Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium

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SUMMARY

We have characterised the functions of the bHLH transcriptional repressors HES1 and HES5 in neurogenesis, using the development of the olfactory placodes in mouse embryos as a model. Hes1 and Hes5 are expressed with distinct patterns in the olfactory placodes and are subject to different regulatory mechanisms. Hes1 is expressed in a broad placodal domain, which is maintained in absence of the neural determination gene Mash1. In contrast, expression of Hes5 is restricted to clusters of neural progenitor cells and requires Mash1 function. Mutations in Hes1 and Hes5 also have distinct consequences on olfactory placode neurogenesis. Loss of Hes1 function leads both to expression of Mash1 outside of the normal domain of neurogenesis and to increased density of MASH1-positive progenitors within this domain, and results in an excess of neurons after a delay. A mutation in Hes5 does not produce any apparent defect. However, olfactory placodes that are double mutant for Hes1 and Hes5 upregulate Ngn1, a neural bHLH gene activated downstream of Mash1, and show a strong and rapid increase in neuronal density. Together, our results suggest that Hes1 regulates Mash1 transcription in the olfactory placode in two different contexts, initially as a prepattern gene defining the placodal domain undergoing neurogenesis and, subsequently, as a neurogenic gene controlling the density of neural progenitors in this domain. Hes5 synergises with Hes1 and regulates neurogenesis at the level of Ngn1 expression. Therefore, the olfactory sensory neuron lineage is regulated at several steps by negative signals acting through different Hes genes and targeting the expression of different proneural gene homologs.

Key words: Mouse, Olfactory placode, Mash1, Prepattern, Notch signalling, Lateral inhibition

INTRODUCTION

The generation of the numerous types of neurons that constitute the adult nervous system from an initially homogenous population of neurectodermal progenitor cells is an extremely complex process, which has been thoroughly investigated, first in Drosophila (Campos-Ortega, 1993; Jan and Jan, 1994) and, more recently, in vertebrates (Tanabe and Jessell, 1996; Chan and Jan, 1999). In vertebrates, the early steps of specification and differentiation of neuronal precursors have been studied in several models, including the primary neurons of lower vertebrates (Chitnis and Kintner, 1995), the spinal cord (Tanabe and Jessell, 1996), the neural crest (Anderson et al., 1997) and the retina (Cepko, 1999). The olfactory sensory epithelium (OE) has also been a useful model to identify transcription factors and extracellular signals regulating neurogenesis in the mouse (Calof et al., 1996). The value of the OE model stems from the simple nature of this tissue, where primary olfactory sensory neurons (OSNs) are the main neuronal type, and from its capacity to renew its neuronal population during adulthood and to regenerate it after damage (Graziadei and Graziadei, 1979; Crews and Hunter, 1994). These properties have facilitated the identification and characterisation of OSN progenitors, which are present in the embryonic as well as in the adult OE. Recent evidence suggests the existence of at least two types of progenitors, representing sequential steps in the OSN lineage. A rare population of slow-dividing cells with the self-renewing capacity of stem cells has been shown to generate a much more abundant population of rapidly dividing precursors committed to neuronal differentiation (Calof and Chikaraishi, 1989; Calof et al., 1996; Mumm et al., 1996).

Several genes of the basic helix-loop-helix (bHLH) class have been implicated in neurogenesis in the olfactory placode (Cau et al., 1997). Mash1 is the first of these genes to be expressed in OSN progenitors and analysis of the OE phenotype of mice mutant for Mash1 shows that it plays a central role during early stages of the OSN lineage (Guillemot et al., 1993; Cau et al., 1997). Loss of Mash1 function results in a lack of expression of the downstream bHLH genes Neurogenin1 (Ngn1) and NeuroD, and in a failure to generate OSN precursors in the OE (Cau et al., 1997; our unpublished
data). Mash1 has therefore a neural determination function in the OE similar to that of its Drosophila counterparts of the achaete-scute complex (AS-C) (Ghysen and Dambly-Chaudiere, 1988; Jan and Jan, 1994). A preliminary analysis of the OE phenotype of Ngn1 mutant mice indicates that Ngn1 is required at a later step, in the differentiation of OSNs (E.C., Q. Ma, D. Anderson and F. G., unpublished data). To get further insights into the regulation of OSN development, we have examined the function of genes that are potential regulators of Mash1 and Ngn1 in this tissue.

