al., 1994). Cells were transfected simultaneously with equal levels of ICD (100 ng/well) relative to the reporter (100 ng/well). (C) Luciferase activity of Esr1/RV co-transfected with increasing (25 ng/well and 100 ng/well) levels of ICD compared with a construct in which all upstream Su(H) sites are mutant (Esr1/RV mS3-5) or the Esr10/Hin3 deletion mutant. Unlike the wild-type reporter, luciferase activity of the Su(H) and Hin3 mutant constructs saturates at low (25 ng) ICD levels. (D) An S4 mutation results in loss of transcription similar to Esr1/RV mS3-5.

by Xngnr1/E47 heterodimers, which was specific and not competed by the mutant E2 oligonucleotide (Fig. 6F). We also observed shifts of E2 by heterodimers containing the atonal homologs mouse NeuroD and Xenopus Ath3 (data not shown). By contrast, under identical conditions, heterodimers of Ath3 (data not shown). Xngnr1/E did not shift an E1 oligo nor did the E1 oligo efficiently compete for Xngnr1/E47 binding to E2 (data not shown). Taken together, these observations indicate that factors encoded by proneural genes drive neural Esr10 expression both by activating Notch signaling and through direct interaction with bHLH-binding sites, most probably the E2 site.

Neural expression of Esr1 does not require intact E-boxes

Esr1/RV has three E-boxes (Fig. 7A, top), two (E1 and E2) conserved in Xt Esr1 and one (E3) that is not. To determine if these motifs mediate synergy between proneural proteins and ICD (similarly to Esr10) we undertook transfection assays. Xngnr1 alone did not activate Esr1/RV-luciferase nor was synergy observed between ICD and Xngnr1 on Esr1/RV or on the Esr10/Hin3 deletion mutant, which includes E2 (Fig. 7A). Interestingly, high levels of ICD drove Esr1/RV luciferase activity approximately 100-fold over reporter alone, levels 10 times greater than those seen in comparable assays of Esr10/Dra and other ICD-responsive Hes genes (Fig. 7B). Such levels approached those seen using multimerized Su(H) site vectors (Fig. 7B). Thus, in cultured cells, Esr1/RV behaves differently from Esr10/Dra, both in lack of direct response to Xngnr1 and responsiveness to ICD.

We next asked whether Esr1/RV E-boxes were required in vivo. E1, E2 and E3 were mutated in Esr1/RV, and the construct (Esr1/RV mE1E2E3) assayed for GFP expression. In contrast to Esr10/Dra, GFP expression in frogs carrying Esr1/RV mE1E2E3 was equivalent to controls (Fig. 8A,B). Likewise, misexpressed Xngnr1 robustly upregulated activity of Esr1/RV mE1E2 (Fig. 8D), similar to controls (Fig. 8C). These observations show that intact E-boxes are not required for Esr1/RV expression, indicating that factors induced by Xngnr1 and directly activating the Esr1 enhancer are probably not bHLH proteins. Overall, these observations, together with the differential activities of the SPS motifs, indicate that although responsive to both Notch and Xngnr1, the activity of proneural enhancers of Esr1 and Esr10 differs mechanistically.

Neural Esr1 expression requires upstream Notch input

Loss of robust responsiveness to ICD seen with the Esr10/Hin3 deletion mutant (Fig. 7A,C) suggests that ICD activates sequences between RV and H3. Three potential Su(H) sites (S3-S5) are clustered in that region (Fig. 7A). Mutating all three (Esr1/RV mS3-5) reduced luciferase activity in cultured cells to a level comparable with that seen with Esr10/Hin3 (Fig. 7C), indicating that at least one of them responds to ICD. Intact S3-S5 sites were also necessary in vivo: transgenic frogs carrying S3-S5 mutations showed highly attenuated GFP expression in neural tissue relative to controls (Fig. 8E), again comparable with the weak activity mediated by Esr10/Hin3 (Fig. 3E). Injection of Xngnr1 mRNA into mS3-5 transgenic embryos failed to rescue GFP expression (Fig. 8F).

Within the S3-S5 cluster, S4 is highly conserved in position and orientation in orthologous genes (Fig. 8H). Mutating S4 alone abrogated enhancer activity both in transfected cells (Fig. 7D) and in vivo (Fig. 8G), indicating that it is required for high levels of Notch-mediated transcription and for enhancer activity in vivo. Taken together, these observations indicate that the distal 216 bp of Esr1/RV are required for Xngnr1 to activate Esr1 enhancer activity. Failure of Xngnr1 to activate the mS3-5 or S4 construct indicates that at least some inputs to that region are activated Notch itself (see Discussion).

