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 co-repressors 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 Hairy and Hes5-like repressors. A *hairy* homolog, *Xenopus Hairy2,* is expressed during gastrulation (Tsuji 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 neurectodermal 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 stained by whole-mount in situ hybridization with digoxygenin-labeled probes (Harland, 1991), including *Esr1* (Wettstein et al., 1997), *Esr7* (Deblandre et al., 1999), *Esr10* (Li et al., 2003), *GFP* and *Hairy2* (Turner and Weintraub, 1994).

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.

Transgenic frogs were generated using standard (Amaya and Kroll, 1999; Sparrow et al., 2000) protocols. In addition, we delayed activation by injecting oocytes in Ca^{2+}-free injection buffer and activating them following injection by incubation in 0.1×MMR plus Ca^{2+} 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 *Dpn*I digestion of the parent plasmid.

Oligonucleotides to mutate *Esr1* were (mutant nucleotides underlined): mS1, GCTAAACGAGTGTGGCAAAGTGTAGCAGGTTTG; mS2, GTAGCAGGTTTGGGAGTCATGCATTAGTATGCG; mS4, GATGGGAATCTCTTTGCCACGTTCTCCCACCTC; mE1, GCCCTATTGTACAACCTCTTGTTATACCAAATTACGTG; mE2a, TGTAACACACTCTCAACCTTCTCCACTGGGAGC; 3xmSu(H), GATTATAGTGATGGCAATCTCTTTGCCACGTTCTGCCACCTC; mE2b, GTGTAACACACTCTGAAGGTTTCCACTGGGAGCAG; mE3, GCTCCACAGCTCATATCCTCTCCAGCACTAGC.

Oligonucleotides to mutate *Esr10* were: m1E1, GTATCTCAGTGTCCGGATTTCCCACACTTC; m1E2, TGTTCAGGGCTCTCCGGACCACCCTTAATG; m2E1, TAGTATCTCAGTGCCAGTCTTTCCCACACTTCCCCTC; m2E2, ATTGTTCAGGGCTCCCGATTCCACCCTTAATGTGACAC; mS1, GCTACTGAGTGTGGCAACCTCTGCTCAGCC; mS2, CTCAGCCTGATCCTGACACATTATTATGCA; mCAAT, CTGCAGGGCTGGGTCGAGCTACTGAGTGTG.

Animal cap assay

RNA injection, preparation of neuralized caps, RNAse protection assay, and probes for *Esr1* and *EF1α* 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 Bam, 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), Xngnr1 (Ma et al., 1996) and E47 (Lee and Pfaff, 2003). In addition to those described in the text, reporters included *Xenopus Hairy2* (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 *lacZ* 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 [^{32}P]dCTP using Klenow to a specific activity of 2×10^6 CPM/pmol. Heterodimers were preincubated 30 minutes at room temperature prior to binding. Binding reactions included $1\text{-}5\times10^5$ 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,

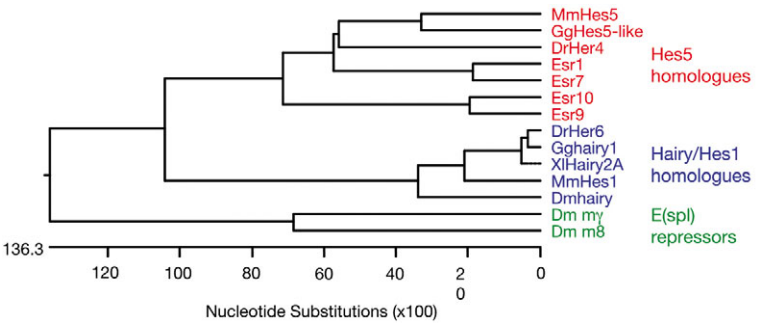


Fig. 1. *Xenopus* Esr proteins are orthologs of HES proteins. Tree compares primary sequence of bHLH domain. Esr1 and Esr7 are orthologs of mouse (Mm) Hes5, zebrafish (Dr) Her4 and a chick (Gg) Hes5-like protein. Esr9 and Esr10 form a subgroup within this family. MmHes1 is homologous to *Drosophila*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

