XSu(H)2 is an essential factor for gene expression and morphogenesis of the Xenopus gastrula embryo

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ABSTRACT The CSL (CBF-1, Suppressor of Hairless, Lag-1) transcriptional factor is an important mediator of Notch signal transduction. It plays a key role in cell fate determination by cell-cell interaction. CSL functions as a transcriptional repressor before the activation of Notch signaling. However, once Notch signaling is activated, CSL is converted into a transcriptional activator. It remains unclear if CSL has any function during early development before neurogenesis, while transcriptional products exist from the maternal stage. Here, we analyzed the function of Xenopus Suppressor of Hairless (XSu(H)) using morpholino antisense oligonucleotides (MO), which interfere with the translation of transcripts. In Xenopus embryos, maternal transcripts of both XSu(H)1 and XSu(H)2 were ubiquitously observed until the blastula stage and thereafter only XSu(H)1 was zygotically transcribed. Knockdown experiments with MO demonstrated that XSu(H)2 depletion caused a decrease in the expression of the Xbrachyury, MyoD and JNK1 genes. Morphological and histological examinations indicated that XSu(H)2 depletion caused abnormal gastrulation, which resulted in severe defects of the notochord and somitic mesoderm. The effect of XSu(H)2-MO was completely rescued by co-injection of XSu(H)2 mRNAs, but not by XSu(H)1 mRNAs. XESR-1, a Notch signaling target gene, inhibited Xbrachyury expression. However, expression of the XESR-1 gene was not induced by depletion of XSu(H)2. Co-injection of the dominant-negative form of XESR-1 could not rescue the suppression of Xbrachyury expression in the XSu(H)2-depleted embryo. These results suggest that XSu(H)2 is involved in mesoderm formation and the cell movement of gastrula embryos in a different manner from the XESR-1-mediated Notch signaling pathway.

KEY WORDS: Suppressor of Hairless, notch signaling, gastrulation, Xbrachyury, XESR-1

Introduction

CSL (CBF-1, Suppressor of Hairless, Lag-1) is a highly conserved transcriptional factor from human to C. elegans (Schweisguth et al., 1992; Amakawa et al., 1993; Tun et al., 1994; Christensen et al., 1996; Wettstein et al., 1997). CSL functions as a transcriptional factor of Notch signaling transduction, which is essential for a variety of developmental processes, including asymmetric cell-fate decision and boundary formation (Artavanis-Tsakonas et al., 1995). When Notch signaling is activated by binding ligands, the Delta and Serrate/Jagged family, the transmembrane domain of Notch receptor is proteolytically cleaved and the Notch intracellular domain (NICD) is released from the membrane. NICD then translocates into the nucleus and transactivates Hairy/Enhancer of Split (E(spl)) by association with CSL. In the absence of NICD, CSL act as transcriptional repressors by associating with Hairless, CtBP, Groucho, Histone deacetylase (HDAC) in Drosophila (Furiols and Bray, 2000; Morel et al., 2001; Barolo et al., 2000; 2002), or with CIR, SMART, SKIP, HDAC1 in mammalian tissues (Chen and Evans, 1995; Horlein et al., 1995; Kao et al., 1998; Hisheh et al., 1999; Zhou and Hayward, 2001); however, binding of NICD causes CSL to convert into transcriptional activators (Artavanis-Tsakonas et al., 1995; Lai, 2002).

Null mutant mice of RBP-Jk, a mouse homologue of CSL, show severe growth retardation at 8.5 days of gestation and defective neurogenesis and somitogenesis at 9.5 days of gestation and then become lethal before day 10.5 of embryogenesis (Oka et al., 1995). This lethal phase of RBP-Jk null mutant mice appears at an earlier stage than in Notch1 null mutant mice.

