Members of the bHLH-PAS family regulate Shh transcription in forebrain regions of the mouse CNS

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SUMMARY

The secreted protein sonic hedgehog (Shh) is required to establish patterns of cellular growth and differentiation within ventral regions of the developing CNS. The expression of Shh in the two tissue sources responsible for this activity, the axial mesoderm and the ventral midline of the neural tube, is regulated along the anteroposterior neuraxis. Separate cis-acting regulatory sequences have been identified which direct Shh expression to distinct regions of the neural tube, supporting the view that multiple genes are involved in activating Shh transcription along the length of the CNS. We show here that the activity of one Shh enhancer, which directs reporter expression to portions of the ventral midbrain and diencephalon, overlaps both temporally and spatially with the expression of Sim2. Sim2 encodes a basic helix-loop-helix (bHLH-PAS) PAS domain containing transcriptional regulator whose Drosophila homolog, single-minded, is a master regulator of ventral midline development. Both vertebrate and invertebrate Sim family members were found sufficient for the activation of the Shh reporter as well as endogenous Shh mRNA. Although Shh expression is maintained in Sim2−/− embryos, it was determined to be absent from the rostral midbrain and caudal diencephalon of embryos carrying a dominant-negative transgene that disrupts the function of bHLH-PAS proteins. Together, these results suggest that bHLH-PAS family members are required for the regulation of Shh transcription within aspects of the ventral midbrain and diencephalon.

Key words: Shh, Sim2, bHLH-PAS, Gene regulation, Central nervous system, Mouse

INTRODUCTION

Generating regional diversity of neuronal progenitors induced by Shh along the two major axes of the CNS depends on at least two mechanisms. Along the dorsoventral axis, a morphogenic gradient of Shh activity has been shown to function in the establishment of diverse neuronal cell fates (Roelink et al., 1995; Marti et al., 1995; Ericson et al., 1996, 1997). In contrast, along the anteroposterior axis cooperative interactions between Shh and other signaling pathways have been shown to generate distinct patterns of differentiated neurons (Dale et al., 1997; Ye et al., 1998). Both of these molecular strategies are reliant on sources of Shh derived from the axial mesoderm (notochord and prechordal plate), as well as the ventral midline of the CNS (floor plate). Presently, the genes coordinating the regulation of Shh expression within these signaling centers remain poorly defined.

Although the expression of Shh in tissues imparting pattern on the ventral neural tube seems continuous, it is nevertheless regulated differentially along the anteroposterior axis of the mouse embryo. Our previous studies have identified distinct regulatory sequences responsible for activating Shh transcription in defined regions along the anteroposterior axis of the mouse embryo (Epstein et al., 1999). For instance, the transcriptional requirements governing Shh expression in the axial mesoderm were found to differ in posterior (notochord) versus anterior (prechordal plate) regions of the embryo. Similarly, sequences regulating Shh transcription in the ventral midline of the CNS were found to differ for regions of the spinal cord, midbrain and forebrain (Epstein et al., 1999).

Additional evidence of the regionalized manner by which Shh transcription is regulated along the anteroposterior neuraxis comes from studies of mice carrying targeted mutations in components of the Shh signal transduction pathway. The activation of Shh expression within the floor plate has been shown to be dependent on Shh signaling derived...
from the underlying axial mesoderm (Chiang et al., 1996; Ericson et al., 1996). An alternative explanation has recently emerged from studies in the chick, which suggest that Shh expression in both notochord and floor plate is acquired through common lineage from cells of the node and chordoneural hinge (Teillet et al., 1998). Regardless of the mechanism, a role for the zinc-finger-containing transcriptional regulator Gli2 in mediating floor plate development has recently been described (Ding et al., 1998; Matise et al., 1998). Analysis of Shh expression in Gli2−/− embryos revealed that ventral midline development is dependent on Gli2 activity caudal to the midbrain-forebrain boundary and that other transcriptional activators regulate Shh expression rostral to this point. The Gli2-dependent activation of Shh expression in the floor plate is likely to be indirect and mediated in part through the action of the winged-helix transcriptional activator Hnf3b (Foxa2 – Mouse Genome Informatics). Functional Gli-binding sites have been identified in Hnf3b regulatory sequences conferring floor plate activity and floor plate expression of Shh has been shown to be directly dependent on Hnf3b (Sasaki et al., 1997; Epstein et al., 1999).