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

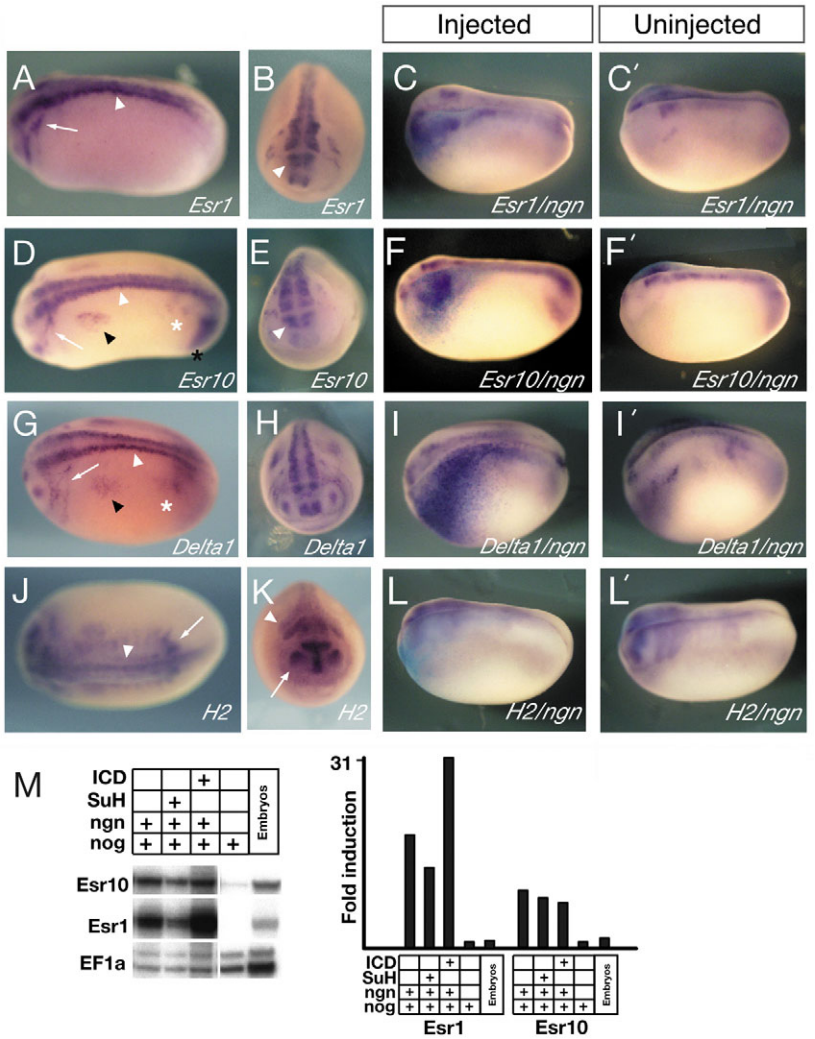


Fig. 2. *Esr1* and *Esr10* are expressed in proneural domains. *Esr1*, *Esr10* and *Delta* are expressed in the neural tube (A,D,G, white arrowhead), cranial ganglia (A,D,G, arrow) and anterior neural tube (B,E, arrowhead). *Esr10* is also expressed in the tailbud (D, black asterisk) and somitomeres (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 uninjected 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.