Abbreviations used in this paper: CSL, CBF-1, Suppressor of Hairless, Lag-1 transcription factor; MO, morpholino antisense oligonucleotide; XSu(H), Xenopus Suppressor of Hairless.
(Conlon et al., 1995), suggesting that CSL has a different function from Notch-dependent signaling. The Notch-independent CSL function has been indicated in the Drosophila mechanoreceptor (Barolo et al., 2000) who reported that Su(H) maintains its own activity by auto-activating the socket cell-specific transcriptional enhancer on the Su(H) gene and this auto-activation does not require continued Notch signaling. Notch signaling is involved in various cell fate decisions such as neurogenesis, somitogenesis and T cell / B cell differentiation (Furukawa et al., 1992; Han et al., 2002; Yamamoto et al., 2003). Expressions of chick Notch1 and Delta are detected at epiblast in early gastrula (Capriol et al., 2002) and X-Delta-1 (Kuroda et al., 1999; Wittenberger et al., 1999), dndeltaC, dndeltaD (Haddon et al., 1998; Smithers et al., 2000) and dnnotch1 (Bierkamp and Campos-Ortega, 1993) are recognized from unfertilized eggs to the gastrula stage. Enriched maternal transcripts of XSu(H)1 and gene expression was detected by RT-PCR. Eggs (E) to stage 35. Enriched maternal transcripts of XSu(H)1 and XSu(H)2 have been cloned. XSu(H)1 has an additional 20 amino acid residues at the N-terminal region of XSu(H)2, but otherwise they have an identical amino-acid sequence. First, we performed semi-quantitative RT-PCR in order to know the expression pattern of XSu(H)1 and XSu(H)2 during the early development of Xenopus. Transcriptional products of XSu(H)1 were detected ubiquitously from the unfertilized egg to the tailbud stage embryo as in a previous report (Fig. 1A, B, Wettstein et al., 1997). Transcripts of XSu(H)2 were also detected ubiquitously during all embryonic stages; however, enriched transcripts of XSu(H)2 were detected maternally until the gastrula stage and thereafter sharply decreased to the same level as XSu(H)1 (Fig. 1A, B). The ubiquitous distribution of XSu(H)1 transcript has been reported (Wettstein et al., 1997), but that of XSu(H)2 is still unknown. In order to elucidate the distribution of XSu(H) transcripts, blastula embryos were dissected into three parts at stage 9 and semi-quantitative RT-PCR was performed for each extract. As shown in Figure 1C, both XSu(H)1 and XSu(H)2 were localized ubiquitously in the blastula embryo. These results suggest that XSu(H) plays a role during the early embryonic stages.

**Results**

*The expression of XSu(H)2 is different from that of XSu(H)1*

In Xenopus, XSu(H)1 and XSu(H)2 have been reported as CSL homologues. XSu(H)1 has an additional 20 amino acid residues at the N-terminal region of XSu(H)2, but otherwise they have an identical amino-acid sequence. First, we performed semi-quantitative RT-PCR in order to know the expression pattern of XSu(H)1 and XSu(H)2 during the early development of Xenopus. Transcriptional products of XSu(H)1 were detected ubiquitously from the unfertilized egg to the tailbud stage embryo as in a previous report (Fig. 1A, B, Wettstein et al., 1997). Transcripts of XSu(H)2 were also detected ubiquitously during all embryonic stages; however, enriched transcripts of XSu(H)2 were detected maternally until the gastrula stage and thereafter sharply decreased to the same level as XSu(H)1 (Fig. 1A, B). The ubiquitous distribution of XSu(H)1 transcript has been reported (Wettstein et al., 1997), but that of XSu(H)2 is still unknown. In order to elucidate the distribution of XSu(H) transcripts, blastula embryos were dissected into three parts at stage 9 and semi-quantitative RT-PCR was performed for each extract. As shown in Figure 1C, both XSu(H)1 and XSu(H)2 were localized ubiquitously in the blastula embryo. These results suggest that XSu(H) plays a role during the early embryonic stages.

**Translational inhibition of XSu(H)2 causes abnormal gastrulation and neural fold disorganization**

We investigated whether XSu(H) is involved in early embryogenesis by using morpholin antisense oligonucleotides (MO). First, we tested whether the MOs prepared against XSu(H)1 and XSu(H)2 specifically inhibit the translation of each transcript (Fig. 2A). The expressions of myc-XSu(H)1 and myc-XSu(H)2 proteins were detected with anti-myc antibody (lanes 2, 6). XSu(H)1-MO and XSu(H)2-MO inhibited the translation of its own target (lanes 3, 7), but XSu(H)1-MO and XSu(H)2-MO did not inhibit the...
XSu(H)2 is essential for gastrulation and Xbrachyury expression

In order to examine the tissue affected by XSu(H)2-MO, histological analysis was performed on XSu(H)2-depleted embryos. Since severe defects of XSu(H)2 cause developmental arrest at the gastrula stage, low-dose XSu(H)2-MO (10 ng per embryo) was used in the histological examination. In stage 35 embryos injected with XSu(H)2-MO in one blastomere at the 2-cell stage, tissue defects were observed in the somite and notochord, but not in the neural tube on the injection side (100%, n=10) (Fig. 3A, B). These tissue defects were not detected either in the control-MO-injected embryo (100%, n=9) or in the XSu(H)1-MO-injected embryo (100%, n=10). Since morphological abnormalities in the XSu(H)2-depleted embryos first appeared during gastrulation, we analyzed the expression of genes essential for development in the early gastrula stage. As shown in Figure 3C, depletion of XSu(H)2 caused a remarkable decrease in Xbrachyury, MyoD, Xvent1, chordin and JNK1 expression, which was not observed in the XSu(H)1-depleted embryo. Even under these conditions, goosecoid showed a normal expression both in XSu(H)1-MO- and XSu(H)2-MO-injected embryos. Overexpression of XSu(H)1 or XSu(H)2 had no effect on the expression of marker genes (Fig. 3C). These results indicate that XSu(H)2 is an essential factor in gastrulation, mesoderm formation and cell movement of gastrula embryos, which is quite different from the role of XSu(H)1.

