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The *Xenopus* homolog of *Drosophila Suppressor of Hairless* mediates Notch signaling during primary neurogenesis

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SUMMARY

The X-Notch-1 receptor, and its putative ligand, X-Delta-1, are thought to mediate an inhibitory cell-cell interaction, called lateral inhibition, that limits the number of primary neurons that form in *Xenopus* embryos. The expression of *Xenopus ESR-1,* a gene related to *Drosophila Enhancer of split*, appears to be induced by Notch signaling during this process. To determine how the activation of X-Notch-1 induces *ESR-1* expression and regulates primary neurogenesis, we isolated the *Xenopus* homolog of *Suppressor of Hairless* (*X-Su(H)*), a component of the Notch signaling pathway in *Drosophila*. Using animal cap assays, we show that X-Su(H) induces *ESR-1* expression, perhaps directly, when modified by the addition of ankyrin repeats. Using a DNA binding mutant of X-Su(H), we show that X-Su(H) activity is required for induction of *ESR-1*. Finally, expression of the DNA binding mutant in embryos leads to a neurogenic phenotype as well as increased expression of both *X-Delta-1* and *XNGNR1*, a proneural gene expressed during primary neurogenesis. These results suggest that activation of X-Su(H) is a key step in the Notch signaling pathway during primary neurogenesis in *Xenopus* embryos.

Key words: RBP-J$_\kappa$/KBF2/CBF1, neurogenic genes, *ESR-1*, transcriptional activation, *Enhancer of split*

INTRODUCTION

The differentiated cell types that constitute neural tissue develop from neural precursor cells that are generated within neurogenic epithelia. In *Drosophila*, these neural precursors arise via the activity of basic Helix-Loop-Helix (bHLH) transcription factors encoded by proneural genes, such as those in the *achaete-scute* gene complex, whose expression within a neurogenic epithelium is thought to promote the formation or differentiation of neural precursors (reviewed by Lewis, 1996). Conversely, a second class of bHLH proteins such as those encoded by the *hairy* gene and by the genes within the *Enhancer of split* complex [E(spl)-C] appear to inhibit the formation of neural precursors. These bHLH proteins, which contain a characteristic tetrapeptide, WRPW, at the carboxy terminus, act as transcriptional repressors (Wainwright and Ish-Horowicz, 1992; Dawson et al., 1995). In vertebrates, similar proneural bHLH genes have been identified that are expressed during the development of the nervous system, and some of these are expressed early on when neural precursors first form (see Ma et al., 1996 and references therein). In addition, the formation of neural precursors during vertebrate neural development is likely to be negatively regulated by genes encoding bHLH-WRPW proteins, as shown by both gain- and loss-of-function experiments with one such gene, called HES-1 (reviewed by Guillemot, 1995).

In *Drosophila*, the proneural bHLH genes and bHLH-WRPW genes regulate neural precursor cell formation as part of a genetic circuit that mediates an inhibitory cell-cell interaction, called lateral inhibition. During this process, expression of a proneural gene within nascent neuroblasts promotes the expression of the membrane bound ligand Delta (Kunisch et al., 1994; Haenlin et al., 1994), which binds and activates the Notch receptor on neighboring cells. Activation of Notch in a cell is thought to upregulate expression of several genes in the E(spl)-C, which encodes inhibitory bHLH-WRPW transcription factors (Jennings et al., 1994). These in turn appear to inhibit cells from adopting a neural fate, perhaps by inhibiting the activity of the proneural bHLH genes. In theory these interactions amplify small differences between cells in their potential to adopt a neural fate, ultimately restricting the number of cells that form neural precursors (Heitzler et al., 1996 and references therein).

Studies on the vertebrate homologs of *Delta* suggest that the formation of neural precursors in vertebrate embryos may also be negatively regulated by lateral inhibition (Henrique et al., 1995; Chitnis et al., 1995). The *Xenopus* homolog of *Delta*, called *X-Delta-1*, is expressed when the neuronal precursors for the primary nervous system form, and the expression of *X-Delta-1* can be promoted by ectopic expression of proneural genes, as in *Drosophila* (Chitnis and Kintner, 1996; Ma et al., 1996). Moreover, the number of primary neurons that form can be decreased or increased, by increasing or decreasing X-Delta-1 activity, respectively (Chitnis et al., 1995). Thus, these results are consistent with a model where the formation of primary neurons is inhibited by X-Delta-1, presumably when

it binds to X-Notch-1 and activates the Notch signaling pathway. To test this model further, we examined the events that occur downstream of Notch activation during primary neurogenesis, and identified *Xenopus ESR-1* as a potential downstream target of Notch signaling (D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished). *ESR-1* encodes a new member of the bHLH-WRPW family in vertebrates that bears similarity to genes in the E(spl)-C, and it is expressed in the regions of the neural plate where primary neurons form (D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished). Moreover, expression of *ESR-1* can be induced in *Xenopus* embryos by ectopic expression of *X-Delta-1*, as well as by activated forms of X-Notch-1 (D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished). Thus, these and other lines of evidence suggested that induction of *ESR-1* expression may be one consequence of activating the Notch signaling pathway during primary neurogenesis.

To determine how X-Notch-1 activates the expression of *ESR-1*, we have followed the lead of recent studies in *Drosophila* which show that Notch appears to regulate E(spl)-C gene expression at least in part via the product of the *Su(H)* gene (Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). *Su(H)* encodes a DNA binding protein, which is highly conserved evolutionarily; essentially identical proteins have been isolated from mouse (RBP-J_K), *Drosophila*, human and *C. elegans* (LAG-1) (Hamaguchi et al., 1989; Schweisguth and Posakony, 1992; Amakawa et al., 1993; Christensen et al., 1996). In *Drosophila*, Su(H) binds to a region, called RAM-23, within the Notch intracellular domain (Tamura et al., 1995), as well as to sites in the upstream regulatory regions of bHLH-WRPW genes in the E(spl)-C (Tun et al., 1994) that are essential for the induction of these genes upon Notch activation (Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). These observations suggest a model where Su(H) is first bound to the Notch intracellular domain and is then released when ligand binding occurs, allowing it to translocate to the nucleus where it acts as a transcriptional activator of genes within the E(spl)-C (Artavanis-Tsakonas et al., 1995). A similar pathway may exist in vertebrates: mouse Su(H) also binds to the RAM23 region of mouse Notch (Tamura et al., 1995; Hsieh et al., 1996) as well as to Su(H) binding sites in the HES-1 gene (Takebayashi et al., 1994; Brou et al., 1994). Moreover, complexes can be formed in vitro between the Notch intracellular domain, Su(H), and Su(H) binding sites (Jarriault et al., 1995). Thus, Su(H) appeared to be a good candidate for mediating X-Notch-1 activation of *ESR-1*, and therefore a likely component of lateral inhibition during primary neurogenesis.

We have isolated a *Xenopus* homolog of *Drosophila* Su(H) (X-Su(H)1). We show that X-Su(H)1 binds to the RAM23 region of X-Notch-1 and that this region along with the ankyrin repeats is required for optimal induction of *ESR-1* expression. We show that X-Su(H)1 can activate the expression of *ESR-1*, when modified by the addition of the ankyrin repeats. Based on this information, we generated an antimorphic form of X-Su(H)1, called X-Su(H)1^{DBM} (for DNA binding mutant) which does not bind DNA but still interacts with X-Notch-1. We show that this form of X-Su(H)1^{DBM} inhibits the activation of *ESR-1* in response to X-Delta-1, suggesting that it is an inhibitor of Notch signaling. Ectopic expression of X-Su(H)1^{DBM} in embryos produces a striking neurogenic phenotype in which more primary neurons form than normal. X-Su(H)1^{DBM} also reverses the inhibitory effects of ectopic X-Delta-1 expression on the formation of primary neurons. Finally expression of X-Su(H)^{DBM} in embryos leads to an increase in the expression of *X-Delta-1* and of *XNGNR1*, a proneural gene expressed during primary neurogenesis. We conclude that X-Su(H)1 is a key component of the Notch signaling pathway, which operates during primary neurogenesis in *Xenopus* embryos.