The regulation of Shh expression in the forebrain is complex and appears to be dependent on multiple factors. Whereas Gli2 has been identified as an important regulator of floor plate development in caudal areas of the CNS, Gli family members has been identified as an important regulator of floor plate and appears to be dependent on multiple factors. Whereas Gli2 point. The Gli2-dependent activation of Shh expression in the floor plate is likely to be indirect and mediated in part through the action of the winged-helix transcriptional activator Hnf3b (Foxa2 – Mouse Genome Informatics). Functional Gli-binding sites have been identified in Hnf3b regulatory sequences conferring floor plate activity and floor plate expression of Shh has been shown to be directly dependent on Hnf3b (Sasaki et al., 1997; Epstein et al., 1999).

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An additional example of the regionalized nature of Shh expression in the forebrain comes from the analysis of embryos deficient in the homeodomain containing transcription factor Nkx2.1 (Titfl – Mouse Genome Informatics). These mutant mice reveal a restricted loss of Shh expression from ventral regions of the telencephalon (Sussel et al., 1998). Interestingly, in Gli1−/−; Gli2−/− double mutant embryos the expression of both Shh and Nkx2.1 is preserved in this region (Park et al., 2000). Taken together, current evidence supports a multifaceted mechanism underlying the transcriptional regulation of Shh along the anteroposterior axis of the mouse CNS. What remains to be determined is the identity of the genes responsible for activating Shh expression in this manner.

In an effort to identify potential regulators of Shh transcription in forebrain regions of the mouse CNS we have assessed the temporal and spatial overlap of known transcription factors with that of a Shh reporter construct expressing lacZ in rostral regions of the CNS. Sim2, a bHLH-PAS domain-containing protein was found to precede Shh expression in a region of the presumptive ventral diencephalon, attesting to its candidacy as an activator of Shh transcription in this brain territory. Misexpression of Sim2 in transgenic mice resulted in the ectopic expression of Shh, demonstrating its role as a sufficient activator of Shh. Examination of Shh expression in Sim2−/− embryos as well as ones expressing a dominant-negative bHLH-PAS transgene suggests a required role for bHLH-PAS family members in the regulation of Shh transcription within the diencephalon.

**MATERIALS AND METHODS**

**Generation of misexpression constructs**

Full-length murine cDNAs encoding Sim1 and Sim2 (Fan et al., 1996) in addition to Drosophila sim (Nambu et al., 1991), were cloned into the EcoRV site of the Wnt1 expression vector (Wexp3) (Danielian et al., 1996). The dominant-negative Arnt cDNA (dnArnt) was created by loop-out site directed mutagenesis using the Gene Editor kit (Promega) with an oligonucleotide (5’TCTGCGGATAAAGAGAGAC-TTCGGAAACAGTACGAGCTTAC3’) flanking both sides of the Arnt basic domain. The first 21 nucleotides of the oligonucleotide correspond to positions 247-267 of the coding region and the second half corresponded to position 304-325 (Reisz-Porszasz et al., 1994; GenBank Accession U20325). The deleted amino acids ARENHESIERRR (amino acid positions 90-101) were previously defined as the basic domain of Arnt and are required for DNA-binding activity. The dnArnt cDNA was subsequently cloned into the Smal site of the Shh expression vector 1 (Sexp1). The Sexp1 vector was constructed by first cloning sequences mediating SBE1 and SPFE2 activity (1.3 kb EcoRI/BgIII) from a genomic subclone of Shh intron 2, upstream of the Shh promoter (1.1 kb EcoRI/SalI) (Epstein et al., 1999). The 2.4 kb promoter/enhancer fragment was then cloned into a promotorless expression vector containing a β-globin intron, polynucleotide sites, lacZ mRNA tag and SV40 poly-adenylation signal (Kimmel et al., 2000).

**Production and genotyping of transgenic mice**

Outbred Swiss-Webster mice (Taconic; Germantown, NY) were used to produce transgenic embryos and mouse lines essentially as described (Hogan et al., 1994). Where indicated, constructs were also injected into fertilized eggs generated by crossing males carrying the SBE1+SPFE2 reporter construct (RC8, Epstein et al., 1999) to wild-type females. Transgenese were prepared for microinjection as described (Epstein et al., 1996). The genotyping of transgenic embryos was carried out by PCR using proteinase K digested yolk sacs as DNA templates. Forward primers directed against the 3’ end of Sim1 (5’CAGACAGGAGAGAGCATCC3’), Sim2 (5’CGGTCTCAT-CATACCAACGGCG3’), Drosophila sim (5’GACGACCTACAG-CGATCGAG3’) or dnArnt (5’GGTCTAGTCCAGTGGAGCAG3’), and a reverse primer directed against lacZ (5’TACCACAGCGG-ATGTTCCGG3’), were used under the following PCR conditions: 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute (for 30 rounds) followed by a final extension at 72°C for 10 minutes. For staging embryos, noon of the day of vaginal plug detection corresponded to 0.5 days post coitus (d.p.c.).