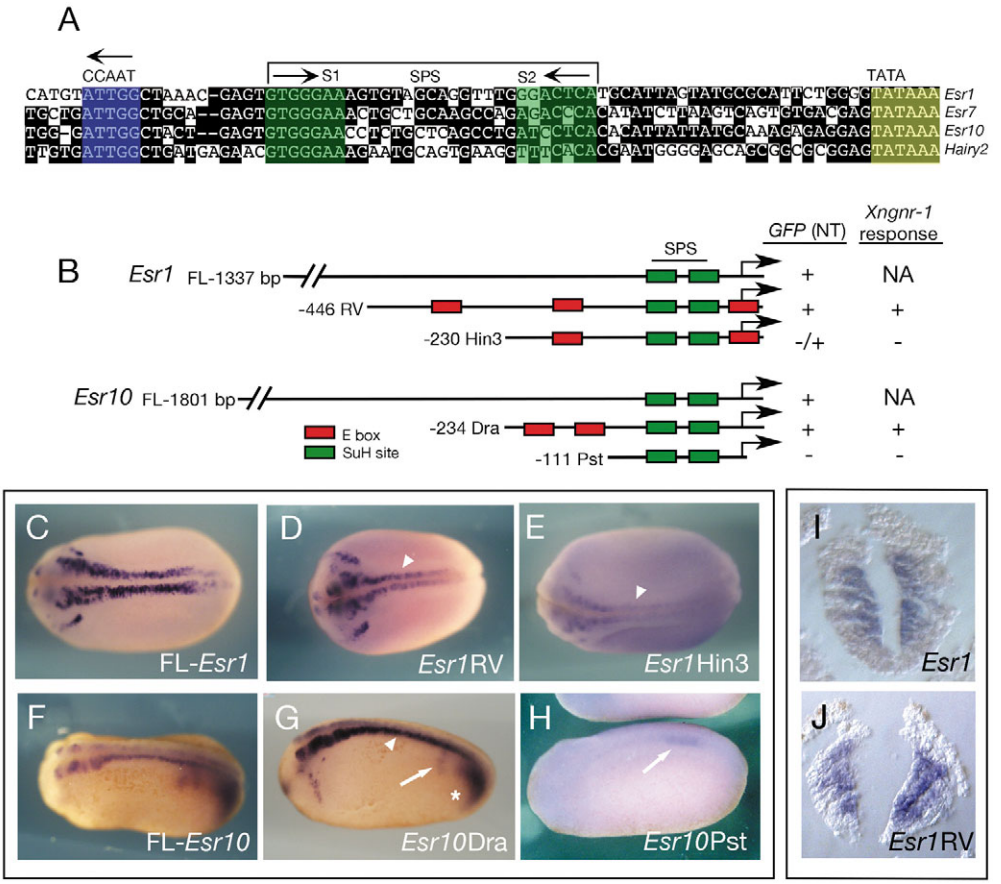


Fig. 3. Compact elements drive neural *Esr* gene expression. (A) Promoters of *Esr1*, *Esr7*, *Esr10* and *Hairy2* (Davis et al., 2001) show high and moderate homology of S1 and S2, respectively, in the SPS (green). All exhibit a conserved CCAAT motif (blue). *GFP* expressed by deletion mutants of *Esr1* (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).

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′) 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 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 *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* (Fig. 3A; see Materials and methods). Each of these sequences exhibits paired Su(H) sites resembling an SPS proximal to the TATA box, as seen in several *E(spl)* genes and vertebrate homologs (Jarriault et al., 1995; Bailey and Posakony, 1995; Nellesen et al., 1999; Gajewski and Voolstra, 2002); the upstream S1 site is highly conserved among *Esr1*, *Esr7*, *Esr10* and *Hairy2* (Davis et al., 2001), which is shown for comparison (Fig. 3A). However, S2 is variable and deviates from the Su(H) consensus site (see below). All SPS elements are flanked by an inverse CCAAT-type motif (Fig. 3A) seen in numerous vertebrate *E(spl)* homologs (Gajewski and Voolstra, 2002). Homology among

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*/Dra) 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*/Dra (data not shown). Further deletion of 216 bp in *Esr1* (*Esr*/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 a 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

Construct	Detectable *GFP**	Total embryos	Relative *GFP* expression†
Esr1-FL	57 (44%)	128	+++
Esr1 (RV)	123 (56%)	219	+++
Esr1 (Hin3)	78 (28%)	277	+
Esr1 (RV) mS1S2	34 (31%)	109	–
Esr1 (RV) mS1	7 (5%)	142	–
Esr1 (RV) mS2	39 (53%)	73	+++
Esr1 (RV) mE1E2	19 (32%)	59	+++
Esr1 (RV) 3xmSuH	10 (5%)	210	+
Esr1 (RV) mE2a	79 (55%)	142	+++
Esr1 (RV) mE2b	120 (68%)	176	+++
Esr1 (RV) mE3	49 (90%)	54	+++
Esr1 (RV) mE123	110 (72%)	152	+++
Esr1 (RV) mS4	22 (18%)	121	+
Esr10-FL	140 (45%)	310	+++
Esr10 (Dra)	146 (45%)	325	+++
Esr10 (Pst)	138 (40%)	344	–
Esr10-FL m1E1E2	15 (12%)	122	–
Esr10 (Dra) m1E1E2	83 (40%)	208	–
Esr10 (Dra) m2E1E2	12 (5%)	226	–
Esr10 (Dra) mS1	0	214	–
Esr10 (Dra) mS2	13 (11%)	120	–
Esr10 (Dra) mCAAT	72 (52%)	140	+++
Esr7	28 (40%)	70	*GFP*+

