Medaka spalt acts as a target gene of hedgehog signaling

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SUMMARY

In vertebrates, pattern formation in the eye, central nervous system, somites, and limb depends on hedgehog activity, but a general target gene controlled by hedgehog in all these signaling centers has remained largely elusive. The medaka fish gene spalt encodes a zinc-finger transcription factor, which is expressed in all known hedgehog signaling centers of the embryo and in the organizer region at the midbrain-hindbrain boundary. We show that the spalt expression domains expand in response to ectopic hedgehog activity and narrow in the presence of protein kinase A activity, an antagonist of hedgehog signaling, indicating that spalt is a hedgehog target gene. Our results also suggest a signaling mechanism for anterior-posterior patterning of the vertebrate brain that controls spalt expression at the midbrain-hindbrain boundary in a protein kinase A dependent manner likely to involve an unknown member of the hedgehog family.

Key words: spalt, hedgehog target gene, medaka, patterning, midhindbrain boundary

INTRODUCTION

Pattern formation in multicellular organisms depends on positional information generated by transient gradients of secreted signaling molecules (reviewed by Lawrence and Struhl, 1996). This graded activity is then permanently established by the differential expression of target genes. In Drosophila, one of the diffusible patterning activities is encoded by the segment polarity gene hedgehog (hh) which mediates pattern formation during segmentation (Lee et al., 1992) and during imaginal disc determination (Tabata and Kornberg, 1994).

Recently several homologues of the Drosophila hedgehog gene have been identified in various vertebrate species, revealing the existence of a hedgehog gene family of secreted signaling molecules (Zardoya et al., 1996). Best characterized is sonic hedgehog (shh), whose spatiotemporal expression is conserved throughout vertebrates. Initially, shh is expressed in the embryonic shield or node during gastrulation, then becomes localized to the ventral midline during axis formation and subsequently to the notochord, floorplate and anteriorly to the prechordal mesoderm. Later shh expression is found in the outgrowing posterior distal limb and the excretory system (reviewed by Hammerschmidt et al., 1997).

Diffusible HEDGEHOG protein emanates from localized sources, referred to as hh signaling centers, and mediates pattern formation over short and long distances. The eyes, the neural tube, the somites, the excretory system and limbs are structures that are organized through the patterning activity of SHH. Patterning in the vertebrate eyes is mediated by SHH emanating from the underlying prechordal mesoderm in the embryonic midline. The finding that shh deficient mice suffer phenotypically from cyclopia (Chiang et al., 1996) indicates that midline signaling by SHH is necessary for a proper separation of the anterior eye domain. Consistently, cyclopia was also observed when midline signaling was antagonized by overexpression of protein kinase A (PKA), which efficiently inhibits signaling of several members of the hedgehog family (Hammerschmidt et al., 1996). Conversely, ectopic overexpression of shh leads to the expansion of proximal eye structures at the expense of distal structures as shown by the expansion of proximal Pax2 expression and suppression of the distal eye marker Pax6 (Ekker et al., 1995b; Macdonald et al., 1995). In the notochord, shh expression leads to the contact dependent induction of floorplate cells in the neural tube which themselves start to secrete SHH and subsequently induce the differentiation of motor neurons (Ericson et al., 1996 and references therein). In addition, lateral diffusion of SHH from midline structures was shown to control the patterning of the paraxial mesoderm (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995). In the excretory system shh is expressed at different sites (Bitgood and McMahon, 1995) and mediates patterning processes and induction events which are currently analyzed in shh deficient mice (Chiang et al., 1996). Finally, during limb development SHH is generated in the posterior part of the outgrowing limbs, the zone-of-polarizing activity (ZPA), which mediates anteroposterior (AP) polarity of the limb. An ectopic source of diffusible SHH in the anterior part of the limb bud mimics a ZPA and leads to mirror-image duplication of digits (Riddle et al., 1993; Chang, 1994; López-Martínez et al., 1995).

Although the important role of shh in patterning processes of vertebrate development is well established, little is known about the transmission of the HEDGEHOG signal. Recently, the transmembrane protein PATCHED (PTC) was discovered to function as SHH receptor releasing the bound
SMOOTHENED (SMO) protein from its inactive state by binding to SHH (Stone et al., 1996; Marigo et al., 1996a). General target genes of HH that mediate hh signaling at the level of transcription, and are activated in all hh signaling centers, remain largely elusive.