**Mice**

Breeding and genotyping of Gli2−/−, Shh−/− and Sim1−/− mutant embryos were performed as described (Mo et al., 1997; Chiang et al., 1996; Michaud et al., 1998). In addition, the SBE1+SPFE2 reporter line was maintained on a Gli2−/− background and intercrossed to produce Gli2−/−; SBE1+SPFE2 embryos. Sim2 mutant mice were generated by homologous recombination in embryonic stem (ES) cells...
Gli2 for their induction. As previously reported, Shh expression in the ventral forebrain is maintained in Gli2−/− embryos, in contrast to the rest of the neural tube, which lacks Shh expression and consequently fails to undergo floor plate induction (Matise et al., 1998; Ding et al., 1998). With this in mind, transgenic mice containing a Shh reporter construct, SBE1+SFPE2 (Shh Brain Enhancer-1, Shh floor plate enhancer 2; Epstein et al., 1999), were crossed onto a Gli2−/− background (Fig. 1). At 10.5 d.p.c., wild-type embryos carrying the transgene showed X-gal staining in the ventral midline of the CNS from the hindlimb level of the spinal cord to an anterior limit within P4 of the diencephalon (Fig. 1A). Staining was also observed in the zona limitans intrathalamica (ZLI). In contrast, X-gal staining in Gli2−/− embryos (n=3) was absent from floor plate regions of the spinal cord, hindbrain and midbrain and was only detected in the ventral midline rostral to the midbrain-forebrain boundary, in addition to the spike of expression in the ZLI (Fig. 1B). This finding identifies a distinct compartment of Shh expression within the diencephalon between prosomeres 1 and 4 that is regulated in a manner independent of Gli2.

Overlapp of SBE1 reporter activity and Sim2 expression

The pattern of X-gal staining that persisted in Gli2−/− embryos appeared remarkably similar to that of Sim2, a BHLH-PAS domain-containing transcriptional regulator whose expression in the diencephalon had previously been described (Fan et al., 1996; Ema et al., 1996). To determine the extent of the temporal and spatial overlap between Shh and Sim2 in the ventral diencephalon, a comparison of their expression patterns was performed at multiple stages of development. In the mouse embryo, the CNS expression of Shh initiated in the ventral midbrain at the 6–8 somite stage, under the direct control of Hnf3β and then progressed into the forebrain primordium by the 10-somite stage (Echelard et al., 1993; Epstein et al., 1999). X-gal staining of embryos from the SBE1+SFPE2 reporter line faithfully recapitulated this early aspect of Shh expression (Fig. 2A,D and Epstein et al., 1999). The onset of Sim2 expression occurred as early as the two-somite stage, within a domain of the presumptive diencephalon immediately anterior to where Shh transcription is initiated a full 8 hours later (Fan et al., 1996). The initial expression of Sim2 was broad along the doroventral axis, but by the 8–10 somite stage Sim2 expression was restricted to the ventral region of the presumptive diencephalon (Fig. 2B,E). As Shh expression advanced rostrally, from the ventral midbrain into the diencephalon, it traversed through cells that were already expressing Sim2 (Fig. 2C,F).

By 9.5 d.p.c., the rostral and caudal boundaries of Sim2 expression became fixed and the first indication of expression within the ZLI begins to emerge from the ventral aspect of the caudal diencephalon (Fig. 3A). At this stage Shh expression had already progressed into the ventral telencephalon whereas, SBE1 reporter activity did not proceed further than the rostral boundary of the Sim2 expression domain (Fig. 3C). Interestingly, Shh expression, as assessed by either whole-mount RNA in situ hybridization or SBE1 reporter activity, was not yet detected in the ZLI at 9.5 d.p.c. (Fig. 3C and Epstein et al., 1999). By 10.5 d.p.c., however, Sim2 and SBE1 activity were both observed in the ZLI (Fig. 3B,D). Another Sim family
member, Sim1, is also expressed in ventral regions of the midbrain and diencephalon (Fig. 3E,F). However, in contrast with Sim2, Sim1 is expressed after the onset of Shh and is not found in the ventral midline but in cell types immediately adjacent (Fan et al., 1996). To summarize, the expression of Sim2 is consistently found to precede and overlap Shh in areas of the diencephalon and ZLI. Moreover, SBE1 reporter activity and Sim2 share the same rostral expression boundary.