MATERIALS AND METHODS

ICD constructs

Oligos 5′-GTGAATTCCATATGAATAAGAAGCGTCGCCG and 5′-GAAGATCTACTTGAAAGCTTCAGGTATG were used in a polymerase chain reaction (PCR) to amplify a 2.3-kb fragment (ICD), which was shuttled into pCS2+MT (Turner and Weintraub, 1994) or pCS2+NLS/MT (Rupp et al., 1994). All deletion constructs were created by double restriction, blunting (Pfu DNA polymerase; Stratagene), and intramolecular religation. For ICD22, 23, 24, and 25, ICD was first restricted with *Spe*I (unique flanking site), then with *Bam*HI (7189), *Ava*II (6715), *Dde*I (6297), or *Bgl*I (5972), respectively. For ICD31, ICD was restricted with *Bgl*I (5972) and *Hph*I (6731). For ICD11, 12, 14, 15, and 16, ICD was first restricted with *Nde*I (unique flanking site), then with *Eco*NI (5689), *Taq*I (6184), *Ava*II (6715), *Bam*HI (7189), or *Bst*NI (7627). Numbering (Coffman et al., 1990) indicates restriction site location.

Oligos 5′-GTGAATTCCATATGGATGTCAATGTCCGT and 5′-GTGGATCCTATTCATCCAGCAGGTGAA were used in a PCR to amplify a 0.7-kb fragment (Ank), which was shuttled into pCS2+ NLS/MT (NLS-Ank). Sequencing (USB) verified PCR products.

Ribonuclease protection assays and whole-mount in situ hybridization

Synthesis and injection of RNA was carried out as described previously (Chitnis and Kintner, 1996). To generate neuralized animal caps, 0.3 ng of RNA encoding Noggin (Lamb et al., 1993) was injected into both sides of a two-cell stage embryo. RNA (1.0 ng/embryo) encoding deleted forms of ICD, different forms of X-Su(H)1, or X-Delta-1, was injected along with the *Noggin* RNA. Animal caps were isolated (see Fig. 3 legend), and *ESR-1* (D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished) expression was analyzed using RNAse protection assay as described previously (Kintner and Dodd, 1991). Quantitation was carried out on a Phosphor Imager (Molecular Dynamics). Protected band intensities (*ESR-1* and *EF-1α*) were integrated and background corrected, and then the *ESR-1* values were normalized for the *EF-1α* (loading control) values of each lane.

The different forms of X-Su(H)1 were expressed in embryos by injecting 1.0 ng of RNA encoding each form along with 0.2 ng of *nlacZ* (Turner and Weintraub, 1994) RNA into one side of a two-cell stage embryos. Analysis of RNA-injected embryos by whole-mount in situ hybridization and X-gal staining was as described previously (Chitnis et al., 1995).

Cloning of *Xenopus Su(H)*

Degenerate oligonucleotide primers 5′-GTIAA(A/G)ATGTT(C/T)TA(C/T)GGIAA and 5′-TCIAC(A/G)TCICC(A/G)AACCAIAC were used in a PCR with a stage 17 (neurula) *Xenopus* embryo library (Kintner and Melton, 1987). This generated a 0.73-kb fragment used to screen the library. Clones encoding two forms of X-Su(H) were isolated, which differed in length at their N-termini but were otherwise identical (X-Su(H)1 and 2, 501 and 481 aa, GenBank accession numbers U60093 and U60094, respectively). X-Su(H)1 was used here. Sequences were compared using Lasergene (DNASTAR).

GST fusion proteins and binding assays

ICD constructs were shuttled into pGEX-4T-1 (Pharmacia), and glutathione S-transferase (GST) fusion proteins were generated as described by Frangioni and Neel (1993). The TNT SP6 Coupled Reticulocyte Lysate System (Promega) was used to synthesize [^{35}S]Met-labeled X-Su(H)1 protein. Equal masses (equivalents) of fusion proteins, as judged by Coomassie staining, were used in binding reactions. In vitro-translated protein (9 μl) was first mixed (pre-cleared) with 9 equivalents of GST protein bound to glutathione-agarose (GA) beads (13.5 μl 50% bead slurry) in 10 mM sodium phosphate pH 7.5, 135 mM KCl, 0.1% Triton X-100, 1 mM DTT, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, 0.1 mM PMSF, and 5 mM $MgCl_2$ (binding buffer; 450 μl) for 1 hour at room temperature (RT). Beads were then precipitated, and 50 μl (one ninth) of the supernatant was transferred to tubes containing one equivalent of specific ICD deletion/GST fusion proteins bound to GA beads (1-12 μl 50% slurries) in 450 μl of binding buffer and incubated for 1 hour at RT. Beads were washed twice with binding buffer, heated in Laemmli buffer, and then electrophoresed on a 10% polyacrylamide gel, which was autoradiographed.

Dexamethasone and cycloheximide treatments

Animal caps were treated with 20 μM dexamethasone (Sigma; Kolm and Sive, 1995) and/or 10 μg/ml cycloheximide (CHX; Sigma; Cascio and Gurdon, 1987). Efficacy of CHX treatment was verified by treating animal caps with CHX for 1 hour, washing them, and then incubating them in [^{35}S]Met (Amersham) for 2 hours, followed by TCA precipitation of cap lysates and scintillation counting. CHX treatment reduced to 22.6% the incorporation of [^{35}S]Met into acid-insoluble material in treated versus untreated caps.

Fusion protein construction and mutagenesis

Oligonucleotide primers 5′-TGAATTCGGACACTACT and 5′-ACTTGTGCCTTTCTC were used in a PCR to replace the terminal stop codon of *X-Su(H)1* with an *Eco*RI site for attachment of the *X-Notch-1* ankyrin repeats to the C terminus of X-Su(H)1 to create X-Su(H)1/Ank. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to create X-Su(H)1^{DBM}, using the following oligos, 5′-GTGGCACTATTTAACGAGCTGGAATCGCAGACAGT-CAGTACTGAATCTCTGCACGTA and 5′-TTCTACGTGCAGA-GATTCAGTACTGACTGTCTGCGATTCCAGCTCGTTAAATAGT. Oligonucleotides 5′-ACTTGTGCCTTTCTC and 5′-TTCTCGAGT-CAACGTAAATTTGGAGT were used in a PCR to generate a carboxy-terminally truncated (amino acid residue 385) form of X-Su(H)1 (X-Su(H)1-Tr). Attachment of the X-Notch-1 ankyrin repeats to the carboxy terminus of X-Su(H)1-Tr (X-Su(H)1-Tr/Ank) does not convert this protein into an inducer of *ESR-1* in neuralized animal caps, and therefore appears to indicate that X-Su(H)1-Tr is non-functional.