Here we describe the cloning of such a target gene, the medaka (Oryzias latipes) homologue of the Drosophila zinc finger transcription factor spalt (sal). In the Drosophila wing imaginal disc sal was shown to be activated by hh expression (Sturtevant et al., 1997) and to mediate patterning (de Celis et al., 1996; Sturtevant et al., 1997). In agreement with this, we find medaka spalt (sal) expressed in all hh signaling centers and in the organizer at the mid-hindbrain boundary (MHB). Medaka sal is activated by ectopic SHH activity. Suppression of hh signaling by overexpression of protein kinase A (PKA) results in suppression of sal expression. Our results indicate that sal is a hh target gene which is positively regulated by HH activity in all known signaling centers. In addition, the sal expression domain at the MHB expands in response to ectopic HH and it narrows in response to PKA activity. Our results are consistent with the proposal that AP patterning of the dorsal vertebrate brain is mediated by hh signaling, although a corresponding member of the HH family that is expressed in the relevant region of the brain has not yet been identified.

MATERIALS AND METHODS

Medaka stocks

Fish were maintained in a constant recirculating system at 28°C on a 14 hours light/10 hours dark cycle. Wild-type Oryzias latipes from a closed stock were originally obtained from Carolina Biological, North Carolina, USA. Pairwise mating was performed and collected embryos were kept at 28°C.

Isolation and sequencing of medaka sal, Pax2 and shh

Medaka sal was isolated by PCR using degenerate oligonucleotides [5': TGYGCGATRTTYTAYAARTGGAC; 3': CTDAT-CATYTTCATGRTTYTC; 40 cycles: 94°C, 1 minute (first: 3 minutes); 49°C, 1.5 minutes; 72°C, 1.5 minutes (final 10 minutes)] specific for conserved regions of the known spalt genes (Hollemann et al., 1996; Ott et al., 1996). A 2.5 kb fragment of coding region was amplified from medaka genomic DNA and subsequently used to screen a neuraula stage (stage 18) CDNA library (stages refer to Iwamatsu, 1994). The longest clone contained a 4.5 kb cDNA insert which was sequenced with internal primers (Applied Biosystems 373A DNA Sequencer). The coding region of this cDNA clone could be extended in the 5' direction by sequencing genomic DNA (accession #U77376).

A 900 bp medaka Pax2 fragment [5': GCIGAYCITTTYWSIGCFI-ATCA; 3': GRTTISWRAAIKCCAICGCYTCRTTRA; 35 cycles: 94°C, 1 minute (first: 5 minutes); 54°C, 30°C; 2 minutes; 72°C, 4 minutes (final 9 minutes)] and a 297 bp medaka shh fragment [5': CCIAAYTAYAAYACYGAYATHTTYAAY; 3': YTCRTARTA-1ACCCARTRAAICC; 35 cycles: 94°C, 1 minute (first: 5 minutes); 54°C, 30°C; 2 minutes; 72°C, 4 minutes (final: 9 minutes)] was isolated by RT-PCR with degenerate primers using RNA from the anterior part of the axis of early neuraula stage (stage 17) embryos and TA cloned into the pCRRII vector (Invitrogen). Identity of the cloned fragments was confirmed by sequencing and comparison to the known vertebrate homologues (EMBL accession numbers are Pax2, Z97020 and shh, Z97019).

In situ hybridization and immunohistochemistry

In situ hybridization was performed as described by Oliver et al. (1996). When double staining was carried out medaka sal in situ hybridization was performed first. Acetylated tubulin was detected using a monoclonal antibody (Sigma #T6793 1:1000 dilution). Secondary anti-mouse antibody coupled to alkaline phosphatase (Jackson ImmunoResearch, 1:200 dilution) was preabsorbed to medaka embryos. Phosphatase activity was detected using Fast Red (Boehringer Mannheim) as substrate. Stained embryos were briefly rinsed in methanol and mounted in 87% glycerol. Intensely stained embryos were embedded in Technovit 7100 (Kulzer), sectioned (6 μm) and mounted in Entellan (Merck).