Effect of XSu(H)2-MO is caused by the specific inhibition of XSu(H)2 function

In order to confirm whether XSu(H)2-MO specifically inhibits the translation of XSu(H)2 but not XSu(H)1, we performed rescue experiments by co-injection of XSu(H)1 or XSu(H)2 together with

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<th>Injected sample (ng)</th>
<th>Total number of embryos</th>
<th>Gastroatriation defect</th>
<th>Unaffected</th>
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<tbody>
<tr>
<td>control-MO (25)</td>
<td>63</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>XSu(H)1-MO (25)</td>
<td>52</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>XSu(H)2-MO (25)</td>
<td>56</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + ∆5'UTR XSu(H)1(2.0)</td>
<td>25</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + ∆5'UTR XSu(H)1(5.0)</td>
<td>25</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + ∆5'UTR XSu(H)2(2.0)</td>
<td>32</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + ∆5'UTR XSu(H)2(15.0)</td>
<td>40</td>
<td>10</td>
<td>90</td>
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Twenty-five ng of morpholino antisense oligonucleotides (MO) were injected into one dorsal blastomere of 4-cell-stage embryo, with or without 5'UTR-deleted mRNA. The injected embryos were cultured at 18°C until stage 11, when gastrulation defect was examined on each embryo.}

Fig. 2. Effect of XSu(H) morpholinos on early embryonic development. (A) Specificity of morpholino oligonucleotides (MO). Five ng of myc-XSu(H)1 or myc-XSu(H)2 mRNA was injected into the animal pole of each blastomere of 2-cell stage with or without 25 ng of XSu(H)1-MO or XSu(H)2-MO. XSu(H)1 or XSu(H)2 protein was detected by anti-c-myc antibody at stage 10.5. 43 kDa actin bands were used as loading controls (Coomassie stained). (B) Phenotype of the MO-injected embryo. Embryos were injected with control-MO (B1, B6), XSu(H)1-MO (B2, B7), XSu(H)2-MO (B3, B8), mRNAs of XSu(H)1 (B4, B9) or XSu(H)2 (B5, B10) into one dorsal blastomere at the 4-cell stage and morphological changes were analyzed at stage 13 (B1-5) or stage 20 (B6-10). To identify the injection side of the embryo, 1 ng of GFP mRNA was co-injected with each mRNA or MO. Upper panels (B1-5) show the vegetal view and lower panels (B6-10) show the dorsal view. XSu(H)2-depleted embryos showed delayed gastrulation (arrow in B3) and defective neurogenesis (arrow in B8), while XSu(H)1-depleted embryos developed normally (stage 10.5). Thereafter, however, the XSu(H)2-MO-injected embryos showed abnormal gastrulation and neural fold disorganization (Fig. 2B3, B8). These effects were not observed in embryos injected with XSu(H)1-MO nor control-MO (Fig. 2B1, B2, B6, B7; Table 1). In contrast, overexpression of XSu(H)2 did not cause any developmental abnormality, which was the same as the overexpression of XSu(H)1 (Fig. 2B4, B5, B9, B10) or XSu(H)1 + XSu(H)2 (data not shown). These results suggest that XSu(H)2 plays an important role from gastrulation to neurogenesis.

translation of XSu(H)2 and XSu(H)1, respectively (lanes 5, 9). The results indicate that these MOs are able to specifically inhibit the translation of each XSu(H). Next, we observed the effect of MO on the early development of embryos. Morpholino oligonucleotide was injected into one blastomere of a four-cell-stage embryo and the injection side of MO was detected by co-injecting with GFP mRNA. Embryos injected with either 25 ng of XSu(H)1-MO or XSu(H)2-MO developed normally until the early gastrula stage
XSu(H)2-MO. Since XSu(H)2-MO recognizes 5'UTR sequences just upstream of the first methionine, 5'UTR-deleted mRNA of XSu(H)1(∆5'UTR-XSu(H)1) was used as a rescue molecule. Abnormal development caused by XSu(H)2-MO injection was completely rescued by co-injection of ∆5'UTR-XSu(H)2 (Fig. 4A1, A2, A4), but not by ∆5'UTR-XSu(H)1 (Fig. 4A3). Specific rescue by co-injection of ∆5'UTR-XSu(H)2 was also confirmed by the recovery of Xbrachyury expression only in the embryo injected with XSu(H)2-MO and ∆5'UTR-XSu(H)2 (Fig. 4B). This recovery occurred in a dose-dependent manner (Table 1). These results suggest that abnormal gastrulation is caused specifically by the depletion of XSu(H)2.