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) m1E1E12 and m2E1E2 are different mutations in *Esr10* E-boxes (see Materials and methods); m1E1E12 showed loss of neural staining but gave ectopic *GFP* in the heart field.

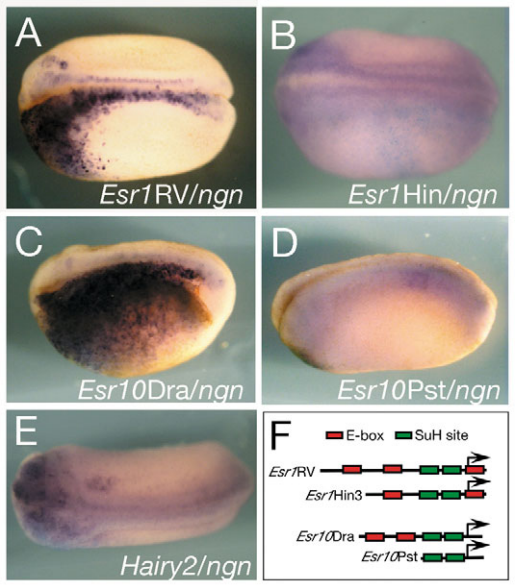


Fig. 4. Proximal elements constitute *Esr1* and *Esr10* proneural enhancers. Sequences driving neural *GFP* expression of *Esr1* and *Esr10* constructs are shown schematically in F. (A-E) *Xngnr1* mRNA (ngn) with a *lacZ* tracer mRNA was injected into embryos made transgenic with sequences flanking *Esr1*, *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 *H2* flanking sequence (E) are not, indicating that they lack elements responsive to proneural input.

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.

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

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

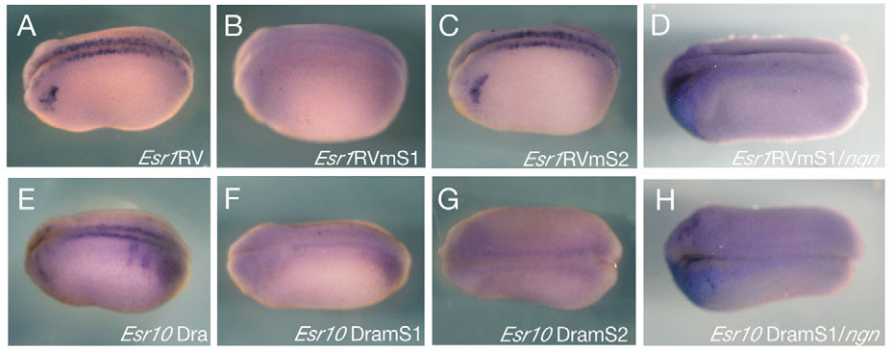
Constructs are described in the text. *Hairy2* is identical to 'H2pm' (Davis et al., 2001).

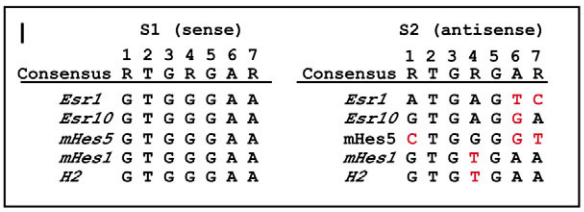
*Total surviving embryos scored transgenic based on *GFP* signals and presence of β-gal (i.e. injected with *Xngnr1* RNA). Except for wild-type *Esr1* (RV), *Esr10* (Dra) and H2A, this number probably greatly underestimates the number of transgenic embryos because (with the exception of the *Esr1*/RV construct mE1E2) deletion and point mutants show minimal *GFP* expression.