RNA injections

Capped RNA for injections was transcribed from linearized pSP64T (Krieg and Melton, 1984) plasmids using the SP6 mMESSAGE mMACHINE Kit (Ambion #1340). Individual blastomeres of 2- to 4-cell stage embryos were injected as described by Wittbrodt and Rosa (1994). Initially different dilutions of mRNA were injected at concentrations of 50, 125 and 250 ng/μl (shh) and 2.5, 5, 10 and 20 ng/ml (PKA*) respectively. Concentration dependent effects observed in injected embryos were comparable to those described previously in that they became increasingly severe with rising mRNA concentrations in the injection solution (Ekker et al., 1995b; Hammerschmidt et al., 1996). Due to moderate morphological effects in injected embryos, concentrations of 125ng/ml (shh) and 2.5 ng/ml (PKA*) respectively were chosen for the injection experiments shown in Figs 5 and 6. Control injections using Xenopus EF1α (Ambion) mRNA were performed at identical concentrations. All injections were monitored with RNA encoding β-galactosidase (5 ng/μl), and subsequently a number of embryos were stained for lacZ activity to visualize distribution and functional integrity of injected RNA (Joore et al., 1996).

RESULTS

Isolation and analysis of the medaka sal gene

Based on the comparison of known vertebrate (Hollemann et al., 1996; Ott et al., 1996) and Drosophila (Kühnlein et al., 1994) spalt sequences we designed degenerate oligonucleotides specific for regions conserved in these spalt homologues. PCR amplification from medaka genomic DNA resulted in a 2.5 kb fragment. This fragment was subsequently used to screen a late neuraula stage (stage 18) medaka cDNA library. Sequence analysis of the longest cDNA clone of 4.5 kb revealed that it contained the complete 3' untranslated region including a polyadenylation consensus but lacked an inframe AUG at the 5' end. The sequence could be extended in the 5' direction using sequence information from genomic DNA. So far we have not been able to further extend the known sequence by RACE-PCR. Sequence comparison with the known vertebrate spalt genes indicates that this clone lacks approximately 70 residues at the amino terminus.

The deduced amino acid sequence of medaka sal shows high overall similarity to the known vertebrate sal genes (68% in comparison to mouse and 66% in comparison to Xenopus, respectively). Medaka sal encodes a protein that contains the three diagnostic double zinc finger domains and a single zinc finger of the C2H2-motif, located adjacent to the second pair of zinc fingers, which are characteristic for all SAL homologues. The entire domain containing the zinc fingers of SAL is highly conserved between vertebrates and far we have not been able to further extend the known sequence by RACE-PCR. Sequence comparison with the known vertebrate spalt genes indicates that this clone lacks approximately 70 residues at the amino terminus.

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specific DNA binding (Barrio et al., 1996), suggest that all sal homologues recognize a highly conserved binding site. At the amino terminus SAL contains an additional single zinc finger of the C2HC-type characteristic for vertebrate SAL proteins (Hollemann et al., 1996). In summary these features identify medaka sal as a homologue of the Drosophila and the known vertebrate spalt genes (Kühnlein et al., 1994; Hollemann et al., 1996; Ott et al., 1996; Kohlhase et al., 1996).

**Spatiotemporal expression pattern of sal during embryogenesis**

In *Drosophila, spalt* functions downstream of the hh signaling pathway (Sturtevant et al., 1997). To investigate whether medaka sal plays a similar role in vertebrates we first examined sal expression during early medaka embryogenesis at the time when *shh* is known to exert its patterning activity. Localization of medaka sal was examined by whole-mount in situ hybridization. Transcripts of sal are detected during development of the medaka embryo in the eye, the central nervous system, the excretory system, the somites and the developing fins.

Initially, sal expression is apparent at neurula stages (stage 18; for stages refer to Iwamatsu, 1994). Expression is detected in the anteroposterior part of the optic vesicle, in the optic stalk and the rostral optic vesicle prior to the formation of the multi-layered retina (Fig. 2A,D). sal expression levels fade when lens embedding and invagination of the optic vesicle occur (stage 21, not shown).

From neurula stages onwards, sal is expressed in the neural tube along the entire anteroposterior (AP) axis of the developing central nervous system (CNS) (stage 18) (Fig. 2D,E). Later, during organogenesis (stage 26), expression in the CNS becomes restricted to single cell clusters in the trunk region of the neural tube, whereas anteriorly in the rhombencephalon sal transcripts are found bilaterally in each rhombomere (Fig. 2C,F). In rhombomere four sal is expressed in commisural cells which cross the midline.