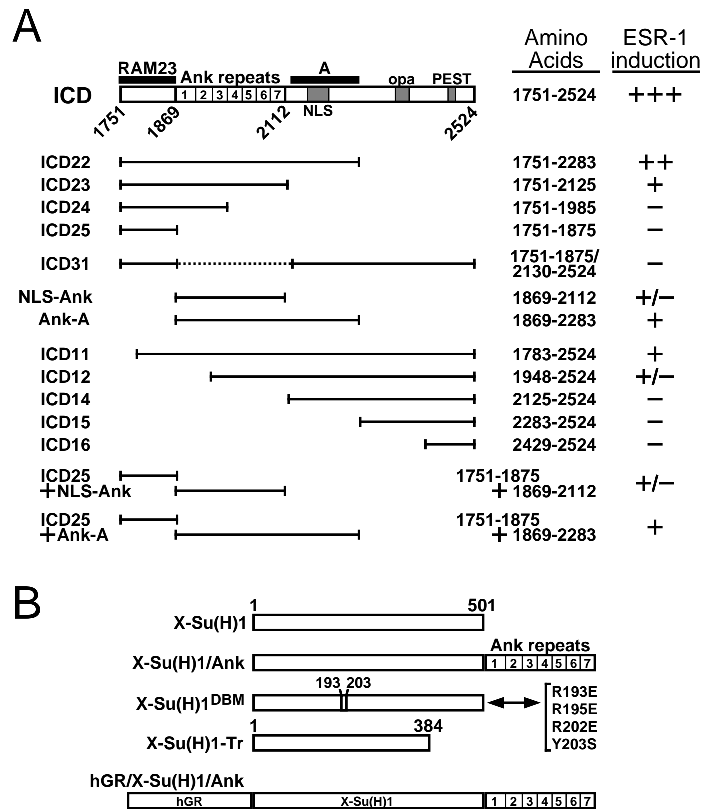

Fig. 1. (A) Deletion analysis of ICD. Diagram of the *X-Notch-1* intracellular domain (ICD) and of the deletions used for mapping *ESR-1* induction. NLS-Ank contains the SV40 NLS at the N-terminus (Rupp et al., 1994). The amino acid residues (Coffman et al., 1990) encompassed by each truncation are indicated. The right column summarizes the data from RNase protection assays, such as those shown in Fig. 3, that measure the ability of each construct to induce *ESR-1* expression in neuralized animal caps (+++, 100%; ++, 20-40%; +, 10-20%; +/–, 1-5%; or –, not detectable). RAM23 (Tamura et al., 1995) is the subtransmembrane region. Addition of the SV40 NLS (Rupp et al., 1994) to the N terminus of ICD22, 23, 24, and 25 did not alter *ESR-1* induction. (B) Different forms of X-Su(H)1. Schematic representation of wild-type X-Su(H)1, a form in which the ankyrin repeats from Notch are fused to the carboxy terminus (X-Su(H)1/Ank), a form in which four residues required for DNA binding, between amino acids 193-203, are altered (X-Su(H)1^{DBM}), a truncated form in which the carboxy terminal 117 amino acids are deleted X-Su(H)1-Tr, and a form of X-Su(H)1/Ank fused to the hormone binding domain of the human glucocorticoid receptor (hGR).

RESULTS

Xenopus Su(H)1 binds to the RAM23 region of ICD

cDNAs encoding *Xenopus* Su(H) (X-Su(H)) were isolated from a neurula (stage 17) cDNA library (see Materials and Methods). The identity between X-Su(H)1 (see Methods) and the homologs of mouse, *Drosophila* and humans is 96%, 72%, and 94%, respectively. Results from whole-mount in situ hybridization indicate that *X-Su(H)1* is ubiquitously expressed in early embryos, including throughout the developing nervous system (data not shown).

In vitro binding assays were used to determine whether X-Su(H)1 binds to the Notch intracellular domain (ICD), as shown in mouse and *Drosophila*. The results show that the minimal region of ICD mediating optimal binding to X-Su(H)1 is the RAM23 region of ICD (Fig. 2; see Fig. 1A for constructs): all constructs containing the RAM23 region bind X-Su(H)1 strongly, while those that lack it bind X-Su(H)1 weakly. For example, X-Su(H)1 binds weakly to a form of ICD in which 32 amino acids containing a portion of the RAM23 region have been deleted from the amino terminus (GST-ICD11), and X-Su(H)1 binds strongly to a form of ICD containing primarily the RAM23 region (ICD25). While the strongest binding occurs at the RAM23 region, X-Su(H)1 also binds weakly to the ankyrin repeats alone (GST-Ank; Fortini and Artavanis-Tsakonas, 1994) and to the carboxy-terminal half of ICD (GST-

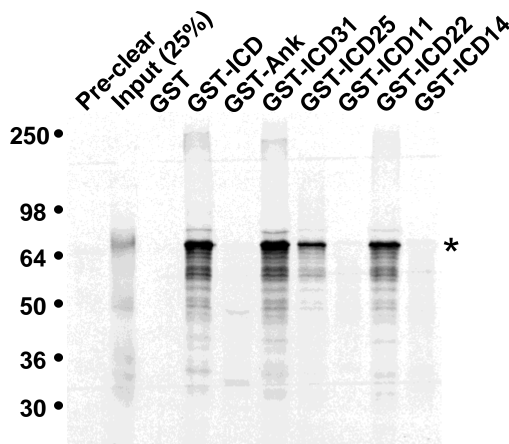

Fig. 2. Regions of the X-Notch-1 intracellular domain required for X-Su(H)1 binding. Lane labels indicate the GST fusion protein (see Fig. 1A) used to precipitate in vitro-translated [^{35}S]Met-labeled X-Su(H)1. The asterisk marks full-length in vitro-translated X-Su(H)1. The lane labeled 'Pre-clear' (see Methods) shows that GST binds no X-Su(H)1. The 'Input' lane contains 25% of the [^{35}S]Met-labeled X-Su(H)1 used in each binding reaction. Approximate migration of protein markers is indicated. Longer exposure of the gel shows weak binding of X-Su(H)1 to GST-Ank, GST-ICD11, and GST-ICD14, while GST alone still shows no binding.

ICD14) (see Fig. 2 legend). These other, weaker binding sites, may allow for cooperative binding of X-Su(H)1 to the RAM23 region, as shown, for example, by the stronger binding of X-Su(H)1 to GST-ICD22 or GST-ICD31 than to GST-ICD25. Thus, these results corroborate those obtained with mouse and *Drosophila* Notch (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995; Hsieh et al., 1996) which show that the RAM23 region is a strong binding site for Su(H).

The region of the X-Notch-1 intracellular domain required for *ESR-1* induction

To examine the role of X-Su(H)1 in the X-Notch-1 signaling pathway further, we asked whether the RAM23 region, where X-Su(H)1 binds, is involved in the induction of *ESR-1* expression by activated X-Notch-1 in a neuralized animal cap assay. In this assay, ICD RNA was injected along with RNA encoding the neural inducer Noggin into two-cell stage embryos. At late blastula stage, the animal caps were removed, cultured to the equivalent of the neural plate stage, and then assayed for the expression of *ESR-1* using RNase protection assay. *ESR-1* is not detectably expressed in animal caps neuralized by Noggin, or in nonneuralized animal caps, but can be induced by the expression of ICD (Fig. 3A; D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished). Using this assay, we analyzed a deletion series to delineate the region of ICD required for the activation of *ESR-1* expression in neuralized animal caps (Fig. 1A). This analysis showed that the ankyrin repeats are a core region required for *ESR-1* expression, any deletion of which abolished gene induction (Figs 1A and 3A). However, though necessary, the ankyrin repeats alone are extremely poor inducers of *ESR-1* (Fig. 3A, lane 12). In addition, optimal induction of *ESR-1* expression is dependent on the RAM23 region, as shown by the marked decrease in *ESR-1* induction when just a portion of this region is deleted (ICD11; Fig. 3A, lane 13). The low level of *ESR-1* expression that is induced without the RAM23 region (i.e. ICD11) may reflect the fact that other sites in the ICD bind X-Su(H)1 weakly (see Fig. 2), or that other parts of ICD weakly induce *ESR-1* expression via a X-Su(H)1-independent pathway. Finally, the RAM23 region (i.e. ICD25, Fig. 1A) does not induce *ESR-1* on its own, or increase the levels of *ESR-1* expression when expressed in *trans* with the ankyrin repeats (Fig. 1A). Thus, these results indicate that the RAM23