XSu(H)2-MO-induced suppression of Xbrachyury cannot be rescued by Notch signaling

It is known that XSu(H)1 functions as a transcriptional factor under Notch signaling during neurogenesis (Wettstein et al., 1997), while it is unknown whether XSu(H)2 functions as a component of Notch signaling. To test the function of XSu(H)2, we made two constructs: XSu(H)2ANK, which is an active form of XSu(H)2 fused with the ankyrin region of Notch intracellular domain (NICD) and XSu(H)2DBM, which is a DNA-binding mutant prepared as a competitive inhibitor of Notch signaling by trapping NICD. Using these constructs, we examined the effect of XSu(H)2ANK and XSu(H)2DBM on the expression of XESR-1, a Notch signaling target gene. As shown in Figure 5A, XSu(H)2ANK activated the expression of XESR-1, whereas XSu(H)2DBM inhibited it as well as XSu(H)2-MO. These results are consistent with the effects of NICD by XSu(H)1 (Wettstein et al., 1997). At the same time, these results indicate that XSu(H)2 functions as a transcriptional factor under Notch signaling. Next, we examined whether Notch signaling is activated via endogenous XSu(H)1 even in the XSu(H)2-MO-injected embryo, because XSu(H)1 exists under depleted conditions of XSu(H)2. As shown in Figure 5A, NICD, the activated form of Notch signaling (Artavanis-Tsakonas et al., 1995; Wettstein et al., 1997), could activate the expression of XESR-1 with the depletion of XSu(H)2, suggesting that Notch signaling can be activated under XSu(H)2-depleted conditions.

If XSu(H)2-mediated Notch signaling has a role in mesoderm formation, there is a possibility that activation of Notch signaling can rescue the decrease of Xbrachyury expression caused by XSu(H)2-MO injection. To test this possibility, we examined whether NICD injection can rescue the decrease of Xbrachyury expression in the XSu(H)2-MO-injected gastrula embryo. In contrast with the complete rescue by co-injection of ∆5'UTR-XSu(H)2 (Fig. 5B1-B3), NICD could not rescue the XSu(H)2-MO-induced suppression of Xbrachyury expression (Fig. 5B5; Table 2). The effect of XSu(H)2-MO on Xbrachyury was examined in an animal

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<th>Injected sample (ng)</th>
<th>Total number of embryos</th>
<th>Xbrachyury expression (%)</th>
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<tr>
<td>control-MO (25)</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>XSu(H)2-MO (12.5)</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>XSu(H)2-MO (25)</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + ∆SUTR-XSu(H)2 (2.0)</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>XSu(H)2-MO (25) + NICD (2.0)</td>
<td>35</td>
<td>91</td>
</tr>
<tr>
<td>XSu(H)1(∆5'UTR) (2.0)</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>XSu(H)2DBM (2.0)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>NICD (2.0)</td>
<td>31</td>
<td>0</td>
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XSu(H)2-MO was injected with mRNAs of XSu(H)2 or NICD into one dorsal blastomere of 2-cell stage embryo. The injected embryos cultured at 18 °C were fixed with 1X MEMFA at stage 10.5 and gene expression of Xbrachyury was examined by whole-mount in situ hybridization.
cap assay, where Xbrachyury expression induced by Xnr2 was suppressed by the injection of XSu(H)2-MO (Fig. 5C). The suppression of Xbrachyury expression by XSu(H)2-MO was rescued by co-injection with Δ5’UTR XSu(H)2 but not with NICD (Fig. 5C). The activation of Notch signaling by NICD injection alone did not affect the expression of Xbrachyury (Fig. 5B). NICD did not induce the expression of Xbrachyury in animal cap without a mesoderm-inducing factor (data not shown). These results show
that the downregulation of Xbrachyury expression caused by XSu(H)2-MO is not recovered by activating Notch signaling.