In the dorsal part of the midbrain, sal is expressed in a sharp stripe (sal stripe) of two to three cell diameters width in the mid-hindbrain boundary (MHB). This expression domain is first seen at early neurula stages (stage 17) and persists in the isthmus until the constriction between mid- and hindbrain has formed and separates mes- and metencephalon (stage 26; Fig. 2).

Strong sal expression is detected in pro- and opisthophores from neurula stages onwards (Fig. 2). In the pronephros, the progenitor of the head nephros, high expression levels of sal are detected throughout embryogenesis. Concomitant with the development of the head nephros to a lymphatic organ, sal expression disappears (not shown). In the opisthophores, expression is restricted to the posteriormost somites and declines from posterior to anterior in a graded manner suggesting a transient requirement of sal expression in the formation of the opisthophores.

**Expression of sal in the trunk is restricted to somitic cells and neurons**

To analyze the expression of sal in the trunk in more detail transverse sections of whole-mount stained embryos were performed. Lower levels of sal expression were detected
medially in the forming somites, the region of the prospective sclerotome (Fig. 3A) which has been shown to be induced by long range signaling of a secreted HH family member (Fan and Tessier-Lavigne, 1994).

In neural structures sal expression is detected at neurula stages in the ventral third of the forming neural tube (stage 19), but is excluded from the ventralmost floorplate cells (Fig. 3). As neuronal differentiation proceeds during somitogenesis (stage 24), small bilateral clusters of sal-expressing cells are found in the ventral part of the neural tube (Fig. 3B). Here sal expression reflects the time course of neuronal differentiation, suggesting that sal transcripts are confined to differentiating neurons. To verify this, double labeling experiments were performed which combined in situ hybridization for sal and immunostaining of the neuronal axons (Chitnis and Kuwada, 1990) with an antibody directed against acetylated tubulin (Piperno and Fuller, 1985). sal-positive cells located ventrally in the neural tube project their tubulin-positive axons to the underlying somites, identifying these cells as motor neurons (Fig. 3C,D). Later, during organogenesis stages (stage 27), sal-expressing cell clusters are detected in intermediate and then in dorsal regions of the neural tube (Fig. 3E, 4E) representing interneurons and sensory neurons respectively. Taken together this shows that sal is expressed in neuronal cells, where its expression identifies differentiating neurons rather than a specific neuronal cell type.

**sal expression is confined to hedgehog signaling centers**

In vertebrates, hedgehog signaling is required for the proper patterning of the eye, neural tube, kidneys, somites and limb buds. Secreted SHH protein, diffusing from localized sources, mediates pattern formation within the hh signaling center over short and long distances. In medaka, as reported for other vertebrates (Krauss et al., 1993; Riddle et al., 1993; Echelard et al., 1993; Roelink et al., 1994), shh is expressed in the notochord and subsequently in the ventral midline of the neural tube (Fig. 4A). Comparison of medaka sal and shh expression shows that in the hh signaling centers, the eye, the ventral part of the neural tube, the kidneys and somites, sal transcripts are found adjacent to shh expression (Fig. 4). In outbudding fins the expression domains of sal and shh are partially overlapping (not shown). At late neurula stages sal transcripts are found in the proximal part of the outbudding optic vesicle (Fig. 4C), which is known to be under the control of hh signaling in vertebrates (Ekker et al., 1995b; Macdonald et al., 1995; Chiang et al., 1996). Transverse sections through the rostral head region reveal sal expression in the optic chiasm (Fig. 4D) and a faint expression in the more distal part of the optic vesicle (Fig. 4D). In the early neural tube sal expression is limited to the ventral region (Figs 3A, 4D) but excluded from the floorplate cells expressing shh (Figs 3A, 4A). This expression of sal is subsequently refined to differentiating neurons and reflects the patterning activity of SHH which mediates at least two phases of patterning. First, medial neural plate cells are converted to ventralized progenitors (expressing sal; Fig. 3A), which are then, in a second step induced directly by SHH to differentiate into (sal positive; Fig. 3B-D) motor neurons (Marti et al., 1995; Tanabe et al., 1995; Ericson et al., 1996). The absence of the second induction step leads to the
induction of interneurons (Fig. 4E), located more dorsally, indicating that SHH acts as a morphogen in the neural tube (Ericson et al., 1996). The finding of sal expression at organogenesis stages in dorsally located sensory neurons (Fig. 3E) might be due to long range diffusion of an HH signal through the cavity of the neural tube as suggested for patched in chicken (Marigo and Tabin, 1996).