**Sim family members are sufficient activators of Shh transcription**

Given the spatial and temporal overlap in their expression patterns, Sim2 proved to be an excellent candidate for activating Shh transcription in the diencephalon. To address the sufficiency of Sim2 in this role transgenic embryos were generated that misexpressed Sim2 in the dorsal CNS under the regulatory control of a Wnt1 enhancer (Wexp3-Sim2). Transgenic embryos collected at 10.5 d.p.c. that were confirmed to be expressing the Wexp3-Sim2 construct (see Materials and Methods) portrayed a consistent dysmorphic cranial CNS phenotype resulting in a dorsal expansion of the midbrain to diencephalic region that in the most severe cases resulted in an open neural tube (compare Fig. 4A with 4B,D). Of the 22 transgenic embryos analyzed, 12 displayed this phenotype, consistent with the predicted frequency (50%) of embryos that normally express Wexp3 constructs (Echelard et al., 1993; Epstein et al., 1996). The appearance of the cranial overgrowth was reminiscent of embryos misexpressing either Shh or a dominant-negative form of Protein Kinase A (PKA).

**Fig. 1.** Shh reporter expression in wild-type and Gli2−/− embryos. (A) Wild-type embryo at 10.5 d.p.c. displaying SBE1+SFPE2 reporter activity in the ventral midline of the CNS from the posterior aspect of the spinal cord to an anterior limit within the caudal diencephalon. A spike of expression is also observed within the ZLI (red arrow). (B) Gli2−/− embryo crossed to the SBE1+SFPE2 reporter line at 10.5 d.p.c. showing an absence of X-gal staining in the floor plate up to the border between the midbrain and diencephalon (black arrowhead). At positions rostral to this boundary midline expression can be detected in the caudal diencephalon and ZLI (red arrow).

**Fig. 2.** Comparative expression of a Shh reporter and Sim2 at 8.5 d.p.c. (A,D) Expression of the SBE1 lacZ reporter construct (salmon-gal) in the presumptive midbrain of an eight-somite stage embryo. (B,E) Sim2 expression in the presumptive caudal diencephalon of an eight-somite stage embryo by whole-mount RNA in situ hybridization. (C,F) Overlapping expression of Shh (magenta) and Sim2 (purple) in a ten-somite stage embryo. As SBE1 lacZ expression progresses rostrally, it advances into Sim2-expressing cells. Panels (D-F) represent dorsal views of cranial regions prior to neural tube closure.

**Fig. 3.** Comparative expression of a Shh reporter and Sim family members at 9.5 and 10.5 d.p.c. (A,B) Sim2 expression in the caudal diencephalon and ZLI by whole-mount RNA in situ hybridization. Sim2 initiates in the ZLI at 9.5 d.p.c. (red arrows). (C,D) Expression of the SBE1-lacZ reporter construct (X-gal) in the midbrain and caudal diencephalon. X-gal staining in the ZLI does not initiate until 10.5 d.p.c. (red arrow). Note that the rostral expression boundary is shared between Sim2 and SBE1-lacZ. (E,F) Sim1 expression initiates in the ventral midbrain subsequent to that of Shh (white arrows). Asterisks mark craniofacial expression of Sim1.
Sim2 is a regulator of Shh transcription, suggesting that the Shh pathway was being ectopically activated in Wexp3-Sim2 embryos (Echelard et al., 1993; Epstein et al., 1996). To determine whether this was indeed the case, embryos carrying the Wexp3-Sim2 transgene were stained for Shh using whole-mount RNA in situ hybridization, while the embryos in (C,D) were stained with X-gal to assess SBE1 reporter activity. Every embryo expressing a Wexp3-Sim transgene was also found to ectopically express Shh in cranial regions of the CNS. The black arrows in B point to regions of cranial overgrowth as compared with the wild-type embryo in A. The red and white arrowheads point to ectopic and endogenous expression of Shh, respectively. Misexpression of Sim genes can result in embryos with open neural tubes as evidenced by frontal views of embryos in panels (D,G) as well as the lateral view in (F).

In order to rule out the possibility that ectopic Shh expression was an indirect consequence of the cranial dysmorphology in Wexp3-Sim2 transgenic embryos, Shh expression was assessed at 9.5 d.p.c., prior to the appearance of the overgrowth phenotype. In the only two 9.5 d.p.c. embryos carrying both the Wexp3-Sim2 and SBE1+SFPE2 transgenes, ectopic X-gal staining was observed in the dorsal CNS in the absence of any alterations to cranial CNS morphology (Fig. 4C). This finding suggests that the ectopic activation of Shh by Sim2 occurs prior to the morphological phenotype and is a likely cause of the cranial overgrowth observed at later stages.