**XSu(H)2 regulates Xbrachyury expression without transactivation of XESR-1**

From the results of Figure 5, XSu(H)2 seems to regulate the expression of Xbrachyury in a different manner from Notch signaling. To test whether XSu(H)2 regulates the expression of Xbrachyury through XESR-1, we investigated the effect of XESR-1 on Xbrachyury expression in the animal cap assay. As shown in Figure 6, overexpression of XESR-1 intensely suppressed Xnr2-induced Xbrachyury expression, showing that XESR-1, a target gene of Notch signaling, is a negative regulator of Xbrachyury expression. Next, we examined whether XSu(H)2 depletion activates XESR-1, which results in the suppression of Xbrachyury expression. In order to test this idea, we constructed DN XESR-1, the dominant-negative form of XESR-1 lacking the C-terminal WRPW motif (Fisher et al., 1996; Giebel and Campos-Ortega, 1997). Prior to using DN XESR-1, we checked the effect of this molecule on primary neurogenesis. The injection of this construct into embryos caused an increased expression of N-tubulin (data not shown). Then, we examined whether DN XESR-1 injection can rescue the decrease of Xbrachyury expression in the XSu(H)2-MO-injected animal cap. As shown in Figure 6, DN XESR-1 could not rescue the downregulation of Xbrachyury caused by XSu(H)2-MO, while it could rescue that caused by XESR-1. These results show that XSu(H)2-MO-induced suppression of Xbrachyury expression is not caused by the activation of XESR-1.

**Discussion**

XSu(H)1 is known to function as a transcriptional factor of Notch signaling that controls the neurogenic pathway. Here, we demonstrated that not XSu(H)1 but XSu(H)2 plays an important role in the gastrulation of Xenopus embryos.

**XSu(H)2 has a different function from XSu(H)1 during early embryogenesis**

XSu(H)2 knockdown embryos showed remarkably abnormal phenotypes, whereas the depletion of XSu(H)1 caused no change in normal development at the gastrula stage (Fig. 2B2, B3, B7, B8). The decrease of Xbrachyury expression caused by XSu(H)2-MO could be rescued by Δ5′UTR XSu(H)2, but not by Δ5′UTR XSu(H)1 (Fig. 4A3, A4, B). Regardless of the same expression pattern of both genes (Fig. 1B), these results revealed that XSu(H)2 may have a different function from XSu(H)1. XSu(H)1 has 20 additional amino acids at its N-terminus, different from XSu(H)2. Moreover, XSu(H)1 has different 5′UTRs from XSu(H)2. Xpygopus-alpha and -beta are transcriptional factors and their structures are similar to XSu(H)1 and XSu(H)2 (Lake and Kao, 2003). As compared with Xpygopus-beta, Xpygopus-alpha has 21 additional N-terminal residues and shows a different temporal expression pattern (Lake and Kao, 2003). In Xenopus, it is likely that these isoforms play different roles during early development, since the additional 20 amino-acid region is able to interact with some factors.

Deficiency of XSu(H)2 protein by XSu(H)2-MO caused a decreased expression of mesodermal markers, which resulted in abnormal gastrulation (Fig. 2B3, B8, 3C). JNK1 is involved in convergent extension of the Wnt/JNK pathway in a mesoderm-independent manner (Yamana et al., 2002). It is reported that Xbrachyury functions as a switch between cell migration and convergent extension (Kwan and Kirschner, 2003). The decrease of JNK1 expression caused by XSu(H)2-MO (Fig. 3C) may result from the reduction of Xbrachyury expression, because it has been reported that Xbrachyury is required for convergent extension movements and functions upstream of Wnt11, a ligand of the Wnt/JNK pathway (Smith, 2000; Tada et al., 2005). Thus, abnormal development in the XSu(H)2-depleted embryo may result from defective mesoderm formation and cell movement caused by the lack of Xbrachyury expression; however, it is also possible that XSu(H)2 regulates both Xbrachyury and JNK expression independently. Further examination is necessary to clarify this possibility. Gastrulation defects are not caused by the inhibition of Notch signaling using a dominant-negative Notch ligand, X-Delta-1<sup>Δ1</sup> (data not shown) and a dominant-negative Notch component, C-terminus-deleted Mastermind (Katada and Kinoshita, 2003). These results suggest that abnormal gastrulation caused by the depletion of XSu(H)2 occurs in a Notch signaling-independent manner.

The injection of XSu(H)1-MO had no effect on gastrulation, but induced the up-regulation of the primary neuronal marker, N-tubulin (data not shown). This is consistent with the inhibition of Notch signaling, which causes excessive primary neurogenesis (Greenwald, 1994; Wettstein et al., 1997; Kiyota and Kinoshita, 2002). These results suggest that XSu(H)1 functions as a down-stream factor of Notch signal transduction during primary neurogenesis, but not at the gastrula stage.