In Drosophila imaginal discs, expression of the AS-C proneural genes is controlled by upstream regulators, or prepattern genes (Ghysen and Dambly-Chaudiere, 1989; Skeath and Carroll, 1994; Simpson, 1996), whose products bind to site-specific enhancers found around the coding sequence of AS-C genes (Gomez-Skarmeta et al., 1995; Modolell, 1997). One of these upstream regulators, hairy, is a direct repressor of achaete expression in the wing and leg imaginal discs (Ohsako et al., 1994; Van Doren et al., 1994). In absence of hairy, the achaete expression domain expands and ectopic neural precursors are generated (Skeath and Carroll, 1991; Orenic et al., 1993). Proneural genes, which are initially expressed in groups of cells, called proneural clusters, become later restricted to single cells which thereby acquire a neural fate. This change in proneural gene expression involves a process of lateral inhibition mediated by the Notch receptor (Simpson, 1997). Interaction of Notch with its ligand Delta activates a signalling pathway resulting in transcriptional activation of the genes of the Enhancer of split (Espl) complex (Jennings et al., 1994; Artavanis-Tsakonas et al., 1995; Bray, 1997; Simpson, 1997). Activity of the Espl genes, which encode bHLH transcriptional repressors structurally related to hairy, inhibits neural development by antagonizing proneural gene function (Ohsako et al., 1994; Van Doren et al., 1994; Nakao and Campos-Ortega, 1996; Jennings et al., 1999).

The components of the Notch signalling pathway are conserved in vertebrate species (Lewis, 1996; Gridley, 1997). Vertebrate Notch, Delta and Serrate/Jagged (which, like Delta, encode Notch ligands), have been implicated in processes of lateral inhibition controlling cell fate specification in numerous tissues, including the neural plate, inner ear, retina, skin and pancreas (Chitnis and Kintner, 1995; Chitnis et al., 1995; Henrique et al., 1997; Crowe et al., 1998; Lanford et al., 1999). Four genes homologous to the Drosophila Hairy and Espl genes, named Hes1-Hes3 and Hes5, have been identified in mouse (Akazawa et al., 1992; Sasai et al., 1992). Genetic analysis have shown that Hes1 and Hes5 in particular have important roles in neurogenesis (Ishibashi et al., 1994, 1995; Tomita et al., 1996; Ohtsuka et al., 1999; reviewed in Kageyama and Nakaniishi, 1997). However it has remained unclear whether these genes were regulating neural development through a process of lateral inhibition, or in a different context.

To better define the functions of Hes1 and Hes5 in neurogenesis, we have examined in details the regulation of expression and function of these two genes, using the development of the olfactory placode as a model. We have analysed the OE phenotype of Hes1 null mutant mice, and we have generated mice null mutant for the gene Hes5 to examine its function in neurogenesis and its possible synergy with Hes1. Our results show that Hes1 activity in OE neurogenesis can be divided in two distinct phases. Hes1 functions initially to delimit a domain of proneural gene expression at the onset of neurogenesis in the placode. Hes1 subsequently functions in a process of lateral inhibition which regulates the recruitment of neural precursors in the OE. Therefore, Hes1 appears to cumulate the functions of a prepattern gene and of a neurogenic gene. Mash1 is a target of Hes1 for these two activities. In contrast, Hes5 appears to function exclusively as a neurogenic gene and it regulates the expression of the differentiation gene Ngn1. Thus, development of the OSN lineage is negatively regulated by Hes genes at several sequential stages.

**MATERIALS AND METHODS**

**Generation of Hes 5 mutant animals**

To generate a Hes5 targeting vector, a PGK-neo cassette was flanked by 5’ and 3’ arms consisting of a 3 kb NotI-SmaI fragment and a 3 kb NotI-SmaI I fragment from the mouse (129/Sv) Hes5 locus (unpublished data), respectively. The mutation introduced by this vector is a deletion of 724 bp, including the coding region from Met1 to Ala76 and comprising the bHLH domain. The vector was electroporated in the ES cell lines H1 (from IGBMC, Strasbourg, unpublished) and R1 (Nagy et al., 1993), and selection was performed as described (Fode et al., 1998). Homologous recombination was detected by Southern blotting of ES cell genomic DNA, using a 0.9 kb SmaI-BamHI fragment and a 0.2 kb DraI-Khol fragment as 3’ and 5’ external probes, respectively. Ten independent targeted ES clones were obtained out of 252 clones analysed. Three of them were used to generate chimeras by blastocyst injection. Germline transmission was obtained by crossing the chimeras with C57bl6 females and the colony was expanded on a CD1 genetic background.