Although our study has shown Sim2 to be sufficient to activate Shh transcription, the molecular mechanism by which this activation takes place was not clear. A previous report identified two independently functioning repression domains within the C terminus of Sim2 and determined its role to be that of a repressor in tissue culture cells (Moffett et al., 1997). However, Sim1 and Drosophila sim, which lack the repression domains found in Sim2, can both function as transcriptional activators (Moffett and Pelletier, 2000; Wharton et al., 1994). Given that we could not exclude the possibility that Sim2 activated Shh transcription through the repression of a negative regulator, we reasoned that if other Sim family members of the Sim family are sufficient activators of Shh transcription. Expression of Shh in wild-type (A) and transgenic embryos ectopically expressing Sim2 (B-D), Sim1 (E) and Drosophila sim (F,G) in the dorsal neural tube under the influence of Wnt1 regulatory sequences (Wexp3). The embryos in (A,B,E-G) were stained for Shh using whole-mount RNA in situ hybridization, while the embryos in (C,D) were stained with X-gal to assess SBE1 reporter activity. Every embryo expressing a Wexp3-Sim transgene was also found to ectopically express Shh in cranial regions of the CNS. The black arrows in B point to regions of cranial overgrowth as compared with the wild-type embryo in A. The red and white arrowheads point to ectopic and endogenous expression of Shh, respectively. Misexpression of Sim genes can result in embryos with open neural tubes as evidenced by frontal views of embryos in panels (D,G) as well as the lateral view in (F).

Fig. 4. Members of the Sim family are sufficient activators of Shh transcription. Expression of Shh in wild-type (A) and transgenic embryos ectopically expressing Sim2 (B-D), Sim1 (E) and Drosophila sim (F,G) in the dorsal neural tube under the influence of Wnt1 regulatory sequences (Wexp3). The embryos in (A,B,E-G) were stained for Shh using whole-mount RNA in situ hybridization, while the embryos in (C,D) were stained with X-gal to assess SBE1 reporter activity. Every embryo expressing a Wexp3-Sim transgene was also found to ectopically express Shh in cranial regions of the CNS. The black arrows in B point to regions of cranial overgrowth as compared with the wild-type embryo in A. The red and white arrowheads point to ectopic and endogenous expression of Shh, respectively. Misexpression of Sim genes can result in embryos with open neural tubes as evidenced by frontal views of embryos in panels (D,G) as well as the lateral view in (F).

Fig. 5. Expression of Shh in Sim2−/− embryos. Expression of Shh in wild-type (A) and Sim2−/− (B) embryos at 10.0 d.p.c. No alteration in the pattern of Shh expression was detected.

Fig. 6. Downregulation of Shh in embryos carrying the Sexp3-dnArnt transgene. Wild-type (A,D) and transgenic (B,C,E,F) embryos were bisected along the midline at 12.5 d.p.c. and stained for either Shh (A,B,D,E) or lacZ (C,F) (to assess transgene expression) by whole-mount RNA in situ hybridization. Panels (D-F) are enlargements of the affected regions in (A-C). Red arrowheads mark the limits of Shh downregulation.
members with known abilities to operate as transcriptional activators could initiate Shh transcription, then it was likely that all Sim family members could operate through a common activation mechanism. All transgenic embryos that expressed either the Wexp3-Sim1 or Wexp3-Drosopila sim constructs had cranial CNS dysmorphology and ectopic Shh expression, similar to those embryos expressing the Wexp3-Sim2 transgene (Fig. 4E-G). Ectopic expression of vertebrate Sim genes was not detected in Wexp3-Drosopila sim embryos, ruling out the possibility that Drosopila sim was acting in an autoregulatory manner with its vertebrate homologues (data not shown). Furthermore, downregulation of Gli3, a known repressor of Shh transcription in the dorsal CNS, was not observed in Wexp3-Sim2 embryos displaying weak expression of Shh in the dorsal CNS (data not shown). Taken together, these findings indicate that each of three Sim family members is sufficient to activate Shh transcription and likely do so through a common mechanism.