Discussion

Both the Hes1-like and the Hes5-like subfamilies of bHLH repressors have been proposed to regulate neurogenesis in vertebrate embryos as Notch targets. Members of these subfamilies, however, show marked differences in their expression patterns in neural precursors, suggesting that they are activated in combination with other inputs according to their function. In Xenopus, the Hes5-like genes, Esr1 and Esr10,
Differential regulation of Notch targets

Differential regulation of Notch targets

Identification of proneural enhancers

Analysis of a mesodermal enhancer of Hairy2A (Davis et al., 2001) and those contained in Esr1/RV and Esr10/Dra indicates that elements required for expression in neural precursors are localized close to the transcription start site. Aligning the proximal sequences of these enhancers reveals a conserved region, situated ~80 nucleotides upstream of the transcription start site, that contains an SPS, or remnant thereof, and an upstream inverted CCAAT motif. This region is seen in both Hes1- and Hes5-like family members in various vertebrate species (Gajewski and Voolstra, 2002), suggesting that an ancestral bHLH repressor gene responded to transcriptional input through these core elements. Despite this common feature, however, the neural enhancers of Hes1- and Hes5-like genes have clearly diverged, resulting in a situation in which this common element interacts with other factors to regulate expression of these genes in neural precursors. For example, the Hairy2 promoter is CpG-rich ChIP by antibodies to the repressor MeCP2 (Stancheva et al., 2003), while the Esr proximal elements exhibit no CpG islands (analyzed using http://cpgislands.usc.edu). Indeed, decreasing MeCP2 activity derepresses Hairy2 but has no effect on Esr1 expression (Stancheva et al., 2003), suggesting that epigenetic regulation is one factor leading to differential expression of bHLH repressors. In addition, we show here that both the conserved SPS as well as flanking sequences have also diverged not only between the Hes1- and Hes5-like enhancers but also between enhancers of genes in the Hes5-like family with similar but distinct expression patterns.

Proneural enhancers of Esr1 and Esr10 exhibit structural hallmarks of Notch targets

Both Esr1 and Esr10 require at least two functional Su(H)-binding sites for expression in neural precursors and to respond to ectopic proneural activity, but differ in how these sites are arranged. In Esr10, these two sites are configured in the classic inverted repeat SPS motif located at ~84, highlighting the importance of this motif to Notch responsiveness. In this aspect, the Esr10 SPS resembles that of Hairy2 (Davis et al., 2001), which also requires both S1 and S2 in the SPS for mesodermal expression within somitomeres. Indeed, Esr10/Pst, which consists primarily of an SPS, drives faint somitomeric reporter expression reminiscent of Hairy2 (Fig. 3H, Fig. 4D), in agreement with the findings of Davis et al. (Davis et al., 2001) that two functioning Su(H) sites in an SPS configuration are sufficient for somitomeric expression.

By contrast, the Esr1 SPS has diverged, such that S1 is conserved while S2 is predicted to not bind Su(H), to not be required for Notch activation in transient transfection assays (Fig. 5J) and to not be required for proneural enhancer activity (Fig. 5C). Instead, we found that an upstream Su(H) site (S4) among a cluster of three potential sites is required in S1 for Esr1 expression (Figs 7, 8) and to respond to proneural activity. Interestingly, S4 is spatially conserved relative to S1 in several Esr1/Hes5 orthologs (Fig. 8H). Furthermore, S2 of mouse Hes5, like that of Esr1, is potentially a suboptimal binding site (Fig. 5L, right), suggesting that Notch activation of Esr1 orthologs may require Su(H) sites in an S4-S1 configuration rather than in the ‘classical’ SPS configuration. It will be of interest to determine whether the spacing and orientation of the S4-S1 Su(H)-binding sites can substitute for Notch in other Hes5 orthologs.

Numerous vertebrate E(spl) genes, including Esr1, Esr7, Esr10, Hairy2, and chick, mouse and fish homologs exhibit inverse CCAAT motifs flanking the SPS, and Sox1 represses
**Hes1** promoter-dependent luciferase activity in transfection assays through this site (Kan et al., 2004). Mutation of the **Esr10** CCAAT resulted in **GFP** expression that was extremely robust (Table 1, mCAAT) but not quantifiably more so than controls. This discrepancy may reflect differences in transcriptional regulation of **Hes1** and **Esr10** or differences in assay sensitivity.

**Esr10 and Esr1 are differentially regulated by bHLH proteins**

Our data indicates that proneural bHLH input to the **Esr10** enhancer is both indirect (through Notch) and direct (Fig. 6). ICD and Xngnr1 synergistically upregulate transcription in transfection assays, Xngnr1 binds to the **Esr10** downstream E-box in vitro, and the **Esr10** proneural enhancer with mutant E-boxes shows marked loss of activity in vivo, which cannot be rescued by exogenous Xngnr1. These findings extend observations in **Drosophila** that proneural proteins synergize with Notch in activating E(spl) genes in larval discs (Kramatschek and Campos-Ortega, 1994; Bailey and Posakony, 1995; Cooper et al., 2000). Our data also support analysis of the **Drosophila** E(spl) gene **m8** (Cave et al., 2005). In that case, E boxes and Su(H) sites in only the configuration of a classical SPS enabled synergy between ICD and bHLH proteins, and enhancer activity was lost when one Su(H) site was mutant or oriented incorrectly. The **Esr10** proneural enhancer behaves similarly in transgenics and provides the first example of such a required architecture among vertebrate Notch targets.