**Generation of compound mutant animals for Hes1, Hes5 and Mash1**

For staging of embryos, mid-day of the vaginal plug was considered as E0.5. The Mash1 and Hes1 mutant mouse lines have been described in Guillemot et al. (1993) and Ishibashi et al. (1995), respectively. Compound Hes1:Hes5 embryos were obtained by intercrossing Hes1+/−;Hes5+− or Hes1−/−;Hes5−/− mice. At E10.5, the 8 expected genotypes resulting from these crosses were obtained with Mendelian ratios (see Results section).

**Whole-mount in situ hybridization**

The Mash1, Ngn1 and SCG10 probes have been described in Cau et al. (1997). The Hes1 probe was transcribed from a 0.9 kb cDNA clone described in Ishibashi et al. (1995). The Hes5 probe was transcribed from a 1.3 kb cDNA clone described in Akazawa et al. (1992). The protocol for whole-mount RNA in situ hybridization has been described in Cau et al. (1997). The protocol for two-colour whole-mount RNA in situ hybridization is available upon request.

**Immunohistochemistry with anti-MASH1 antibody**

Detection of the MASH1 protein was performed on 14 m sections of E10.5 embryos frozen in OCT, using a monoclonal anti-MASH1 antibody (Lo et al., 1991). After postfixation in 4% paraformaldehyde at room temperature for 10 minutes, and 3 washes of 5 minutes in PBS with 0.05% Tween-20, immunohistochemistry was performed as Porteus et al. (1994).

**RESULTS**

**Expression of Hes genes in the embryonic olfactory epithelium**

In an effort to understand how neurogenesis is regulated in the embryonic OE, we examined the expression in this tissue of
genes belonging to the Hes (Hairy and Enhancer of split) family of transcriptional repressors. We performed this study at E12.5, a stage when the OE has begun to acquire an organisation in cell layers characteristic of the mature tissue. At this stage, the OE contains two populations of dividing progenitors, located respectively on the apical and basal sides of the epithelium. Cells dividing apically are already present in the olfactory placode, whereas basal progenitors appear in the OE only at around E12.5. As development of the OE proceeds, the proportion of basal progenitors then increases progressively, and that of apical progenitors decreases (Smart, 1971; Cuschieri and Bannister, 1975). Two genes of the Hes family, Hes1 (Sasai et al., 1992) and Hes5 (Akazawa et al., 1992), are expressed in the embryonic OE with distinct patterns. At E12.5, Hes1 transcripts are present in most cells on the apical side of the OE and are absent from the basal side (Fig. 1G). Hes5 in contrast is expressed in scattered cells, which are present only on the basal side of the OE (Fig. 1I). The two genes are already expressed with different patterns when neurogenesis begins in the olfactory placode. At E10.5, Hes1 expression is widespread throughout the placode, but is highest at the periphery and lower in a large central area (Fig. 1A). The area of high Hes1 expression abuts the more centrally located placodal territory, which contains neuronal precursors marked by expression of the neural determination gene Mash1 (Fig. 1C; Cau et al., 1997). In contrast to the broad expression of Hes1, Hes5 expression is restricted to cell clusters (Fig. 1D), which are either intermingled with Mash1-expressing cells or have a more central position (Fig. 1F). At this early stage, there is no distinct cell layering in the olfactory placode, and Hes1-positive cells and Hes5-positive cells are found in both apical and basal positions (data not shown). At E15.5, Hes5 OE expression is not detectable and that of Hes1 is low and restricted to a few apical cells (data not shown).

Hes1 and Hes5 have been shown to be important effectors of the Notch signalling pathway in the embryonic central nervous system (Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999). If Hes genes are also regulated by Notch signalling during OE neurogenesis, their expression patterns should be affected by a mutation in Mash1 (Sasai et al., 1992), which results in an early block in OE neurogenesis (Cau et al., 1997). Indeed, expression of Hes1 (Fig. 1H) and Hes5 (Fig. 1J) is absent in most of the OE of Mash1 mutant embryos at E12.5. Therefore, activation of these genes is part of a program of neurogenesis in the OE.