All known Sim family members have been shown to bind as heterodimers to asymmetrical E-box sites containing an ACGTG core (reviewed in Crews, 1998). To date, the only known dimerization partner for any of the Sim proteins are members of the aryl hydrocarbon receptor nuclear translocator (Arnt) family. Even though we have shown Sim2 to be capable of activating SBE1-directed lacZ reporter expression there are no sequences matching the reported Sim/Arnt consensus within the 532 bp fragment conferring SBE1 reporter activity. As the possibility remained that Sim/Arnt dimers could bind DNA outside of the known consensus, we assessed their ability to bind sequences contained in the SBE1 fragment. Sim2 and Arnt heterodimers that were able to bind DNA sequences containing a known consensus binding site were unable to bind sequences within the 532 bp SBE1 fragment (data not shown). Based on these results, we surmise that Sim activation of Shh transcription is mediated indirectly or, alternatively, that an additional cofactor, present in the embryo, is missing from the Sim2/Arnt binding complex.

**Requirement for Sim family members in the regulation of Shh transcription**

Of the known vertebrate bHLH family members our expression studies pointed to Sim2 as the likely candidate to be regulating Shh transcription in the ventral diencephalon. Sim1, which may also share some overlap in expression with Shh in lateral but not ventral regions of the midbrain and diencephalon, was deemed less likely to be playing a role, given that the onset of Sim1 expression lags behind that of Shh (Fig. 3E,F and Fan et al., 1996). Moreover, Sim1−/− embryos show no obvious alterations to the pattern of Shh (Michaud et al., 1998 and data not shown). To address whether Sim2 was required for the regulation of Shh transcription in the ventral diencephalon we assessed the expression of Shh in Sim2−/− embryos. Surprisingly, at no stage of development examined between 8.5 d.p.c. and 13.5 d.p.c. did Shh expression appear to be altered in Sim2−/− embryos compared with wild-type littermates (Fig. 5A,B and data not shown). In fact, Sim2−/− embryos exhibit no discernible defects in embryonic patterning despite the observation that the mice die shortly after birth from unknown causes (J. L. M. and C.-M. F., unpublished). As the possibility existed that Sim1 was compensating for the loss of Sim2 function, we examined Shh expression in Sim1−/−;Sim2−/− embryos between 9.5 d.p.c. and 13.5 d.p.c. Once again however, Shh expression was found to be unperturbed (data not shown). We also carefully examined Sim2−/− embryos at the 10-12 somite stage and found that not only was Shh expression initiated correctly but that there was no premature activation of Sim1 to compensate for the absence of Sim2 (data not shown).

To determine whether any potentially unknown members of the Sim gene family are required for the activation of Shh transcription in cranial regions of the mouse CNS, we expressed a dominant-negative form of Arnt (dnArnt) under SBE1 and SFPE2 regulation in transgenic mouse embryos (Sexp1-dnArnt). The dnArnt cDNA was created by generating an in frame deletion of the basic domain, resulting in a protein that can dimerize with its partners but is unable to bind DNA. Naturally occurring bHLH inhibitors of a similar design have been described that can perturb MyoD function during muscle differentiation (Jen et al., 1992). Remarkably, five of six embryos expressing the Sexp1-dnArnt transgene at 12.5 d.p.c. showed a consistent downregulation of Shh expression in regions of the rostral midbrain and caudal diencephalon (compare Fig. 6A,C with 6B). These results are consistent with where Sim2 and Shh expression overlap in the mouse CNS. The one embryo that did not result in a loss of Shh transcription was only weakly expressing the transgene (data not shown). Interestingly, Shh expression was not affected in the ZLI of transgenic embryos, suggesting that other factors may be required for the regulation of Shh in this domain (compare Fig. 6D,F with 6E). The loss of Shh expression appears to be specific for rostral midbrain and caudal diencephalic regions of the neural tube, given that hindbrain and spinal cord regions, which also strongly expressed the transgene, were unaffected (Fig. 6B, C). Taken together, our data support the hypothesis that bHLH-PAS family members are required regulators of Shh transcription in the diencephalon. Although we cannot prove definitively that Sim2 is involved in this process, our data are
consistent with the view that in Sim2\(^{-/-}\) embryos an unidentified member of the bHLH-PAS family is able to compensate for Sim2 in regulating the diencephalic expression of Shh.