In the study of somitogenesis, overexpression of XSu(H)1 leads to the defective segmentation of somites and alters the
segmented expression pattern of XESR-5 and X-Delta-2 (Gautier-Courteille et al., 2004). Inhibition of Notch signaling by expressing a dominant-negative mutant of XSu(H)1, XSu(H)1DBM also caused segmentation defects and decreased the expression of XESR-5 and X-Delta-2 (Jen et al., 1999). In this study, however, regardless of the clear knockdown effect induced by MO, overexpression of both XSu(H)1 and XSu(H)2 did not show a remarkable phenotype (Fig. 2B4, B5, B9, B10). Since CSL is thought to function as a mediator of transcriptional co-activators or transcriptional co-repressors (Lai, 2002), excessive co-factors other than XSu(H) are probably needed to induce the remarkable phenotype in the overexpression of XSu(H)1 and XSu(H)2.

**Regulation of Xbrachyury by XSu(H)2**

We showed that XSu(H)2 is involved in the regulation of Xbrachyury expression (Fig. 4A3, A4, B). In Ciona, CSL homologue Ci-Su(H) binds the CSL binding site on the brachyury promoter and directly activates the transcription of brachyury (Corbo et al., 1998). This is consistent with our result that Xbrachyury expression is repressed by the depletion of XSu(H)2. Notochordal cells in Ciona increase in number when X-Notch-1 is overexpressed in notochords using the forkhead promoter of Ciona (Corbo et al., 1998). In our experiment, however, overexpression of NICD could not increase the gene expression of Xbrachyury (Fig. 5B4), suggesting that XSu(H)2 is necessary but not enough to induce Xbrachyury expression.

In recent studies, the activation of Notch signaling by NICD decreases notochordal markers, such as Xbrachyury and chordin expression, in the dorsal midline during the Xenopus neurula stage (Lopez et al., 2003). Other studies indicate that Notch signaling is involved in the midline structure in zebrafish (Appel et al., 1999; Latimer et al., 2002). In this study, however, we examined the effect of XSu(H)2-MO on Xbrachyury expression not in the midline but in the entire marginal zone (presumptive mesodermal cells) at the early gastrula stage. XSu(H)2 at the gastrula stage may have a different function from Notch signaling at a later stage. Further study is required to understand the role of XSu(H)2 in Xbrachyury expression.

**Function of XSu(H)2 in germ layer formation**

RBP-Jk null mutant mice show abnormal somitogenesis and neurogenesis and die before day 10.5 of embryogenesis (Oka et al., 1995), indicating that CSL is an essential factor in early embryogenesis. RBP-Jk null mutant mice die much earlier than Notch1 null mutant mice (Conlon et al., 1995), suggesting that CSL plays an important role in early embryogenesis through a different pathway from Notch signaling. Recently, it was revealed that Notch signaling is involved in the cell fate determination of endomesoderm and mesoderm and is essential for mesoderm formation. In sea urchin embryos, it has been reported that activation of Notch signaling increased the non-skeletogenic mesoderm and the suppression of Notch signaling caused complete defect of the non-skeletogenic mesoderm (Sheerwood and McClay, 2001; Sweet et al., 2002). If this is the case in Xenopus embryos, abnormal gastrulation caused by XSu(H)2-MO may come from mesoderm defects induced by the suppression of Notch signaling. In Xenopus embryos, however, overexpression of NICD did not increase Xbrachyury expression (Fig. 5B4) and suppression of Notch signaling by XSu(H)1/2DBM could not affect the gene expression of Xbrachyury (data not shown). These results suggest that XSu(H)2 plays a role in Xenopus gastrulation in a Notch-independent manner.

In Xenopus, X-Delta-1 and XMyoD are expressed in the marginal zone of early gastrula embryos. XMyoD stimulates X-Su(H)1DBM (Gautier-Courteille et al., 1998) and complementary Ci-Su(H) binds the CSL binding site on the Xbrachyury promoter and directly activates the transcription of Xbrachyury (Fig. 4A3, A4, B). In our experiment, however, regardless of the clear knockdown effect induced by MO, overexpression of both XSu(H)2 and XSu(H)2DBM did not show a remarkable phenotype (Fig. 2B4, B5, B9, B10). Since CSL is thought to function as a mediator of transcriptional co-activators or transcriptional co-repressors (Lai, 2002), excessive co-factors other than XSu(H) are probably needed to induce the remarkable phenotype in the overexpression of XSu(H)1 and XSu(H)2.