Examination of Hes1 expression in Mash1 mutant embryos at E10.5 revealed that, in contrast with its later expression (Fig. 1H), the early expression of Hes1 in the olfactory placode is largely unaffected by the loss of Mash1 (Fig. 1B). This result suggests that different mechanisms regulate Hes1 expression at the onset of neurogenesis and at later stages in the OE. As expected, expression of Hes5 is severely reduced in the Mash1 mutant olfactory placode (Fig. 1E), except in a small domain of the placode where neurogenesis is not affected by loss of Mash1 (Cau et al., 1997).

Fig. 1. Hes1 and Hes5 expression in the olfactory epithelium requires Mash1 function, whereas Hes1 expression at the placodal stage is independent of Mash1. (A,B,D,E) Flat mounts of olfactory placodes from wild type (A,D) and Mash1 mutant (B,E) E10.5 embryos hybridized with cRNA probes for Hes1 (A-C) and Hes5 (D-F). Medial is to the left, lateral to the right; dorsal is up and ventral is down. In the placode, Hes1 is expressed broadly (A) and this pattern is not affected in a Mash1 mutant placode (B), while Hes5 expression is restricted to cell clusters (D) and requires Mash1 function (E). (C,F) Flat mounts of wild-type E10.5 placodes double labelled for (C) Mash1 (brown) and Hes1 (blue) and (F) Mash1 (blue) and Hes5 (brown). The periphery of the placode is to the left, the center to the right. The domain of high Hes1 expression is peripheral to the Mash1-positive cell clusters (C) and Hes5-positive cell clusters are either intermingled with, or more centrally located than, Mash1-positive cells (F). (G-J) Sections of wild type (G,I) and Mash1 mutant (H,J) OE at E12.5 hybridized with Hes1 (G,H) and Hes5 (I,J) cRNA probes. At this stage, both expression of Hes1 in apical cells (G) and of Hes5 in basal cells (I) require Mash1 function (H,J). Hes1 is also expressed in the mesenchyme surrounding the OE (G,H).
**Table 1. Clustering of MASH1-positive cells in Hes1 mutant olfactory placodes**

<table>
<thead>
<tr>
<th>MASH1-positive cells</th>
<th>WT (n=3)</th>
<th>Hes1&lt;sup&gt;−/−&lt;/sup&gt; (n=3)</th>
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<tbody>
<tr>
<td>Cells isolated or in pairs</td>
<td>92±6.6</td>
<td>68±2.6</td>
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<tr>
<td>‘small clusters’ (3-4 cells)</td>
<td>7±6.6</td>
<td>27±1±3.7</td>
</tr>
<tr>
<td>‘large clusters’ (5-8 cells)</td>
<td>0.0±0.0</td>
<td>4.7±3.8</td>
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To quantify the clustering of MASH1-positive cells in Hes1 mutant and wild-type placodes, the percentage of MASH1-positive cells falling in the following three categories: cells isolated or in pairs, ‘small clusters’ (3-4 cells) and ‘large clusters’ (5-10 cells), are shown. In wild-type olfactory placodes, most MASH1-positive cells are either isolated or in pairs, and a small fraction is grouped in small clusters of less than 5 cells. In Hes1 mutant placodes, there is a larger percentage of cells in clusters, and the size of clusters reaches up to 8 cells. The percentages of cells are averages ± s.d, and the numbers of animals counted are indicated in parenthesis. For each animal, three sections were counted using a minimum of 50 cells.

**Hes1 mutant phenotype in the olfactory epithelium**

The distinct expression patterns of Hes1 and Hes5 in the developing OE suggested that these genes may regulate distinct steps in OE neurogenesis. To address this possibility, we examined the loss-of-function phenotypes of Hes1 and Hes5 in the OE, starting with the study of embryos carrying a null mutation in Hes1 (Ishibashi et al., 1995).

Previous studies have shown that loss of Hes1 results in premature neuronal differentiation and upregulation of Mash1 in the forebrain and in morphological defects affecting with a low penetrance the cranial neural tube and the eye (Ishibashi et al., 1995; Tomita et al., 1996). The first indication that lack of Hes1 affects neurogenesis in the OE was a marked increase in the number of Mash1-positive cells in the olfactory placodes of Hes1 mutant embryos. Expression of Mash1 starts in wild-type olfactory placodes at the onset of neurogenesis (E10.0 or 25 somites) and, by E10.5, Mash1 is expressed in clusters of cells located in the medial side and to a lesser extent in the lateral side of the placode (Fig. 2A; Cau et al., 1997). In Hes1 mutant placodes, ectopic Mash1 expression was observed in ventral and dorsal areas, which normally do not express the gene at this stage (Fig. 2B). There was also a marked increase in number of Mash1-positive cells in the lateral side of the placode, and scattered Mash1-positive cells in the center of the placode. This enlargement of the Mash1-positive area in Hes1 mutant placodes shows that Hes1 is involved in defining the area of the placode that activate Mash1 expression.