The pronephros shows abundant sal expression throughout embryonic development (Figs 2, 4E), which is consistent with the suggested role for shh in kidney formation in higher vertebrates (Fig. 4E; Bitgood and McMahon, 1995; Chiang et al., 1996).

In the pectoral fin buds (not shown) and subsequently in the outgrowing pectoral fins, sal transcripts are localized in the posterior half of the distal progress zone (Fig. 4F), an area analogous to the zone of polarizing activity (ZPA) of higher vertebrates. Here shh is required for proper AP-patterning of the limb (Riddle et al., 1993; Chang, 1994).

Thus, in all tissues known to be patterned by HH activity, expression of sal and shh coincide temporally and spatially, suggesting that sal expression is activated in response to HH activity.

Ectopic HH activity expands sal expression domains

We have tested this inference using two complementary experimental strategies. First, we have examined the sal-inducing activity of SHH by injecting different amounts of shh mRNA (Krauss et al., 1993) into one blastomere at the 2-4 cell stage. Due to the limited stability of the RNA injected (data not shown) we focused our analysis on early effects and therefore analyzed embryos up to stage 22. Functional expression of ectopic shh was monitored by the altered size and shape of the developing eyes (Fig. 5C,H) (Ekker et al., 1995b; Macdonald et al., 1995). Already moderate levels of ectopic SHH activity expanded the sal expression domains significantly. We did not, however, observe ectopic de novo expression isolated from the wild-type sal expression domains. Fig. 5C shows that in shh injected embryos sal expression covers the entire eye, which is consistent with the observation that hh overexpression causes an expansion of proximal eye fates (Ekker et al., 1995b; Macdonald et al., 1995). In the neural tube and in the brain of injected embryos the ventral wild-type sal expression domain (Fig. 5B,D) expands towards the dorsal side (Fig. 5C,E), however, sal expression was never detected in the dorsal part of the mesencephalon (Fig. 5C). In the somites (Fig. 5D) and the pronephros (Fig. 5B), the mesodermal sal expression domains, shh injection causes a significant enlargement of the sal domains (Fig. 5C,E).

These results show that sal expression, which expands in hh signaling centers in response to increased HH activity, can indeed be activated by hh signaling. The finding that no de novo sal expression domains are observed, indicates that additional competence factors determine the response to ectopic HH activity which, in the case of sal, allow only an expansion of the wild-type expression domains.

Ectopic PKA* activity reduces the size of the sal expression domains

To further investigate the regulation of sal expression through the hh signaling pathway we have assayed the effects of reduced HH activity. In Drosophila it was shown that protein

Fig. 3. Medaka sal expression is in the neural tube is confined to neurons. (A, B) Transverse sections through the trunk region of whole-mount stained embryos. (A) sal expression is restricted to the ventral third of the neural tube (stage 19). No sal expression is detected in the floorplate (arrowhead). In somites flanking the notochord faint sal expression is detected. (B) Ventral sal expression in the neural tube is restricted to ventral, bilateral clusters of 8-10 cells (stage 25). (C,D) Axons of primary motoneurons (red), visualized by an antibody directed against acetylated tubulin, project from sal-positive cell bodies (blue) to the underlying somites and identify sal-positive cells (in B) as motoneurons. (E) sal staining in a transverse section through the trunk region of an embryo at organogenesis stages (stage 27). In addition to ventral clusters of sal-positive cells in the neural tube staining becomes apparent at intermediate positions. mn, motoneurons; n, notochord; nt, neural tube; sm, somite; fp, floorplate
kinase A (PKA) acts to suppress the expression of hh target genes (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995). To interfere with hh signaling we made use of a constitutively active form of the catalytic subunit of protein kinase A (Orellana and McKnight, 1992; PKA*) previously shown to antagonize known hh signaling activities in vertebrates (Hammerschmidt et al., 1996; Concordet et al., 1996).

