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

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**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

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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 Hairp and Hes5-like repressors. A hairy homolog, *Xenopus Hairy2*, is expressed during gastrulation (Tsugita 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 *Hairy2* expression has been characterized (Davis et al., 2001). That element drives *Hairy2* 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 *Hairy2* regulatory element, *Esr* gene enhancers are upregulated by *Xngnr1*, 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 staged according to Nieuwkoop and Faber, 2003). In addition to those described in the text, reporters included *Xenopus Hairy2* (Davis et al., 2001), *Hes1* (Jarriault al., 1995) and multitemplated Su(H)-binding sites (Ling et al., 1994). Transfection efficiency was assessed using either co-transfected lacZ expression vectors and ONPG substrate (Sigma) or tk-REnilla reporters.

**Identification of promoter elements and transgenic methods**

Proximal elements were obtained as described (Moreno and Kintner, 2004) and cloned upstream of GFP in a vector containing the 700 bp *Hairy2* 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.

**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. 2.I). At early neurula stages, Hairy2 is also expressed in the neural
tube in a narrow stripe of progenitors located along the dorsoventral axis (data not shown).

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'). 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 neuralized 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 *Esr10* 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*, *Esr10* and *Hairy2* (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 somitomeric (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 somitomeric 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 GFP as a reporter (Fig. 3B). Each genomic fragment carried 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-Esr1 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 –234 in Esr10 (Esr10/RV) drove neural (and in Esr10, mesodermal) GFP expression indistinguishable in pattern and intensity from FL constructs (Fig. 2A,D; Fig. 3C,D,F,G; 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/RV (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 – is likely that Esr10/RV 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 (Koyanova-Nakagawa et al., 1999) (Fig. 2C,F). Therefore, we injected mRNA encoding Xngnr1 and a β-galactosidase tracer into one blastomere of two-cell embryos that were transgenic for Esr1/RV or Esr10/RV, and asked whether embryos showed ectopic GFP expression. In both cases, GFP expression was expanded, although, in general, Esr10/RV 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>
<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%) 128</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV)</td>
<td>123 (56%) 219</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (Hin3)</td>
<td>78 (28%) 277</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mS1S2</td>
<td>34 (31%) 109</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mS1</td>
<td>7 (5%) 142</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mS2</td>
<td>39 (53%) 73</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mE1E2</td>
<td>19 (32%) 59</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) 3xmSuH</td>
<td>10 (5%) 210</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mE2a</td>
<td>79 (55%) 142</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mE2b</td>
<td>120 (68%) 176</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mE3</td>
<td>49 (90%) 54</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mE123</td>
<td>110 (72%) 152</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr1 (RV) mS4</td>
<td>22 (18%) 121</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Esr10-FL</td>
<td>140 (45%) 310</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra)</td>
<td>146 (45%) 325</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Pst)</td>
<td>138 (40%) 344</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10-FL m1E1E2</td>
<td>15 (12%) 122</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra) m1E1E2</td>
<td>83 (40%) 208</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra) m2E1E2</td>
<td>12 (5%) 226</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra) mS1</td>
<td>0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra) mS2</td>
<td>13 (11%) 120</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Esr10 (Dra) mCAAT</td>
<td>72 (52%) 140</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Esr7</td>
<td>28 (40%) 70</td>
<td>GFP+</td>
<td></td>
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</tbody>
</table>
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/RV and Esr10/Dra 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 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) 3xmSuH</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 (rCAGSTG) 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
Development

Three potential Su(H) sites (S3–S5) are clustered in that region (Fig. 7A). Mutating S3–S5 mutations showed highly attenuated activity in vivo. Taken together, these observations indicate that factors induced by proneural genes drive neural *Esr1* 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 *Esr1/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/RVmE1E2E3*) assayed for GFP expression. In contrast to *Esr10/Dra*, GFP expression in frogs carrying *Esr1/RVmE1E2E3* was equivalent to controls (Fig. 8A,B). Likewise, misexpressed *Xngnr1* robustly upregulated activity of *Esr1/RVmE1E2* (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 *Esr1/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/RVmS3–S5*) reduced luciferase activity in cultured cells to a level comparable with that seen with *Esr1/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 similar with the weak activity mediated by *Esr1/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*,
probably function in lateral inhibition, which operates downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in embryos and neuralized ectoderm in response to ectopic differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. 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Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. Accordingly, these genes are upregulated in downstream of proneural proteins to limit neuronal differentiation. According to 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 McCP2 (Stancheva et al., 2003), while the Esr proximal elements exhibit no CpG islands (analyzed using http://cpgislands.usc.edu). Indeed, decreasing McCP2 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.

Proencephal 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 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 are also crucial for response to 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...
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 Xnegr1 synergistically upregulate transcription in transfection assays, Xnegr1 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 exogenously Xnegr1. 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 Xt, 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 Xnegr1, 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 Xnegr1) and neural factors not dependent on Xnegr1. 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 (Duvi 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.

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