**XSu(H)2 regulates mesoderm formation without activation of XESR-1**

Recent studies show that CSL interacts with NICD through a hydrophobic pocket on the beta-trefoil domain (BTD) of CSL (Nam et al., 2006; Wilson and Kovall, 2006). BTD is conserved between XSu(H)1 and XSu(H)2; therefore, it is thought that XSu(H)2-DBM can inhibit the Notch signaling pathway by trapping NICD, as shown in XSu(H)1DBM in a previous study (Wettstein et al., 1997). In fact, XSu(H)2-DBM could decrease XESR-1 expression (Fig. 5A). In our study, either the activation of Notch signaling by NICD (Fig. 5B4) or the suppression of Notch signaling by XSu(H)2DBM (data not shown) showed no effect on Xbrachyury expression. Nevertheless, the XSu(H)2-knockdown embryo showed the downregulation of Xbrachyury expression and severe gastrulation defects. Judging from these results, it is likely that endogenous XSu(H)2 can regulate the transcription of Xbrachyury without NICD. Since endogenous XSu(H)2 exists under the XSu(H)2DBM-injected condition, Xbrachyury expression must be induced even in the XSu(H)2DBM-injected embryo. These results suggest that XSu(H)2 regulates Xbrachyury expression through a mechanism other than Notch signal transduction.

Overexpression of XESR-1, a target gene of Notch signaling, decreased Xbrachyury expression (Fig. 6), whereas NICD showed no effect (Fig. 5B4). We cannot explain why NICD showed no effect on the expression of Xbrachyury, because NICD could induce the gene expression of XESR-1 (Fig. 5A). In contrast, XSu(H)2-MO suppressed Xbrachyury expression without the gene expression of XESR-1 (Fig. 5A). DN XESR-1, a dominant-negative form of XESR-1, could rescue the decrease of Xbrachyury expression induced by XESR-1, but not by XSu(H)2-MO (Fig. 6). These results suggest that XSu(H)2 regulates Xbrachyury expression in a different manner from the XESR-1-mediated Notch signaling pathway. Further examination is necessary to clarify mechanism of XSu(H)2. Interestingly, in mouse embryos, NICD induces T-cell differentiation without the gene expression of HES1 and HES5, mouse homologues of XESR-1, but its interference with B lymphocytes is partly mediated by HES genes (Kawamata et al., 2002). Both HES-dependent and HES-independent mechanisms must be
involved in the cell fate decision during animal development.

Materials and Methods

Eggs and embryos

*Xenopus* eggs were obtained by injecting human chorionic gonadotropin, gestron (Denka Seiyaku, Japan) into *Xenopus laevis* female and were fertilized with the testis isolated from *Xenopus laevis* male by surgical operation. Embryos were dejellied with 1% sodium thiglycolate and were cultured in 0.1X MMR (Marc’s Modified Ringers, [10 mM NaCl; 0.2 mM CaCl<sub>2</sub>; 0.5 mM HEPES, pH 7.5]). The developmental stages of embryos were determined by referring to normal table of *Xenopus laevis* (Nieuwkoop and Faber, 1967). Animal cap injected samples were excised from the blastula stage (stage 8), cultured in 1X MMR including 100 µg/ml kanamycin until stage 11 or 15.

Constructs and morpholino antisense oligo nucleotides

XSu(H)<sub>1</sub>/XSu(H)<sub>2</sub> (GeneBank accession number U60093 and U60094, respectively) including only open reading frame was amplified by polymerase chain reaction (PCR) with a *Xenopus* neurula library (a kind gift from Prof. D.A. Melton) using the following primer set; XSu(H)<sub>1</sub>/XSu(H)<sub>2</sub> DNA-binding mutant (XSu(H)<sub>1</sub>/2DBM) was generated by PCR as a template of XSu(H)<sub>1</sub>/2DBM was subcloned into pCS2+ vector at BamHI/Xhol site. XSu(H)<sub>1</sub>/2 antisense oligo nucleotides were designed as the following primer set; 5'-ATGATCTAGATGAAGTTGGGAGAGG and 5'-AATCTAGATGATGGCATCCTGCT and both of isolated XSu(H)<sub>1</sub> and XSu(H)<sub>2</sub> fragments were subcloned into pCS2+ vector (Turner and Wientraub, 1994) at BamHI/Xhol for XCl site, respectively. XSu(H)<sub>2</sub> DNA-binding mutant (XSu(H)<sub>2</sub>/2DBM) was subcloned into plasmid XSu(H)<sub>1</sub>/2DBM by PCR with the following primer set: 5'-ATGATCTAGATGAGTTGGGAGAGG and 5'-AATCTAGATGATGGGACACTG and were subcloned into pCS2+ vector at BamHI/Xhol site. XESR-1 antisense was generated by PCR with a *Xenopus* neurula library (a kind gift from Prof. D.A. Melton) using the following primer set: 5'-ATGATCTAGATGAACTGGGAGAGG and 5'-AATCTAGATGATGGCATCCTGCT. Both of isolated XSu(H)<sub>1</sub> and XSu(H)<sub>2</sub> fragments were subcloned into pCS2+ vector (Turner and Wientraub, 1994) at BamHI/Xhol or ClaI/XbaI site, respectively.