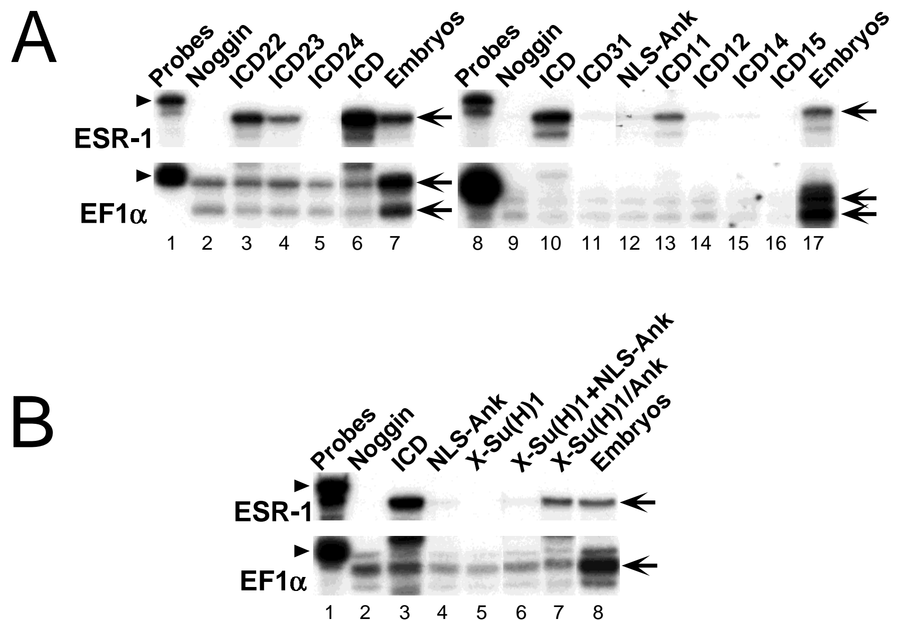

Fig. 3. Induction of *ESR-1* in neuralized animal caps by ICD and by different forms of X-Su(H)1. Test RNAs (1.0 ng/embryo) along with *Noggin* RNA (0.3 ng/embryo) were injected into two-cell stage embryos. Ectoderm was excised from blastula embryos (stage 10), cultured for approximately 6 hours (equivalent to the neural plate stage), and then assayed by RNase protection assay for expression of *ESR-1* RNA and *EF-1α* RNA (loading control). Each assay includes the probes without RNase treatment (Probes), animals caps injected with just *Noggin* RNA (Noggin) and stage-matched embryo RNA (Embryos). Position of probe fragments are marked with an arrowhead, while those of protected fragments are marked with arrows. (A) *ESR-1* RNA levels in neuralized animal caps expressing different regions of ICD as shown in Fig. 1A. Note that those lacking any portion of the ankyrin repeats fail to or barely induce detectable *ESR-1* expression (lanes 5, 11, 14, 15, and 16). Removing the RAM23 region (ICD11, lane 13) markedly reduces the induction of *ESR-1* expression relative to ICD (lane 10). Deletion of sequences downstream of the ankyrin repeats (compare lane 4 to 3) also reduces the induction of *ESR-1* expression. (B) *ESR-1* RNA levels in neuralized animal caps expressing X-Su(H)1 and X-Su(H)1/Ank (see Fig. 1B). Note that X-Su(H)1 has undetectable *ESR-1*-inducing activity (lane 5) and the ankyrin repeats from ICD (NLS-Ank) have very little inducing activity (lane 4), while X-Su(H)1/Ank (lane 7) induces *ESR-1* expression to levels found in embryos (lane 8).

region is required for optimal induction of *ESR-1* expression, and that this region only works in *cis* with the ankyrin repeats. Notably, other regions of ICD, particularly the region downstream of the ankyrin repeats (region A in Fig. 1A) contribute to the induction of *ESR-1* expression by ICD (Fig. 3A, lane 3 to 4).

Overexpression of *X-Su(H)1* does not induce *ESR-1*

The role of X-Su(H)1 in the induction of *ESR-1* expression was tested further by determining whether *ESR-1* could be induced in neuralized animal caps by *X-Su(H)1* RNA. Neuralized animal caps from embryos injected with *X-Su(H)1*, however, did not express *ESR-1* at detectable levels (Fig. 3B, lane 5). To ensure that the exogenous X-Su(H)1 was produced in this experiment, myc-tagged versions of X-Su(H)1 were introduced into embryos by RNA injection. Staining of these embryos with an anti-myc epitope antibody showed cytoplasmic and punctate nuclear staining (data not shown). Thus, together these results indicated that ectopic expression of X-Su(H)1 is not sufficient to activate *ESR-1* expression.

Studies in other systems suggest that Su(H) can bind DNA but cannot promote transcription unless it complexes with another protein, which is a transcription activator (Hsieh and Hayward, 1995; Jarriault et al., 1995). Thus the results described above could be explained if X-Su(H)1 only induces *ESR-1* expression when it associates with an activator, which is limiting in the neuralized animal cap assay. To test this idea, we asked whether we could modify X-Su(H)1 to convert it into an activator of *ESR-1* expression. Specifically, our approach was based on the knowledge that the ankyrin repeats are an active part of the Notch ICD with respect to *ESR-1* activation, but only when attached to the RAM23 region, which binds X-Su(H)1 (Fig. 1A). Therefore, we generated a fusion protein in which the ankyrin repeats of X-Notch-1 were coupled to the carboxy terminus of X-Su(H)1 (X-Su(H)1/Ank in Fig. 1B), and tested it for *ESR-1* induction using the neuralized animal cap assay. As shown above, *ESR-1* is not detectably induced by *X-Su(H)1* (Fig. 3B, lane 5) and is only poorly induced by the ankyrin repeats in neuralized animal caps (Fig. 3B, lane 4). In contrast, animal caps from *X-Su(H)1/Ank*-injected embryos express levels of *ESR-1* comparable to those obtained in embryos (Fig. 3B, lanes 7 and 8). Coexpressing *X-Su(H)1* and the ankyrin repeats (*NLS-Ank*) in animal caps also induced only low levels of *ESR-1* expression (Fig. 3B, lane 6), demonstrating that the ankyrin repeats and X-Su(H)1 must be physically coupled to activate *ESR-1* expression. Thus, these results support the idea that X-Su(H)1 can activate *ESR-1* expression, but that to do so it must associate with other proteins, such as the X-Notch-1 ICD, for example.

Inducible forms of *Xenopus* Su(H)1/ankyrin or ICD

The results described above indicate that *ESR-1* can be induced by either Notch ICD or X-Su(H)1/Ank in neuralized animal caps. To determine whether these proteins are acting as direct transcriptional activators of *ESR-1*, the ligand binding domain of the human glucocorticoid receptor (hGR) was fused to their amino termini (hGR/ICD22 and hGR/X-Su(H)1/Ank; Fig. 1B). The ligand binding domain of steroid hormone receptors is a transferable unit that, when fused to transcription factors, confers hormone inducible nuclear translocation (Hollenberg et al., 1993; Kolm and Sive, 1995). The inducibility of