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





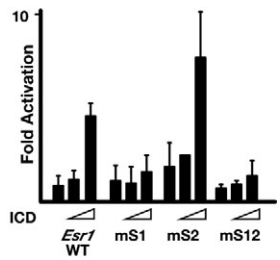


Fig. 5. An intact SPS is required for *Esr1* and *Esr10* expression. S1 (I, left) is highly conserved in *Esr1, Esr10* and homologous genes, and matches the optimal RTGRGAR consensus determined by Tun et al. (Tun et al., 1994). S2 of *Esr1*, *Esr10* and several *E(spl)* homologs is less conserved (mismatches 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.

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

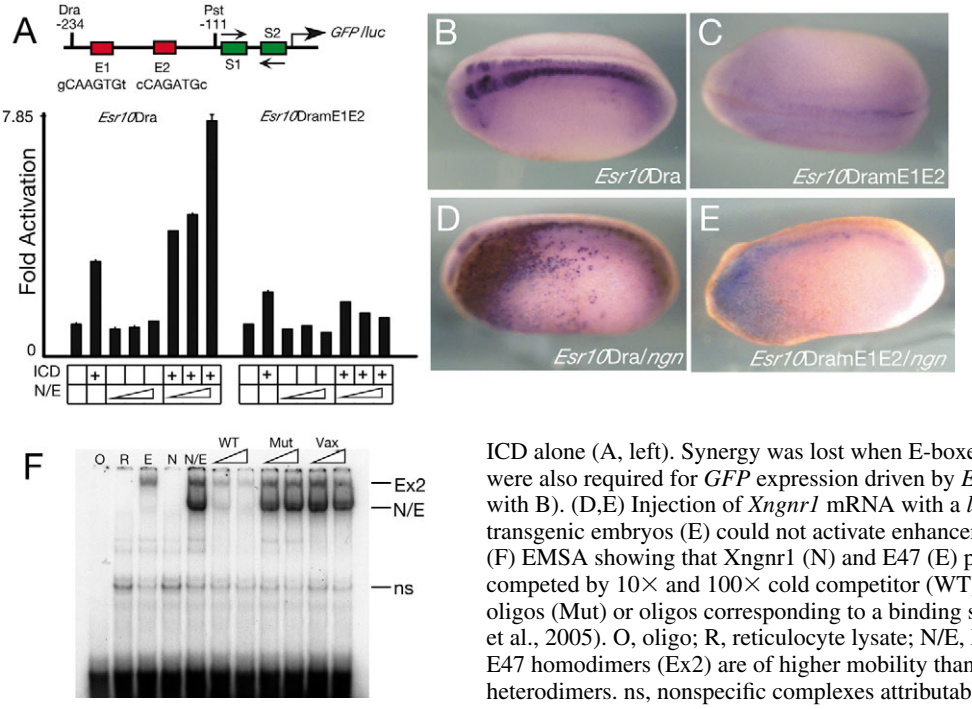


Fig. 6. *Esr10* proneural enhancer activity requires intact E-boxes. HeLa cells were transfected with expression vectors for *ICD*, *Xngnr1* and *E47* (N/E), or both, and with luciferase vectors driven by wild-type (*Esr10*/Dra) or mutated (*Esr10*/DramE1E2) elements, shown schematically above (A). In the case of *Esr10*/Dra, ICD and N/E synergistically activate transcription approximately three times more over 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*/DramE1E2 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.