The injection of PKA* mRNA causes fusion of the eyes (Fig. 6B,D), indicating that PKA* activity is functional in antagonizing midline signaling mediated by HH activity as reported previously (Hammerschmidt et al., 1996; Concordet et al., 1996). While ectopic SHH was expanding the sal expression domains, the expression of PKA* had the opposite effect. We observed a concentration dependent, strong reduction of sal expression in all hh signaling centers. In the eye, minute traces of sal expression are detected (Fig. 6B). sal expression in the ventral CNS is almost eliminated upon PKA* injection (Fig. 6B). Only weak residual sal transcription is detected in the pronephros (Fig. 6B) and somites. Thus, sal expression is reduced in all sal expression domains confined to hh signaling centers, when hh signaling is antagonized by the expression of PKA*.

In addition to its expression in the known hh signaling centers described so far, sal is also detected at organogenesis stages in dorsally located neurons within the neural tube and already at the neurula stage in a stripe at the midbrain hindbrain boundary (MHB; Fig. 4B). While the limited stability of the mRNA injected did not allow the investigation of the effect of ectopic shh and its antagonist PKA* respectively on dorsal sal expressing neurons, this issue could be addressed for the sal expression at the MHB.

sal expression at the MHB responds to ectopic shh and its antagonist PKA*

The MHB was previously identified as a patterning center required for the establishment of an anterior-posterior polarity in the midbrain (reviewed by Joyner, 1996). shh or other members of the hh family have not been identified as an essential signaling activity in this dorsal brain region. However, the sal stripe at the MHB clearly expands in response to ectopic shh expression (Fig. 5C). It expands from only a few cell diameters width in wild-type embryos (Fig. 5B) up to five fold in the AP direction in response to ectopic HH activity (Fig. 5C). Conversely, inhibition of hh signaling in PKA* injected embryos, which efficiently suppresses sal transcription in all hh signaling centers, also prevents the sal stripe expression at the MHB (Fig. 6B).

Fig. 4. Medaka sal expression is confined to hh signaling centers. (A) Lateral view of a stage 24 embryo (early somitogenesis) stained for medaka shh. shh is detected in prechordal mesoderm and along the AP-axis in the ventralmost part of the CNS (floorplate). More caudally expression is detected in the notochord. (B-F) sal expression. (B,C) sal is detected in proximal part of optic vesicle, in optic stalk and in optic chiasm, revealed by transverse sections at the level of the eye in stage 19 embryos (D). sal transcripts are detected in forming neural tube (arrowheads in B) restricted to its ventral part (D) and subsequently in differentiating neurons (E; see also Fig. 3). (B,E) sal expression is found in the mesodermally derived pronephros. (F) In outgrowing pectoral fins sal expression is confined to the posterior part. In addition to known hh signaling centers sal is also expressed in the MHB (B,C) where no shh expression is detected (A). ey, eye; fp, floorplate; MHB, mid-hindbrain boundary; n, notochord; nt, neural tube; oc, optic chiasm; os, optic stalk; ot, otic vesicle; ov, otic vesicle; pn, pronephros.
As hh signaling has not been found to be required for AP-patterning in the dorsal midbrain, we asked whether the experimentally induced HH effect on sal stripe expression is an indirect one, such as an HH-induced overproliferation of the sal-expressing cells. To clarify this, we examined the expression domain of Pax2, a cellular marker for the size of the MHB area (Krauss et al., 1992; Brand et al., 1996). Figs 5H, 6D show that Pax2 expression in the eye depends on HH activity, as reported previously. But neither ectopic shh nor PKA* significantly altered the dimension of the MHB Pax2 expression domain, indicating that the expansion of the sal stripe is not caused by cell proliferation events in response to ectopic HH activity. This suggests that sal expression in the MHB also depends directly on hh signaling.