RNA synthesis and microinjection

All capped mRNAs were synthesized from linearized plasmids using SP6 RNA polymerase (Epicentre Technology). Capped mRNAs were made using mCAP RNA synthesis kit (Gibco BRL) according to the manufacturer’s instructions. Fertilization, culture and microinjection were performed as described previously (Moon and Christian, 1989, Asashima et al., 1995). One blastomere of a two- or four-cell-stage embryo was injected with 5 nL mRNA or MO solution (see text and tables).

Whole-mount in situ hybridization analysis

Whole-mount in situ hybridization was performed according to the improved method of Shain and Zuber (Shain and Zuber, 1996). Hybridized probes were visualized according to the Röch Diagnostics FIG protocol, with the minor alteration that 0.45 µl NBT (75 mg/ml in dimethyl formamide) and 3.5 µl BCIP (Röch Diagnostics) were added to 1 ml AP buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgSO<sub>4</sub>, 0.1% Tween 20, 2.5 mM levamisole]. The antisense RNA probe of XBrachyury (a kind gift from Prof. J. C. Smith) was prepared as described (Smith et al., 1991) by linearizing with EcoRI and transcribed with T7 RNA polymerase. β-galactosidase mRNA was produced from pCMV-SPORT β-gal (Stratagene).

RT-PCR analysis

Total RNA was extracted from embryos or animal caps using the isogen solution (Nippongene). Oligo (dT)-primed first strand cDNA was prepared from 0.5 µg of total RNA using Reverscript (Wako, Japan). Each PCR was performed with this cDNA as a template. The RT-PCR program was 95°C for 2 min, 55°C for 2 min and 20-30 cycles of 72°C 1 min, 95°C 30 sec, 55°C 30 sec. Several primer sequences are as follows; XSu(H)<sub>1</sub> upstream, 5'-GTTCAAGACTTCTTCTTTCTG-3' and downstream, 5'-AGAACTATATGACTTGGTCC-3'; XSu(H)<sub>2</sub> upstream, 5'-AAAGCTCGAGAGTTAGGAGA-3' and downstream, 5'-TCAGCTGCTGACTTCTTG-3'; Histone H4 upstream, 5'-CGGGATAAACATTCAAGGTTCATC-3' and downstream, 5'-ATCATGCGGTTGTGATTCCTC-3'; Xbrachury upstream, 5'-CGCTGGAGATGTGAATTGG-3' and downstream, 5'-TCATTCTGATGCGGATCG-3'; MyoD upstream, 5'-GACGACCCCCGTGTTCAATC-3' and downstream, 5'-GGTATTGTTGGATGATC-3'; Xvent1 upstream, 5'-TTCCTCAGCATGTTCAAC-3' and downstream, 5'-GCATCTCCCTGGCATATTGG-3'; chordin upstream, 5'-AATCTGCCAGACTGATGTG-3' and downstream, 5'-GCCAGATTTAGGTTGCTC-3'; JNK1 upstream, 5'-CAAAGAGGCTTATCGGGAAC-3' and downstream, 5'-TCCCAAGACTGATCTGAGAC-3'; goosecoid upstream, 5'-ACACCTACGAGTTGGTCA-3' and downstream, 5'-ACTTCATGGTACTGCTG-3'; X-ESR-1 upstream, 5'-CAAGAGGCTTATCGGGAAC-3' and downstream, 5'-GCCAGATTTAGGTTGCTC-3'; negative control (-RT) was performed using the same program without reverse transcriptase. These cycle numbers located within the linearity of the growth curve prior to saturation.

Western blotting

Synthetic RNA of the myc-fused construct was injected into both blastomeres of a two-cell stage embryo, which was sampled at the gastrula stage (stage 10.5). XSu(H)<sub>1</sub> and XSu(H)<sub>2</sub> protein with 6myc epitope tags were detected using anti-myc mouse monoclonal antibody (Molecular Probes) as the primary antibody and Alexa Fluor®680-conjugated anti-mouse IgG (H+L) antibody (Molecular Probes) as the secondary antibody. The fluorescent bands were detected using anti-myc mouse monoclonal antibody, anti-6myc mouse monoclonal antibody, and anti-myc mouse monoclonal antibody, respectively. As the internal control, actin bands were detected using anti-actin mouse monoclonal antibody and Fluor®680-conjugated anti-mouse IgG (H+L) antibody as the secondary antibody. The fluorescent bands were detected using anti-actin mouse monoclonal antibody, respectively.

Histology

The embryos were fixed with 4% paraformaldehyde for 12 hours at 4°C. They were then dehydrated through a graded series of methanol, cleared in xylene, embedded in TissuePrep (Fisher Scientific, U.S.A.) and sectioned serially at 10 µm. The sections were stained with hematoxylin and eosin.

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