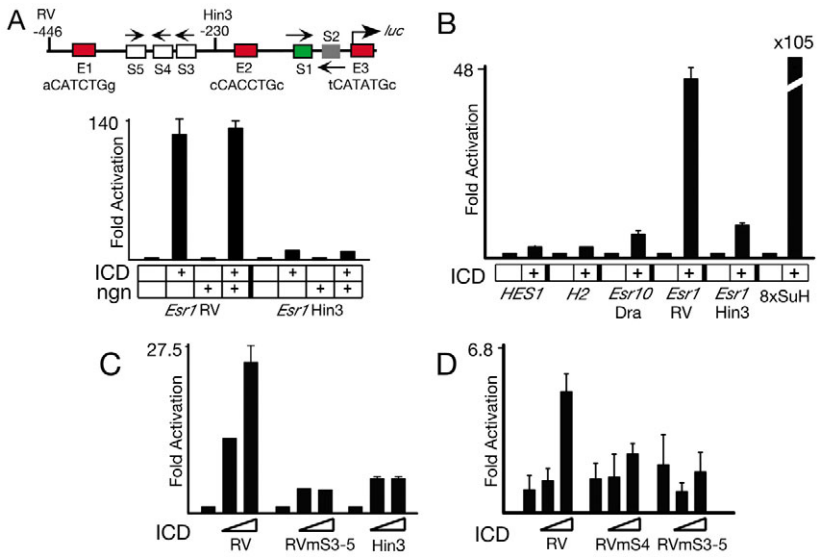


Fig. 7. The *Esr1* enhancer does not require E-boxes and responds to Notch through two loci. (A) Luciferase activity of *Esr1*/RV and Hin3 fragments co-transfected with activated Notch (ICD) plus or minus *Xngnr1* (ngn). (B) Luciferase activity of *Esr1*/RV and *Esr1*/Hin3 vectors co-transfected with 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*/RVmS3-5) or the *Esr1*/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*/RVmS-35.

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 Xngnr1/E did not shift an *Esr10* 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 *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 comparable 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*,

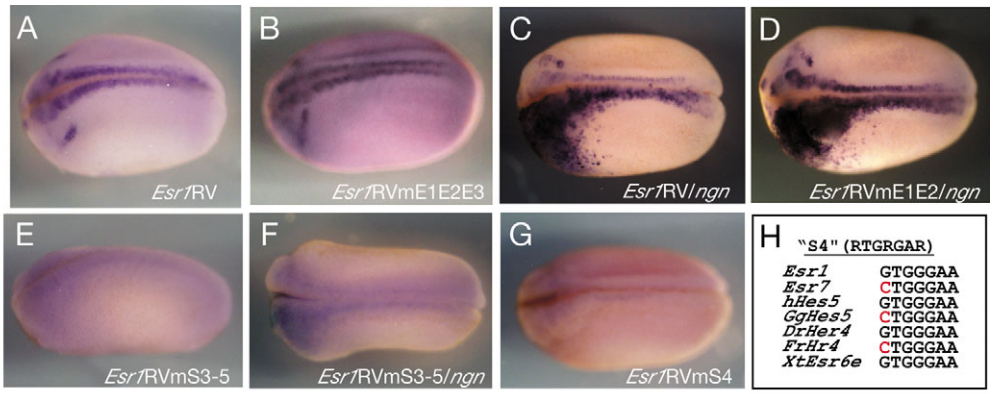


Fig. 8. *Esr1* enhancer activity requires upstream Su(H) sites in vivo. (A,B) *GFP* expression in frogs transgenic with enhancer elements containing mutant E-boxes (*Esr1*/RVmE1E2E3) versus wild-type controls. Wild-type (C) and E1E2 mutant (D) embryos were injected with mRNA encoding *Xngnr1* (ngn) and stained for *GFP*. *GFP* expression in frogs with mutant enhancers is unchanged relative to controls. (E,F) Transgenic frogs bearing *Esr1* enhancer elements mutant in upstream Su(H) sites (*Esr1*/RVmS3-5) show greatly attenuated *GFP* activity (E) relative to controls (A), and activity is not inducible following *Xngnr1* injection (F). (G) Within the S3-5 cluster, mutations within S4 (H), which is conserved in sequence and position in numerous Esr1 homologs, greatly attenuate enhancer activity.

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 Xngnr1, whereas the *Hes1*-like gene *Hairy2* is not. Here, we examine the mechanistic basis for this difference by dissecting the elements required for expression of *Esr1* and *Esr10* in neural precursors in response to proneural activity, and which therefore constitute proneural enhancers. Our results indicate that Esr genes are differentially regulated during lateral inhibition, both in terms of proneural input and the architecture of Notch-responsive motifs in their enhancers.

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 with 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. 5I, 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

Hes1 promoter-dependent luciferase activity in transfection assays through this site (Kan et al., 2004). Mutation 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 *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 *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* (Davis 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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