**Is Sim2 a target of Shh signaling?**

Given that Shh secreted from the axial mesoderm has been proposed to be the signal mediating the induction of Shh in the overlying neural plate, we were interested in determining whether in the caudal diencephalon Sim2 played a role in this homeogenetic signaling process. In regions of the ventral neural tube outside of the forebrain such a role has been attributed to Hnf3b, where it is both a downstream target of Shh signaling from the notochord and an upstream regulator of Shh expression in the floor plate (Ericson et al., 1996; Epstein et al., 1999). To determine whether notochord derived Shh is required for the initiation of Sim2 we assayed for Sim2 expression in Shh\(^{-/-}\) embryos at somite stages prior to the normal onset of Shh in the CNS. Interestingly, the expression of Sim2 in the caudal diencephalon was still detected in Shh\(^{-/-}\) embryos at the seven-somite stage, albeit in a restricted fashion (Fig. 7). Instead of its normally broad domain of expression along the dorsoventral axis at this stage, Sim2 was limited to the ventral most region of the caudal diencephalon in Shh\(^{-/-}\) embryos (compare Fig. 7C with 7D). By 9.5 d.p.c., however, Sim2 was completely absent from the diencephalon of Shh\(^{-/-}\) embryos (data not shown). These observations suggest that the onset of Sim2 expression is not completely dependent on Shh signaling, whereas the maintenance of Sim2 is dependent on Shh.

**DISCUSSION**

**Functional redundancy for Sim2 in the regulation of Shh transcription**

Establishing ventral properties of the vertebrate CNS is dependent on the Shh signaling pathway. This process is regulated in a regionalized manner along the anteroposterior axis of the mouse CNS (Epstein et al., 1999). Within the spinal cord, for instance, Gli2 is required for floor plate development, whereas, in areas rostral to where a morphological floor plate is recognized, other transcriptional regulators appear to be required for this process (Matise et al., 1998; Sussel et al., 1998; Park et al., 2000). We show here, that within ventral regions of the diencephalon, members of the bHLH-PAS family can serve as potent activators of Shh transcription, a read-out of ventral midline development.

Although many bHLH-PAS family members have been identified in vertebrates, Sim2 is the best candidate to be regulating Shh given the striking temporal and spatial similarities in their patterns of expression within the caudal diencephalon. Nevertheless, that Shh expression is maintained in Sim2\(^{-/-}\) embryos, but lost from the caudal diencephalon of those carrying a dominant-negative transgene that disrupts the function of bHLH-PAS proteins, suggests that additional bHLH-PAS family members can compensate for the loss of Sim2. Furthermore, given that Sim2 is expressed in multiple tissues during development and that Sim2\(^{-/-}\) embryos show no overt signs of patterning defects, it is likely that the function of Sim2 is redundant in the rest of the embryo as well.

At present, the identity of the bHLH-PAS protein that is able to initiate Shh transcription in the diencephalon in the absence of Sim2 activity remains unknown. The CNS expression of at least five other bHLH-PAS family members has been described (Fan et al., 1996; Zhou et al., 1997; Jain et al., 1998; Brunskill et al., 1999). Sim1, which partially overlaps Sim2 in the diencephalon, can be excluded from playing a role, based on the observation that Shh expression appears normal in Sim1\(^{-/-}\):Sim2\(^{-/-}\) double mutant embryos. HIF1\(_{\alpha}\) (also known as EPAS1), which is expressed ubiquitously at early stages of mouse embryogenesis, can also be ruled out as a candidate regulator of Shh, given that its core binding sequence differs from that of Sim2 and that its activation is dependent on hypoxic conditions (reviewed in Crews and Fan, 1999). Of the three members of the Npas subgroup, only Npas3 shows CNS expression, albeit ubiquitous, at an early enough stage of development that is coincident with the onset of Shh transcription (Brunskill et al., 1999). The decisive answer as to whether Npas3, or some unknown bHLH-PAS family member, can compensate for Sim2 in the activation of Shh transcription will have to await the assessment of Shh expression in Npas3 (or another bHLH-PAS gene) and Sim2 double mutant embryos.

**Convergence of independent pathways in the regulation of Sim2 and Shh transcription**

Our finding that the expression of Sim2 is downregulated in Shh\(^{-/-}\) embryos suggests that Sim2 is a target of Shh signaling. Like the role of Hnf3b in the spinal cord, where it is both a downstream effector of notochord derived Shh and an activator of Shh expression in the floor plate, Sim2 appears to participate in the homeogenetic induction of Shh in the caudal diencephalon. However, two additional observations suggest that Shh signaling is not all that is required for the initiation of Sim2 transcription. First, Sim2 expression is partially maintained in Shh\(^{-/-}\) embryos suggesting the existence of an alternate pathway that functions to initiate transcription of this marker in the diencephalon. Interestingly, a parallel pathway to that of Shh which relies on retinoid signaling has been implicated in the determination of V0 and V1 interneuron fates in the spinal cord (Pierani et al., 1999). Secondly, on its own, Shh does not appear sufficient for the activation of Sim2 expression, since Sim2 is not activated by the ectopic Shh in Wesp3 Drosophila sim embryos. A likely scenario then, would implicate both Shh-dependent and -independent pathways in the initiation of Sim2 expression in the diencephalon.