DISCUSSION

**sal is a general target of hh signaling**

Signaling by hh and the vetebrate hedgehog gene family is a patterning mechanism involved in many different developmental processes in *Drosophila* and vertebrates. The secreted HH protein diffuses and binds to its receptor complex consisting of at least two multipass membrane spanning proteins, SMOOTHENED and PATCHED (Stone et al., 1996; Marigo et al., 1996a). The receptors patched in chicken and ptc1 in zebrafish, which are the vertebrate homologues of *Drosophila* patched, are expressed in an hh-dependent manner in hh signaling centers adjacent to known sites of hh expression (Marigo and Tabin, 1996; Concordet et al., 1996). In vertebrates, target genes that can mediate hh patterning on the transcriptional level have been described only for a few signaling centers. Here we report the identification of a novel hedgehog target gene, the medaka gene sal. It encodes all features diagnostic for a DNA-binding zinc finger protein and thus, like its *Drosophila* homologue, it is likely to act as a transcriptional regulator. In contrast to other transcription factors which act as hh targets in a subset of the hh signaling centers, sal expression is found in all known hh signaling centers. sal expression expands in response to increased HH activity and is repressed in response to PKA* and thus, it
Fig. 6. Suppression of hh signaling prevents sal expression. Detection of sal (A,B) and Pax2 transcripts (C,D) in lateral views of stage 22 embryos. Compare expression patterns of control (XEF16, ambiom; A,C) and PKA* injected (B,D) embryos. (A) Wt sal expression in the eye and ventral neural tube (arrowheads) as well as in the pronephros (arrow) is strongly reduced or completely absent in PKA* injected embryos (B). No sal stripe expression is detectable in the MHB. (C) Pax2 expression in control and (D) PKA* injected embryos. Expression in the eye is suppressed by PKA* whereas expression in the MHB is unaffected. Axial mesoderm (asterisk) underlying the neural tube is extended towards anterior upon PKA* injection. Abbreviations as in Fig. 5.

appears to be a general target of hh in the medaka fish. The finding of a competence area surrounding the wild-type sal expression domains provides some evidence for an immediate control of sal by hh although, at the moment, we cannot decide whether this control by hh signaling is direct or mediated.

sal expression is observed in all known hh signaling centers including the optic stalk and anterior optic vesicle where shh acts in proximodistal patterning. This domain of sal expression is expanded upon ectopic HH activity whereas blocking of hh signaling by PKA* prevents its expression. In the proximal part of the eye, nk2.2 (Barth and Wilson, 1995) and Pax2 have been reported as hh target genes (Ekker et al., 1995b; Macdonald et al., 1995). Unlike sal, which is hh dependent in all hh signaling centers, the expression of nk2.2 is limited to the proximal parts of the developing eye and the ventral part of the neural tube. Pax2 shows a responsiveness to hh signaling only in the eye expression domain (Ekker et al., 1995b; Macdonald et al., 1995 and our results), while its expression in the MHB and at other locations does not depend on hh signaling.

sal expression in the neural tube, somites and limb buds is reminiscent of the expression pattern of the zinc-finger transcription factor GLI in chick (Marigo et al., 1996b) which was shown to be controlled by shh in the limb. In the neural tube SHH exerts its patterning activity in two phases. First, naive neural plate cells are converted to ventralized progenitors (positive for sal and GLI). In a second step SHH emanating from the floor plate directly induces motoneurons. Peak concentrations of SHH, which acts as a morphogen in the neural tube, induce exclusively the expression of floor plate markers (e.g. HNF3β/axial), while slightly lower concentrations induce expression of motoneuron specific markers and intermediate concentrations induce the expression of interneuron markers (Ericson et al., 1996). This patterning activity is reflected by the expression of sal which is initially present over the entire ventral part of the neural tube (excluded from the floorplate), but is subsequently refined to motoneurons and is later also detected in interneurons. In contrast, GLI is expressed in the ventral neural tube without further refinement (Marigo et al., 1996b). Late dorsal sal expression in sensory neurons could likely represent a long range induction through SHH secreted from the floorplate diffusing up through the lumen of the neural tube, as suggested for patched in chicken (Marigo and Tabin, 1996). This induction might also be due to the activity of other members of the hh family expressed in dorsal regions of the spinal chord like banded hh in Xenopus (Ekker et al., 1995a). Alternatively, dorsally expressed BMP4 (Liern et al., 1995), the vertebrate homologue of Drosophila dpp, which activates spalt in the Drosophila wing imaginal disc (Lecuit et al., 1996; Nellen et al., 1996; de Celis et al., 1996), may be required for late sal transcription in sensory neurons.