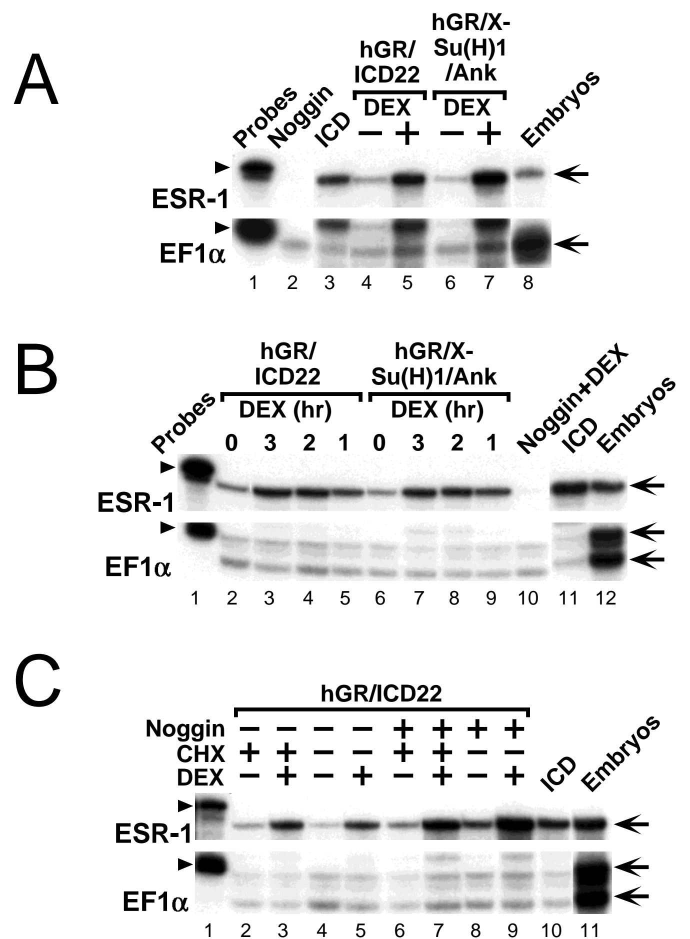

Fig. 4. Inducible forms of X-Su(H)1/Ank and ICD. hGR/ICD22 or hGR/X-Su(H)1/Ank RNA (1.0 ng/embryo) were injected along with *Noggin* RNA (0.3 ng/embryo) into two-cell stage embryos and the induction of *ESR-1* expression was measured in animal cap assays as described in Fig. 3. (A) Neuralized animal caps expressing the indicated constructs were either left untreated (–) or exposed (+) to dexamethasone (DEX) for 3 hours. Note that *ESR-1* expression increases following dexamethasone induction. Both forms also induced *ESR-1* expression in the absence of dexamethasone, probably as a result of expressing them at levels high enough to overwhelm mechanisms for cytoplasmic retention. (B) Neuralized animal caps expressing the indicated constructs were either left untreated (0) or exposed to dexamethasone for three (3), two (2), or one (1) hour before harvesting. As a control, neuralized animal caps were treated for 3 hours with dexamethasone (Noggin+DEX). (C) Animal caps from embryos injected with *hGR/ICD22* RNA were (+) or were not (–) neuralized (Noggin) and had (+) or had not (–) been treated with either cycloheximide (CHX) and/or dexamethasone (DEX), as indicated. This experiment was carried out both in the presence and absence of Noggin, because we knew that *ESR-1* expression can be induced to higher levels in neuralized versus nonneuralized animal caps, and we were concerned that the ability of noggin to neuralize animal caps would be blocked by cycloheximide. Consistent with this notion, the induction of *ESR-1* in neuralized animal caps appeared to be affected to a small degree by the addition of cycloheximide (lane 9 versus 7), while no such effect was observed in nonneuralized caps (lane 5 versus 3). *ESR-1* RNA expression levels in lanes 2-9 after normalizing to *EF-1α* are: 59, 174, 24, 96, 73, 180, 89, and 300.

hGR/ICD22 or *hGR/X-Su(H)1/Ank* was tested in neuralized animal caps that were excised at the beginning of gastrulation, incubated in the presence or absence of dexamethasone (a synthetic ligand for hGR) for 3 hours, and assayed for the induction of *ESR-1* expression. The results show that both hGR/ICD22 and hGR/X-Su(H)1/Ank activate *ESR-1* expression following dexamethasone treatment (Fig. 4A).

Using these inducible forms, we then measured the time course of *ESR-1* induction. Both hGR/ICD22 and hGR/X-Su(H)1/Ank upregulate *ESR-1* expression in animal caps after as little as 1.0 hour exposure to dexamethasone, suggesting they may directly activate *ESR-1* expression (Fig. 4B). To confirm this, we asked whether hGR/ICD22 could induce the expression of *ESR-1* in the presence of cycloheximide, a protein synthesis inhibitor (Cascio and Gurdon, 1987). Animal caps expressing *hGR/ICD22* were excised at the blastula stage, incubated in cycloheximide for 1 hour, exposed to dexamethasone for another 2 hours, and then assayed for expression of *ESR-1*. Control caps showed that this exposure to cycloheximide reduced protein synthesis approximately 5-fold, as measured by incorporation of [^{35}S]Met into acid insoluble material. However, dexamethasone-induced expression of *ESR-1* by hGR/ICD22 reached the same levels in the presence of cycloheximide as it did in the absence (Fig. 4C). In sum, these results are consistent with the idea that hGR/ICD22 rapidly induces *ESR-1* expression upon moving to the nucleus and that this function does not require synthesis of additional proteins.

A *Xenopus* Su(H)1 DNA binding mutant blocks induction of *ESR-1*

The results presented above suggest that activation of *ESR-1* by X-Notch-1 is mediated by X-Su(H)1, once X-Su(H)1 has formed a complex with another protein. Based on this interpretation, we sought to generate a dominant-negative form of X-Su(H)1 by engineering mutations that interfere with DNA binding, based on previous studies of mammalian Su(H) (Chung et al., 1994). Four point mutations were introduced at conserved residues (R193E, R195E, R202E, and Y203S), resulting in a mutant form of X-Su(H)1, called X-Su(H)1^{DBM} (DNA binding mutant; Fig. 1B). X-Su(H)1^{DBM} lacks the ability to bind to a RBP-J$_K$ binding site in gel shift assays, in agreement with previous studies, and in vitro-translated X-Su(H)1^{DBM} bound to ICD with the same profile as wild-type X-Su(H)1 (data not shown). Since X-Su(H)1^{DBM} cannot efficiently associate with DNA, but still associates with proteins such as ICD, we predicted that it should act as a dominant-negative by forming nonfunctional complexes. This prediction was tested by expressing *X-Su(H)1^{DBM}* alone and in combination with *X-Delta-1* in animal caps. Assay for *ESR-1* induction showed that X-Su(H)1^{DBM} alone (data not shown) does not induce *ESR-1* expression and that it inhibits the ability of X-Delta-1 to activate *ESR-1* expression (Fig. 5A, lane 4). A carboxy-terminal truncation of X-Su(H)1 (X-Su(H)1-Tr; Fig. 1B), which appears to be non-functional (see Methods) was not effective at blocking X-Delta-1-mediated *ESR-1* induction (Fig. 5A, lane 5). Thus, a DNA-binding mutant form of X-Su(H)1, when expressed in the context of X-Delta-1-mediated Notch signaling, will interfere with induction of *ESR-1*.

As a control, we also examined whether ectopic expression of *X-Su(H)1* would affect *ESR-1* expression when induced in

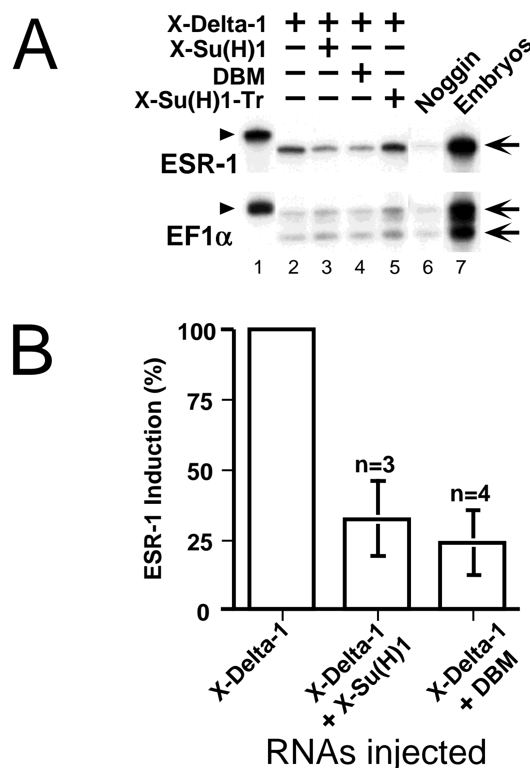

Fig. 5. *ESR-1* expression is blocked by *X-Su(H)1^{DBM}*. Test RNAs (1.0 ng/embryo), as indicated above each lane, along with *Noggin* RNA (0.3 ng/embryo) were injected into two-cell stage embryos, and the induction of *ESR-1* expression was measured in animal cap assays as described in Fig. 3. (A) Expression of X-Delta-1 in neuralized animal caps induces the expression of *ESR-1* (compare lanes 2 and 6). This induction is reduced by the inclusion of *X-Su(H)1^{DBM}* (DBM) or *X-Su(H)1* but not by *X-Su(H)1-Tr*, a carboxy-terminally truncated form of X-Su(H)1 (see Fig. 1B). Quantitation of the *ESR-1* signal in lanes 3-6 is: 91, 24, 24, and 60, respectively. (B) Compilation of inhibition of X-Delta-1-mediated *ESR-1* induction by X-Su(H)1 and X-Su(H)1^{DBM} (DBM). The number of data points is indicated (*n*=) and error bars indicate the true population standard deviation.

animal caps by X-Delta-1. Surprisingly, the results show that coexpression of *X-Su(H)1* along with *X-Delta-1* also inhibits the induction of *ESR-1*, in a manner similar to that of X-Su(H)1^{DBM} (Fig. 5A, lanes 3 and 4; Fig. 5B). Although surprising, this result is still consistent with a model where X-Su(H)1 needs to complex with other proteins, such as the intracellular domain of Notch, to activate *ESR-1* expression. In this model overexpression of the free form of X-Su(H)1 might compete with functional, complexed forms of X-Su(H)1, thus inhibiting the induction of *ESR-1* expression (see Discussion). Thus these in vitro studies are consistent with idea that X-Su(H)1 is involved in the induction of *ESR-1* expression following activation of the Notch signaling pathway. However, because these in vitro assays may represent an artificial situation in terms of how the pathway is normally stimulated, we then asked whether X-Su(H)1 affected the Notch signaling pathway in vivo during primary neurogenesis.