In addition to this enlargement of the Mash1-expressing area, Hes1 mutant placodes also present a change in level of Mash1 expression in the medial domain of the placode, where Mash1 is normally expressed. This defect is characterised by an increase in Mash1 expression, in particular in cells that normally express the gene at low level, so that Mash1 reaches a rather uniformly high expression (compare insets in Fig. 2A and B). To examine this phenotype with a better resolution, we studied Mash1 expression at the protein level, on sections of olfactory placodes. In wild-type placodes, cells were mostly isolated or in groups of two, whereas larger clusters of up to eight MASH1-positive neighbouring cells were observed in Hes1 mutant placodes (Fig. 2C,D; Table 1). The increase in level of Mash1 expression and the presence of clusters of MASH1-positive cells in the neurogenic domain of Hes1 mutant placode suggest that, in addition to an early function in defining the limits of the neurogenic domain of the placode, Hes1 is also involved in a process of lateral inhibition that regulates the number and density of Mash1-positive OSN precursors generated in this domain.

We then examined whether the generation of extra Mash1-positive precursors leads to an increase in the number of neurons differentiating in Hes1 mutant placodes. Using SGC10 as a pan-neuronal marker, scattered neurons were found in the center of wild-type placodes at E10.5 (Fig. 2E; Cau et al., 1997). There was no apparent change in number of neurons in Hes1 mutant placodes at the same stage (Fig. 2F), whereas a significant increase was observed at E12.5 (Fig. 2J). The delay between the appearance of additional Mash1-positive precursors and of additional SGC10-positive neurons suggests that ectopic Mash1 expression may not be sufficient to promote neurogenesis in Hes1 mutant placodal cells and that negative regulators other than Hes1 may inhibit neurogenesis at a step downstream of Mash1 expression. Hes5 is expressed in the olfactory placode and OE (Fig. 1D,F) and is upregulated in Hes1 mutant placodes (Fig. 2G,H), suggesting that a functional compensation could take place between Hes1 and Hes5.

**Lack of neural defects in Hes5 mutant embryos**

We generated a null mutation in the Hes5 gene to study its function and possible interaction with Hes1 in the OE and other parts of the nervous system (Fig. 3). The protein-coding sequence of the Hes5 gene is divided in three exons (Takebayashi et al., 1995). We built a targeting construct in which the first two exons of Hes5, which encode the bHLH domain, and part of the third exon are deleted and replaced by a PGK-neo cassette. This mutation, which should result in a null allele, was introduced in ES cells and clones carrying the targeted mutation were used to generate three independent mutant mouse strains (see Materials and Methods). Animals heterozygous for the Hes5 null mutation were viable and fertile. When interbred, they generated progenies with a normal ratio of homozygous mutant mice (32 out of 149, or 21.5%). These animals do not present defects, are fertile and have a normal life span. Histological examination of their brain did not reveal any abnormality (data not shown).

To determine if neurogenesis in the OE is affected in absence of Hes5, we examined the expression of Mash1 and SGC10 in the placode and OE of Hes5 mutant embryos. No difference in number and distribution of Mash1-positive OSN progenitors and SGC10-positive neurons could be detected between wild-type and mutant placode and OE (Fig. 4G,H and data not shown), suggesting that lack of Hes5 alone is not sufficient to significantly perturb neurogenesis in the OE.

**Hes1 and Hes5 synergize to regulate neurogenesis in the OE**

Hes1 and Hes5 have similar activities of transcriptional repressors and are both expressed in the embryonic nervous system, including the OE (Fig. 1; Akazawa et al., 1992; Sasai et al., 1992). Therefore these genes could functionally compensate for one another in animals mutant for one of the two genes. To examine this possibility, embryos carrying both Hes1 and Hes5 mutations were generated. When the progeny of Hes1<sup>−/−</sup>-Hes5<sup>−/−</sup> and Hes1<sup>−/−</sup>-Hes5<sup>−/−</sup> intercrosses were harvested at E10.5, the eight possible genotypes were obtained
Table 2. Synergy between Hes1 and Hes5 in regulation of brain morphogenesis