Ectopic expression of shh leads to an expansion of the wild-type sal expression domains and does not cause de novo expression of sal. In contrast the hh target genes zp50 (Hauptmann and Gerster, 1996) and axial (Strähle et al., 1993) are expressed at ectopic locations upon shh injection, indicating different competences in the hh response. In shh injected embryos zp50 is expressed in ectopic domains with a drastic loss of wt expression domains (Hauptmann and Gerster, 1996). axial, like sal, upon shh injection exhibits a widening of its ventral neural tube expression. In addition ectopic de novo expression was detected in the MHB region (Krauss et al., 1993) indicating a competence for hh signaling. The widening of the sal expression domains upon shh injection is best explained by assuming that the ectopic shh adds to the endogenous concentration of HH and thereby increases the critical concentration thresholds of HH in the signaling centers. The finding that no de novo sal expression domains are observed indicates that additional competence factors determine the response to ectopic HH activity, which allow sal activation only in the competent tissue. Taken together, these results indicate that sal is indeed a specific and general target of hh signaling.

Does the sal stripe define a new hh signaling center?

In addition to the known hh signaling centers we found that the sal expression domain at the MHB expands in response to hh signaling. Ectopic transplantation studies in chick and quail have demonstrated that the MHB, the junction of mid- and hindbrain, acts as an organizer that has a strong polarizing
activity on midbrain and hindbrain (Bally Cuif et al., 1992; Otero et al., 1993). So far the expression pattern and functional analysis of known hedgehog family members is consistent with a role in dorsoventral patterning of the CNS. However, no member of the hh family has been shown to be expressed in an area around the MHB nor has it been shown that HH activity is involved in AP-patterning at the MHB.

Our results, however, indicate that the sal stripe expression domain responds directly to ectopic hh signaling. This finding and the notion that sal is expressed in all known hh signaling centers, suggest that the sal stripe expression depends on non-detectable amounts of known members of the HH family of secreted molecules or on an unknown member of the family. This argument is consistent with the finding that ectopic PKA* represses sal expression in all these domains, including the MHB.

A distinct HH competence region could be established by cell migration events in the neural keel at the level of the MHB. In this view, a cluster of cells which is initially in contact with HH emanating from the ventral midline could migrate dorsally in a highly coordinated manner and give rise to the MHB region expressing sal in response to the initial SHH activity in the ventral midline. We have not observed dorsal migration of sal-expressing cells within the corresponding region and thus, such a mechanism underlying HH-dependent sal expression at the wild-type MHB appears unlikely.

A second possibility that may account for HH-dependent sal expression at the MHB is a localized dorsal competence responding to long range shh signaling after its ventral expression in the neural tube. The expression pattern of patched, one of the receptor components of the hh signaling cascade in the midbrain-hindbrain boundary of chicken (Marigo and Tabin, 1996), is consistent with this proposal. Additional evidence in support of this model is provided by the observation of ectopic MHB expression of another hh target gene, axial (Strähle et al., 1993) in response to ectopic shh (Krauss et al., 1993). axial is normally expressed at the ventral midline of the neural tube and in the diencephalic-mesen-cephalic boundary (Strähle et al., 1993). Upon injection of shh mRNA, axial is ectopically expressed in the region of the MHB (Krauss et al., 1993) indicating a localized competence to hh signaling in the MHB. In contrast to sal, which is detected by the early neurula stage in the dorsal part of the MHB area, the dorsal patched expression seems to evolve from a more ventrally localized expression domain. Marigo and Tabin (1996) suggest that patched expression in the MHB is likely to represent a long range action of the ventral SHH signal, facilitated through diffusion of SHH in the lumen of the neural tube. At the time when the sal stripe is first detected in the prospective MHB, a neural tube has not yet formed because of fish secondary neurulation (Papan and Campos-Ortega, 1994). This property of fish development makes it unlikely that long range diffusion of ventral SHH through the lumen of the neural tube would be the cause for the sal stripe expression, but we cannot rule out long range signaling through the neural keel itself.

A third scenario, based on the proposal of a local HH competence in the MHB, involves an additional novel source of hh. This proposes a novel member of the family, as none of the hh family members described so far has been shown to be expressed dorsally in the MHB. This could be induced by FGF8, a diffusible molecule prominently involved in the patterning at the MHB (Crossley et al., 1996a) and a member of the fibroblast growth factor family of secreted factors. FGF8 is required for AP patterning of the midbrain and it also mediates AP patterning in the developing limb where it induces shh expression in the ZPA (Crossley et al., 1996b). This link between the two signaling pathways in the limb and the dependence of the MHB sal stripe on hh signaling consistently argues for an unknown member of the hh family to be involved in the sal induction and pattern organizing activity at the MHB.

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