The effects of X-Su(H)1 on primary neurogenesis

To examine the effects of X-Su(H)1 on Notch signaling in vivo,

we injected *X-Su(H)1* RNA along with a *lacZ* tracer into one side of *Xenopus* embryos at the two-cell stage, while the other side served as an uninjected control. At the neural plate stage, the embryos were fixed and stained with X-gal, to mark the injected side, and then double-stained by whole-mount in situ hybridization for RNA encoding a type II neuronal-specific tubulin gene, called *N-tubulin* (Oschwald et al., 1991). *N-tubulin* expression marks the appearance of primary neurons which form within three longitudinal domains of the neural plate.

In five independent experiments, overexpression of X-Su(H)1 alone appeared to have little or no effect on the number of primary neurons, relative to the uninjected side of embryos (Fig. 6A and Table 1). On average, about one third of the embryos expressing X-Su(H)1 showed some decrease in the number of primary neurons that form, indicating that X-Su(H)1 has weak antineurogenic effects. As before, embryos injected with a myc-tagged version of X-Su(H)1 were stained with an anti-myc epitope antibody to show that the injected RNA was translated in cells in the neural plate (data not shown). Thus, these results indicate that overexpression of X-Su(H)1 on its own does not appear to significantly alter Notch signaling in vivo. Conversely, embryos injected with *X-Su(H)1/Ank* RNA showed complete loss of primary neurons that formed on the injected side (Fig. 6B and Table 1), consistent with the idea that this represents an activated form of X-Su(H)1, as shown by its ability to induce *ESR-1* in neuralized animal cap assays. Thus, these results indicate that *X-Su(H)1* expression on its own weakly affects Notch signaling in vivo, while *X-Su(H)1/Ank* activates the Notch signaling pathway.

We then examined the effect of *X-Su(H)1^{DBM}* on the formation of primary neurons. A molecule which interferes with Notch signaling, and therefore lateral inhibition, would be expected to give rise to an increased number of primary neurons. As predicted, X-Su(H)1^{DBM} behaved as a dominant negative mutant such that in regions of the neural plate expressing *X-Su(H)1^{DBM}*, primary neurons formed at a much higher density than observed on the opposite, uninjected side (Fig. 6C,F and Table 1). Importantly, X-Su(H)1^{DBM} only appeared to increase the number of primary neurons that formed within each longitudinal domain, suggesting that it blocked a mechanism for limiting the number of primary neurons (lateral inhibition) rather than affecting where in the neural plate primary neurons form. Indeed, the effect of X-Su(H)1^{DBM} on primary neurons is identical to that observed using a dominant-negative form of X-Delta-1 which is also thought to block Notch signaling (Chitnis et al., 1995). To examine whether X-Su(H)1^{DBM} is indeed acting as a dominant-negative mutant, we asked whether the coexpression of *X-Su(H)1* or *X-Su(H)1/Ank* would rescue the neurogenic phenotype produced by X-Su(H)1^{DBM}. The results show that embryos injected with both *X-Su(H)1^{DBM}* and *X-Su(H)1* RNA did not give an increase in the number of neurons as seen with *X-Su(H)1^{DBM}* alone (Fig. 6D and Table 1). Moreover, injection of *X-Su(H)1/Ank* along with *X-Su(H)1^{DBM}* RNA completely reversed the neurogenic phenotype, resulting again in embryos with a loss of primary neurons (Fig. 6E and Table 1). Thus, these experiments suggest strongly that *X-Su(H)1^{DBM}* is blocking the Notch signaling pathway during lateral inhibition by acting as a dominant negative mutant.

Ectopically expressing *X-Delta-1* in embryos leads to a marked reduction of primary neurons, an antineurogenic phenotype, presumably as a result of increased Notch signaling (Chitnis et al., 1995; Fig. 6G and Table 1). Thus, to extend on the results above, we asked if X-Su(H)1^{DBM} blocks Notch signaling when signaling is artificially stimulated in *X-Delta-1* RNA-injected embryos. If *X-Su(H)1^{DBM}* is expressed along with *X-Delta-1*, primary neurons not only form, but they form at a density higher than that observed on the control uninjected side of the embryo (Fig. 6I and Table 1). Thus, these results indicate that the effects of X-Su(H)1^{DBM} on the formation of primary neurons is epistatic to those observed with X-Delta-1,

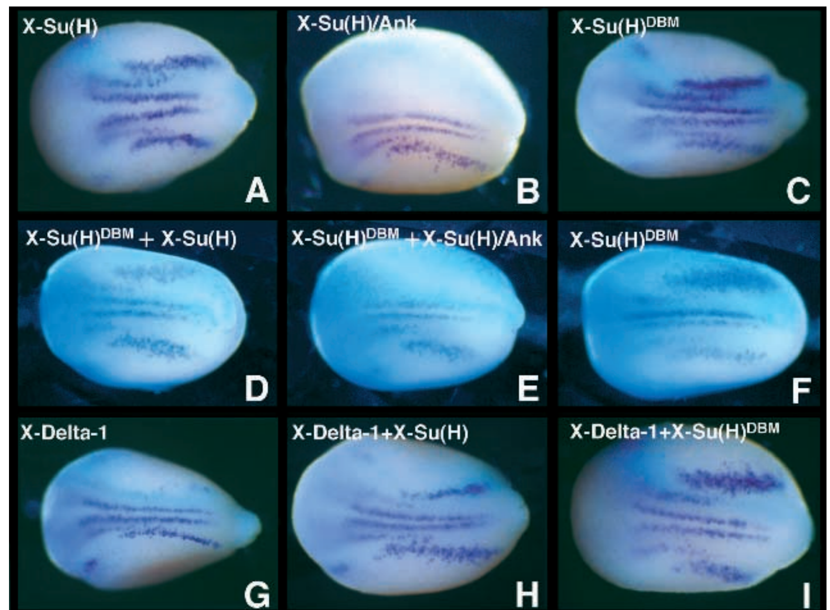

Fig. 6. The effects of X-Su(H)1, X-Su(H)1/Ank and X-Su(H)1^{DBM} on primary neurogenesis. Albino *Xenopus* embryos were injected once at the two-cell stage with test RNAs (1.0 ng/embryo) along with *lacZ* RNA (0.2 ng/embryo). At neural plate stages, the embryos were processed both for β-galactosidase expression (light blue) to mark the injected side, and for the expression of *N-tubulin* (dark purple) to mark the formation of primary neurons. Primary neurons arise in three stripes on either side of the midline of the neural plate: a long medial stripe, a short more anterior intermediate stripe, and a wide lateral stripe. Shown are dorsal views, with anterior to the left and the injected side oriented up in each panel. Embryos shown in each row are from the same experiment. Expression of: (A) wild-type *X-Su(H)1* has little or no effect on the formation of primary neurons; (B) *X-Su(H)1/Ank* abolishes primary neurons; (C,F) *X-Su(H)1^{DBM}* produces a neurogenic phenotype; (D) *X-Su(H)1* along with *X-Su(H)1^{DBM}* ameliorates the neurogenic phenotype; (E) *X-Su(H)1/Ank* along with *X-Su(H)1^{DBM}* completely reverses the neurogenic phenotype; (G)*X-Delta-1* reduces the number of primary neurons; (H) *X-Su(H)1* along with *X-Delta-1* partially reverses the effects of *X-Delta-1*; and (I) *X-Su(H)1^{DBM}* along with *X-Delta-1* not only reverses the effects of *X-Delta-1* but also results in a neurogenic phenotype.