Cooperative interactions may also explain the nature by which Shh signaling from the notochord acts in conjunction with Sim2 in the regulation of Shh transcription in the diencephalon. We have previously shown that Hnf3b, a target of notochord derived Shh signaling, is required for the direct regulation of ventral midline but not lateral aspects of SBE1 reporter function in the midbrain and diencephalon (Epstein et al., 1999). Further characterization of the crucial sequences mediating SBE1 activity will be required to better understand the contributions made by bHLH-PAS proteins in the regulation of Shh transcription in the diencephalon. To this end we have identified an octamer binding site in sequences mediating SBE1 function and found it to be highly conserved across mouse, human and chicken phyla. In light of previous studies showing that Pou domain proteins such as Brm2 (Pou3f2 – Mouse Genome Informatics) and drifter mediate aspects of Sim1 and Drosophila sim function respectively, the
presence of this octamer-binding site in SBE1 sequences may prove significant (Michaud et al., 1998; Anderson et al., 1995).

Regulators of the ventral CNS: conservation by chance or function?
The ventral midline of the Drosophila nerve cord has been compared with the ventral midline of the vertebrate CNS (for a review, see Arendt and Nubler-Jung, 1999). From a functional perspective, midline cells from both species serve as signaling centers in the generation of neuronal pattern and are providers of axon guidance cues. Increasing data support the view that many of the molecular mechanisms underlying axon path finding and dorso-ventral patterning of the CNS are conserved between the species. Netrin and slit are two factors that promote the attraction and repulsion of neurons to and from the ventral midline respectively, and do so in a similar manner in both vertebrates and invertebrates (Zinn and Sun 1999; Arendt and Nubler-Jung 1999). Moreover, the expression of several transcription factors belonging to the Nkx, Gsh and Msx families appear conserved along the dorsal/ventral neuraxis in both mouse and Drosophila implying a conservation of function as well (Weiss et al., 1998).

Significant differences have also been identified between the two species in the use of pathways that mediate ventral midline induction and downstream signaling properties. For instance in Drosophila, sim is expressed in cells fated to make up the ventral midline and is required for their formation (Nambu et al., 1990). Sim functions by regulating a number of midline-specific genes including spitz, a secreted TGFα-like molecule that operates in a graded distribution in the ectoderm to establish distinct cell fates (Nambu et al., 1990, 1991; Kim and Crews 1993; Schweitzer et al., 1995; Skeath 1998). This contrasts with floor plate induction within the spinal cord of higher vertebrates, which appears to be independent of Sim function and reliant on graded Shh signaling for the specification of distinct neuronal fates (Roelink et al., 1995; Marti et al., 1995; Ericson et al., 1996, 1997). hedgehog is expressed in the Drosophila neuroectoderm, however it is localized to transverse stripes and does not play a role in signaling from the ventral midline (Taylor et al., 1993). Although, the genes involved in ventral midline induction differ between the two organisms, the employment of a patterning strategy that relies on the graded response to a factor secreted from the ventral midline is a feature common to both.

Given the many similarities between ventral midline cells of the CNS in Drosophila and mouse, it is rather intriguing that a role for Sim2 in ventral midline determination has re-emerged in vertebrates through its ability to regulate Shh expression in the ventral diencephalon. Whether this points to a conserved role for Sim2 or an example of convergent evolution remains to be determined.

Is Shh upregulated in individuals with Down syndrome?
The mapping of Sim2 to the Down syndrome crucial region on human chromosome 21 suggested that an increased dosage of Sim2 might contribute to manifestations of a subset of phenotypes observed in individuals with Down syndrome (Dahmane et al., 1995; Fan et al., 1996; Ema et al., 1997). In addition to the CNS, Sim2 is expressed in several tissues affected in individuals with Down’s syndrome, including craniofacial structures, the axial skeleton and a subset of muscle cells. Trisomy 21 causes an upregulation of Sim2 transcription during embryogenesis, then an increase in Shh expression would be expected to result as well. Subtle alterations in Shh signaling are known to impact significantly on the establishment of neuronal pattern throughout the CNS (Goodrich et al., 1999). Further studies will be required to determine whether an increase in Shh signaling within the ventral diencephalon could contribute to some of the symptoms observed in individuals with Down syndrome.

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Sim2 is a regulator of Shh transcription

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