By contrast, **Esr1** is not directly regulated by proneural proteins. Although **Esr1/RV** has three E-boxes, E3 is not conserved in Xr, E1 is not conserved in the proneural enhancer of the closely related **Esr7** gene (E.L. and C.K., unpublished), and neither E1 nor E3 fits the RCAGSTG consensus required for high-affinity binding of **Drosophila** proneural proteins to E-boxes (Van Doren, 1991). However, the CACCTG motif seen in E2 is targeted by **Drosophila** proneural proteins (Powell et al., 2004), a CACCTG E-box is required for retinal expression of **Xenopus** Ath5 (Hutcheson et al., 2005), and CACCTG binds MyoD in vitro and in vivo (Yutzey and Konieczny, 1992). Furthermore, E2 is embedded in a 13-base homology extending beyond the E-box in numerous **Hes5** orthologs, although it is not seen in the **Esr10** promoter. We mutated E2 using two strategies and saw no effect on transgene expression in vivo (see Materials and methods and Table 1 (oligos mE2a and mE2b). Further mutation may be required to evaluate the contribution of this motif to **Esr1** expression. Nonetheless that E2 is contained within **Esr1**/Hin3 (Fig. 6A) rules out the possibility that any factor binding to E2 is sufficient (with Notch acting through S1) to activate robust enhancer activity.

We have not identified sites required for proneural **Esr1** expression other than Su(H) sites. Su(H) sites could be sufficient to activate **Esr1**, and tissue-specific responses to Notch might be due either to tissue-specific repressors or to the spacing of Su(H) sites providing a distinct platform for co-activators. Alternatively, Su(H) sites in the **Esr1** enhancer could synergize with heterologous (non-bHLH) factors induced by Xngnr1, which, unlike direct bHLH input to either **Esr10** or **m8**, interact with Notch through an S1-S4 configuration of Su(H) sites. Finally, enhancer activity could require input from both Notch (dependent on Xngnr1) and neural factors not dependent on Xngnr1. Although all three scenarios are possible, observation of attenuated but spatially appropriate **GFP** expression driven by **Esr1**/Hin3 argues against Su(H) site spacing as the sole determinant of specificity and suggests rather that tissue specific input to **Esr1** requires sequences downstream of Hin3.

**Why does transcriptional regulation of Esr1 and 10 differ?**

Although regulation of **Esr10** reflects **Drosophila** models of E(spl) regulation, **Esr1** represents a novel paradigm by which effectors of lateral inhibition are regulated differently both in terms of Su(H) configuration and direct bHLH input. The lack of dependence of the **Esr1** enhancer on direct E-box input may in fact indicate that the S1-S4 configuration precludes interactions of Notch with E-box-binding proteins. Why such similarly expressed genes should be differentially regulated is unclear.

A fundamental difference between **Esr1** and **Esr10** is that **Esr10** is also expressed in the presomitic mesoderm. Our observations and mechanistic analysis of **Hairy2** (Davies et al., 2001) indicates that in these genes, enhancers responsible for expression in differing developmental contexts are spatially intermixed on very short genomic stretches rather than being entirely separable on dispersed elements. Mesodermal **Esr10** expression could also require combinatorial input from bHLH factors and Notch. Data reported here indicates that tailbud **Esr10** expression is abolished in E-box mutants (Fig. 6). We also observed synergistic interaction of mesodermal bHLH proteins with ICD in luciferase assays (E.L. and C.K., unpublished). Alternatively, E-box/Su(H)/Notch interactions may be required for cyclic transcription of **Esr10**. In either case, combinatorial interactions required for mesodermal **Esr10** transcription could have been co-opted in neural contexts. Those same interactions would not be necessary for genes such as **Esr1**, which are expressed in a predominantly neural context.

Alternatively, Esr genes could play different roles in lateral inhibition. Direct regulation of E(spl) genes by bHLH proteins is counterintuitive, given that for a cell to be inhibited from adopting any fate requires downregulation of factors regulating that fate (Heitzler et al., 1996). Therefore a different subset of Notch effectors (such as **Esr10**) might be required to initiate an inhibited state, while others (such as **Esr1**) could maintain it. Such a scenario is analogous to the apparent sufficiency of low levels of bHLH activators to broadly upregulate **Delta** prior to its restriction to selected cells (Kooh et al., 1993; Karp and Greenwald, 2003). Support for this hypothesis will require a single-cell comparison of **Esr1** and **Esr10** expression at high temporal resolution during the process of lateral inhibition, a challenging problem technically. Nonetheless, we observe differences in how **Esr1** and **Esr10** respond transcriptionally to both proneural and Notch input in transfection assays (Fig. 6) and in animal cap assays (Fig. 2). Further analysis of these differences and how these enhancers are tuned to respond to Notch will be important for ultimately understanding their function during neurogenesis and segmentation.

We thank Andy Stevenson for technical assistance; Drs Anne Bang, Christy Fryer, Brian Mitchell, Tanya Moreno and Jennifer Stubbs for discussions and critical reading of the manuscript; Dr Jim Posakony
Developmental regulation of Notch targets


reveals that anteroposterior patterning controls the timing of neuronal differentiation in *Xenopus* neurectoderm. *Development* 122, 3409-3418.