Table 1. Effect of forms of ICD, X-Su(H)1, and X-Delta-1 on primary neurons

RNA injected:	*N-tubulin* expression			
	More	Unchanged	Less	Total
lacZ (5)	6 4%	117 82%	19 13%	142 100%
X-Su(H)1 (5)	14 10%	83 58%	47 33%	144 100%
X-Su(H)1/Ank (3)	1 1%	1 1%	81 98%	83 100%
DBM (4)	118 81%	22 15%	6 4%	146 100%
DBM+ X-Su(H)1 (2)	8 10%	35 45%	34 44%	77 100%
DBM+ X-Su(H)1/Ank(2)	0 0%	1 1%	88 99%	89 100%
X-Delta-1 (1)	0 0%	9 29%	22 71%	31 100%
X-Delta-1+ DBM (1)	25 61%	14 34%	2 5%	41 100%
X-Delta-1+ X-Su(H)1 (1)	1 2%	21 47%	23 51%	45 100%

Embryos with X-gal staining in the neural plate were scored by comparing numbers of *N-tubulin*-expressing cells on the uninjected (control) and injected sides. The number of experiments from which the data were pooled is indicated in parentheses. All embryos were coinjected with *lacZ* RNA as a tracer. DBM, DNA-binding mutant X-Su(H)1^{DBM}.

suggesting that X-Su(H)1^{DBM} blocks activation of the Notch signaling pathway by X-Delta-1.

Because coexpression of wild-type *X-Su(H)1* and *X-Delta-1* in the animal cap assay above had revealed an unexpected inhibitory phenotype of X-Su(H)1, we also looked at the effect on primary neurons of expressing both *X-Su(H)1* and *X-Delta-1*. Interestingly, coexpression of wild-type *X-Su(H)1* with *X-Delta-1* partially reversed the inhibitory effects of *X-Delta-1* (Fig. 6H and Table 1). Whereas 71% of embryos expressing X-Delta-1 showed a decrease in primary neurons on their injected side, this number decreased to 51% when *X-Su(H)1* is expressed along with *X-Delta-1* (Table 1). Thus these results mirror those seen in the animal cap assay. When X-Delta-1 is used to artificially stimulate the Notch signaling pathway, this pathway can be partially inhibited by overexpression of *X-Su(H)1*.

X-Su(H)1^{DBM} increases the expression of *X-Delta-1* and *XNGNR1*

The results with X-Su(H)1^{DBM} suggested that more primary neurons form when Notch signaling is blocked. To extend these results further, we examined the expression pattern of *X-Delta-1* and of *XNGNR1*, a bHLH gene which promotes the formation of primary neurons. The expression of both *X-Delta-1* and *XNGNR1* is known to decrease when Notch signaling is increased with the X-Notch-1 ICD and conversely to increase when Notch signaling is inhibited with X-Delta-1^{STU} (Chitnis et al., 1995; Ma et al., 1996). In embryos injected with *X-Su(H)1^{DBM}* (Fig. 7C,F), but not with wild-type *X-Su(H)1* (Fig. 7B,E), there is an increase in *X-Delta-1* and *XNGNR1* similar to the increase in the density of primary neurons (Table 2). Thus, these results are consistent with the idea that X-Su(H)1^{DBM} blocks lateral inhibition, and provides evidence that X-Su(H)1 is required for Notch signaling in vivo.

Table 2. Effect of forms of X-Su(H)1 on *X-Delta-1* and *XNGNR1* expression

RNA injected:	*X-Delta-1* expression			
	More	Unchanged	Less	Total
lacZ (1)	0 0%	15 79%	4 21%	19 100%
X-Su(H)1 (1)	3 14%	19 86%	0 0%	22 100%
DBM (1)	29 78%	8 22%	0 0%	37 100%
RNA injected:	***XNGNR1* expression**			
	More	Unchanged	Less	Total
lacZ (1)	1 4%	23 96%	0 0%	24 100%
X-Su(H)1 (1)	6 21%	21 75%	1 4%	28 100%
DBM (1)	29 67%	13 30%	1 2%	43 99%

Data presented and tabulated as in Table 1 except that embryos with X-gal staining in the neural plate were scored by comparing numbers of *X-Delta-1*- or *XNGNR1*-expressing cells on the uninjected (control) and injected sides.

DISCUSSION

In *Drosophila*, *Su(H)* behaves genetically as an activator of the Notch signaling pathway (Schweisguth and Posakony, 1992). Thus, loss-of-function mutations in *Su(H)* disable lateral inhibition, producing a neurogenic phenotype, while overexpression of *Su(H)* through heat shock of transgenes appears to give an antineurogenic phenotype (Schweisguth and Posakony, 1994). Our data strongly support the idea that X-Su(H)1 also acts as a positive regulator of the Notch signaling pathway in *Xenopus* embryos. Expression of a dominant negative form of X-Su(H)1 (X-Su(H)1^{DBM}) in vivo leads to an increase in the number of primary neurons that form, thus producing a neurogenic phenotype. Coexpression of *X-Su(H)1^{DBM}* with *X-Delta-1* also results in an increase in primary neurons, completely counteracting the antineurogenic effects produced by expression of *X-Delta-1* alone. In the animal cap assay, X-Su(H)1^{DBM} inhibits the induction of *ESR-1* mediated by X-Delta-1. These results are consistent with X-Su(H)1^{DBM} blocking Notch signaling and suggest that X-Su(H)1 is downstream of X-Delta-1 in the signaling pathway.

Since X-Su(H)1^{DBM} appears to block Notch signaling, one might expect that overexpression of *X-Su(H)1* should give the same effects as activating Notch signaling. However, when *X-Su(H)1* is overexpressed, it does not induce *ESR-1* expression in the animal cap assay and it weakly inhibits the formation of primary neurons. Furthermore, when *X-Delta-1* is overexpressed in embryos or in the animal caps assays, coexpression of *X-Su(H)1* has a slight inhibitory effect on Notch signaling. Thus, overexpression of *X-Su(H)1* alone does not appear to be sufficient to markedly stimulate Notch signaling, but under some conditions will inhibit it.

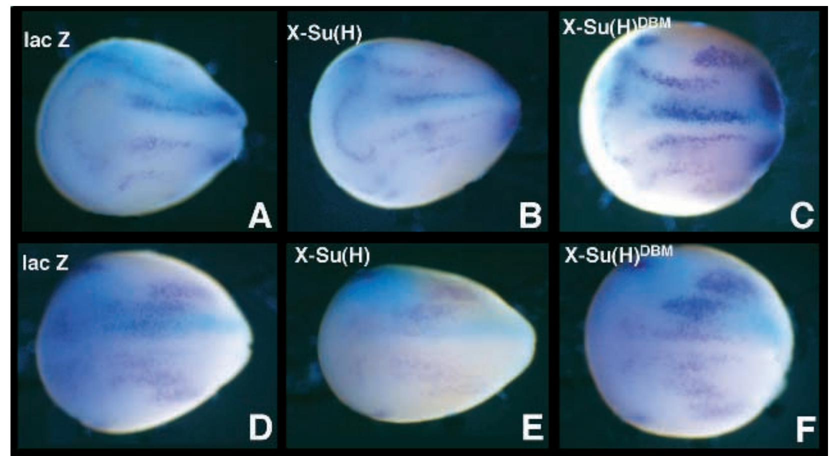

Fig. 7. Forms of X-Su(H)1 affect the pattern of expression of *X-Delta-1* and *XNGNR1*. The effects of test RNAs on the formation of primary neurons was assayed as described in Fig. 6. Embryos were injected with *lacZ* alone (A,D), *X-Su(H)*1 (B,E), or *X-Su(H)1*^{DBM} (C,F) and analyzed for the expression of *X-Delta-1* (A-C) or *XNGNR1* (D-F). Note that the X-Su(H)1^{DBM} increases the expression of both *XNGNR1* and *X-Delta-1.*

These observations are consistent with a model where X-Su(H)1, as a DNA binding protein, can only mediate Notch signaling and activate the expression of target genes, such as *ESR-1*, when it associates with other proteins that provide an activator domain. The precedent for this model comes from studies of EBNA2, a transcriptional activator encoded by the Epstein-Barr virus (EBV), which binds to human Su(H) (called CBF1) and drives immortalization of EBV-infected B cells. EBNA2 endows CBF1/EBNA2 complexes with an activation domain, which promotes transcription at responsive elements where CBF1 is bound (Hsieh and Hayward, 1995). By analogy, X-Su(H)1 may only activate target genes such as *ESR-1* when it associates with another polypeptide generated during Notch signaling. Thus, under normal conditions, the activator is limiting, and overexpression of X-Su(H)1 does not affect Notch signaling. This model can also explain the slight inhibitory effects that X-Su(H)1 has in situations where the Notch signaling pathway is artificially stimulated by overexpression of X-Delta-1. In this case, we imagine that the concentration of X-Su(H)1 exceeds that of an activator, resulting in a situation where the free X-Su(H)1 competes with X-Su(H)1/activator complexes for binding to DNA, and this would block signaling. This model is also consistent with the observation that X-Su(H)1 can be converted into a very strong transcriptional activator of *ESR-1* by attaching ankyrin repeats (Fig. 3A). Finally, we have found that expression of *EBNA2* in neuralized animal caps induces *ESR-1* (D. A. W. and C. K., unpublished results). Thus, all observations thus far support the conclusion that X-Su(H)1 activates ESR-1 expression but only after association with another protein that provides an activator domain.

Our experiments have not resolved the question of how Su(H) normally becomes activated during Notch signaling. One possibility is that Su(H) becomes activated by forming a complex with the Notch ICD which is cleaved off following receptor activation (reviewed by Kopan et al., 1996). In this model the Notch ICD/Su(H) complex translocates to the nucleus where it activates target genes, such as *ESR-1*. While in vitro studies have lent some support to this model (Jarriault et al., 1995), processed ICD-like forms of Notch have not been detected in the nucleus in response to ligand binding. Thus, at present it remains a formal possibility that activation of Notch leads to the activation of another protein, such as one like EBNA-2, which then associates with Su(H) to form a transcriptionally active complex.

Su(H) and lateral inhibition

The main finding of our studies is that X-Su(H)1 is required for Notch signaling in vivo, where the number of neurons that form during primary neurogenesis appears to be regulated by lateral inhibition. Thus, X-Su(H)1/Ank blocked formation of primary neurons, which was predicted by its ability to activate the Notch signaling pathway as evidenced by upregulating *ESR-1* expression. In contrast to the results with X-Su(H)1/Ank, the DNA-binding mutant X-Su(H)1^{DBM} increased the number of primary neurons, as expected if this mutant were interfering with Notch signaling. The ability of X-Su(H)1^{DBM} to inhibit X-Notch-1 signaling was confirmed by showing that it also blocked the inhibitory effects that ectopic *X-Delta-1* expression has on primary neurogenesis. As in previous experiments with an antimorphic form of X-Delta-1 (Chitnis et al., 1995), the inhibition of X-Notch-1 signaling by X-Su(H)1^{DBM} led to a higher density of primary neurons in each longitudinal ‘proneural’ domain, while the extent of these domains remained largely unchanged. Moreover, X-Su(H)1^{DBM} leads to a higher density of expression of *X-Delta-1* and *XNGNR1*, providing further evidence that Notch signaling regulates the activity of other genes that mediate lateral inhibition via Su(H). These data, along with functional analysis of *ESR-1* (D. L. Turner, L. Snider, R. A. W. Rupp, H. Weintraub and C. Kintner, unpublished), suggest that the Notch signaling pathway used in lateral inhibition is conserved during neurogenesis in vertebrates and invertebrates. It will be of interest to determine whether the same pathway is conserved in other cases where Notch signaling appears to regulate developmental processes in the vertebrate embryo.

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REFERENCES

Amakawa, R., Jing, W., Ozawa, K., Matsunami, N., Hamaguchi, Y., Matsuda, F., Kawaichi, M. and Honjo, T. (1993). Human Jk recombination signal binding protein gene (IGKJRB): comparison with its mouse homologue. *Genomics* **17**, 306-315.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of Hairless directly activates transcription of Enhancer of split Complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Brou, C., Logeat, F., Lecourtois, M., Vandekerckhove, J., Kourilsky, P., Schweisguth, F. and Israël, A. (1994). Inhibition of the DNA-binding activity of Drosophila Suppressor of Hairless and of its human homolog, KBF2/RBP-J kappa, by direct protein-protein interaction with *Drosophila* hairless. *Genes Dev.* **8**, 2491-2503.

Cascio, S. and Gurdon, J. B. (1987). The initiation of new gene transcription during *Xenopus* gastrulation requires immediately preceding protein synthesis. *Development* **100**, 297-305.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embyros. *Development* **122**, 2295-2301.

Christensen, S., Kodoyianni, V., Bosenberg, M., Friedman, L. and Kimble, J. (1996). *lag-1*, a gene required for *lin-12* and *glp-1* signaling in *Caenorhabditis elegans*, is homologous to human CBF1 and *Drosophila* Su(H). *Development* **122**, 1373-1383.

Chung, C.-N., Hamaguchi, Y., Honjo, T. and Kawaichi, M. (1994). Site-directed mutagenesis study on DNA binding regions of the mouse homologue of Suppressor of Hairless, RBP-J kappa. *Nucl. Acids Res.* **22**, 2938-2944.

Coffman, C., Harris, W. and Kintner, C. (1990). Xotch, the Xenopus homolog of Drosophila Notch. *Science* **249**, 1438-1441.

Dawson, S. R., Turner, D. L., Weintraub, H. and Parkhurst, S. M. (1995). Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol. Cell Biol.* **15**, 6923-6931.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The Suppressor of Hairless protein participates in Notch receptor signaling. *Cell* **79**, 273-282.

Frangioni, J. V. and Neel, B. G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Analyt. Biochem.* **210**, 179-187.

Guillemot, F. (1995). Analysis of the role of basic-helix-loop-helix transcription factors in the development of neural lineages in the mouse. *Biol. Cell* **84**, 3-6.

Haenlin, M., Kunisch, M., Kramatschek, B. and Campos-Ortega, J. A. (1994). Genomic regions regulating early embryonic expression of the Drosophila neurogenic gene *Delta*. *Mech. Dev.* **47**, 99-110.

Hamaguchi, Y., Matsunami, N., Yamamoto, Y. and Honjo, T. (1989). Purification and characterization of a protein that binds to the recombination signal sequence of the immunoglobulin J kappa segment. *Nucleic Acids Res.* **17**, 9015-9026.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in *Drosophila*. *Development* **122**, 161-171.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hollenberg, S. M., Cheng, P. F. and Weintraub, H. (1993). Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. *Proc. Natl. Acad. Sci. USA* **90**, 8028-8032.

Hsieh, J. J. and Hayward, S. D. (1995). Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* **268**, 560-563.

Hsieh, J. J.-D., Henkel, T., Salmon, P., Robey, E., Peterson, M. G. and Hayward, S. D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol. Cell. Biol.* **16**, 952-959.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Jennings, B., Preiss, A., Delidakis, C. and Bray, S. (1994). The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* **120**, 3537-3548.

Kintner, C. R. and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm. Implications for the action of the organizer in neural induction. *Development* **113**, 1495-1505.

Kintner, C. R. and Melton, D. A. (1987). Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Kolm, P. J. and Sive, H. L. (1995). Efficient hormone-inducible protein function in Xenopus laevis. *Dev. Biol.* **171**, 267-272.

Kopan, R., Schroeter, E. H., Weintraub, H. and Nye, J. S. (1996). Signal transduction by activated mNotch: Importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci. USA* **93**, 1683-1688.

Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene Delta is enhanced by proneural proteins. *Proc. Natl. Acad. Sci. USA* **91**, 10139-10143.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the Enhancer of split complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6**, 3-10.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Oschwald, R., Richter, K. and Grunz, H. (1991). Localization of a nervous system-specific class II beta-tubulin gene in Xenopus laevis embryos by whole-mount in situ hybridization. *Int. J. Dev. Biol.* **35**, 399-405.

Rupp, R. A., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8**, 1311-1323.

Schweisguth, F. and Posakony, J. W. (1992). Suppressor of Hairless, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell* **69**, 1199-1212.

Schweisguth, F. and Posakony, J. W. (1994). Antagonistic activities of Suppressor of Hairless and Hairless control alternative cell fates in the Drosophila adult epidermis. *Development* **120**, 1433-1441.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S. and Kageyama, R. (1994). Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. *J. Biol. Chem.* **269**, 5150-5156.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T. and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-Jκ/Su(H). *Curr. Biol.* **5**, 1416-1423.

Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T. and Kawaichi, M. (1994). Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucl. Acids Res.* **22**, 965-971.

Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.

Wainwright, S. M. and Ish-Horowicz, D. (1992). Point mutations in the Drosophila hairy gene demonstrate in vivo requirements for basic, helix-loop-helix, and WRPW domains. *Mol. Cell Biol.* **12**, 2475-2483.

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