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<tbody>
<tr>
<td></td>
<td>n=54</td>
<td>(n=82)</td>
<td>(n=55)</td>
<td>(n=48)</td>
<td>(n=129)</td>
<td>(n=59)</td>
</tr>
<tr>
<td>Hes5−/−</td>
<td>0%</td>
<td>0%</td>
<td>27.3%</td>
<td>0%</td>
<td>7.75%</td>
<td>71.2%</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>0%</td>
<td>27.3%</td>
<td>0%</td>
<td>7.75%</td>
<td>71.2%</td>
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The numbers are percentages of embryos with neural tube defects (open anterior neural tube and reduced size of telencephalic vesicles) in the progeny of intercrossed Hes1:Hes5 double mutant animals at E10.5. Hes1−/− mutant embryos exhibit with a low penetrance defects in closure of the neural tube and in brain morphology, as previously reported (Ishibashi et al., 1995). The penetrance of these defects is strongly increased in double Hes1:Hes5 homozygous mutant embryos. In addition, Hes1+/−:Hes5−/− embryos present at low frequency neural tube defects which are never observed in Hes1+/+ embryos. Numbers of embryos of each genotype are shown in parenthesis.

Table 3. Increase in number and clustering of Ngn1-positive cells in Hes1 and Hes1:Hes5 mutant olfactory placodes.

<table>
<thead>
<tr>
<th>Ngn1-positive cells</th>
<th>WT</th>
<th>Hes1+/+</th>
<th>Hes1−/−</th>
<th>Hes1+/+;Hes5+/+</th>
<th>Hes1−/−;Hes5−/−</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated cells</td>
<td>49.5±5.4</td>
<td>33.5±3.68</td>
<td>17.8±3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'small clusters' (2 to 5 cells)</td>
<td>50.6±5.4</td>
<td>56.2±4.3</td>
<td>49.2±4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'large clusters' (6 to 20 cells)</td>
<td>0±0.0</td>
<td>10.2±2.67</td>
<td>33.0±2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To quantify the clustering of Ngn1-positive cells in Hes5 mutant placodes, the percentages of Ngn1-positive cells falling in the following three categories: isolated cells (1 cell), 'small clusters' (2 to 5 cells) and 'large clusters' (6 to 20 cells), are shown. In wild-type placodes, a similar number of Ngn1-positive cells is found isolated or in small clusters, and no cluster contains more than 5 cells. In Hes1 single mutants, the proportion of cells in small clusters is the same but there are less isolated cells and larger clusters are found. In Hes1:Hes5 double mutant placodes, the number of cells in small clusters is again the same, but there are even less isolated cells and more cells in large clusters. The percentages of cells are averages, and the numbers of embryos analysed are in parenthesis. For each animal, three sections at different levels of the placode (rostral, medial and caudal) were analysed, and at least 50 cells were counted per section.

DISCUSSION

We have examined the role of two related transcriptional repressors, HES1 and HES5, in neurogenesis in the olfactory epithelium, by examining the phenotype in this tissue of Hes1 and Hes5 single and double mutant embryos. Together, our results suggest the following interpretation of the Hes5 olfactory placode phenotypes. Loss of Hes1 results in an increase in number of cells expressing the determination gene Mash1 in the placode, and a higher level of Mash1 expression in placodal cells that normally express it. The additional Mash1-positive cells activate expression of the downstream differentiation gene Ngn1, with limited efficiency in absence of Hes1 only, and in more cells in absence of both Hes1 and Hes5. The
increase in \textit{Ngn1} expression then results in additional neurons in the mutant placodes. These data suggest that both \textit{Hes1} and \textit{Hes5} negatively regulate neurogenesis, but at different stages in the OSN lineage. The main level of \textit{Hes1} control is on \textit{Mash1} expression, which marks an early stage in olfactory epithelium neurogenesis. In contrast, \textit{Hes5} does not appear to regulate \textit{Mash1} expression, but it synergizes with \textit{Hes1} at a downstream step, the regulation of \textit{Ngn1} expression, to control neuronal production. The observation that \textit{Mash1} and \textit{Hes1} are both expressed in apical cells whereas \textit{Ngn1} and \textit{Hes5} are expressed in basal cells in the OE (Fig. 1; Cau et al., 1997) supports the interpretation that \textit{Hes1} and \textit{Hes5} act at distinct steps in OE neurogenesis and have distinct molecular targets. Fig. 5 presents our current interpretation of the interactions taking place between the negative regulators \textit{Hes1} and \textit{Hes5} and the positive regulators \textit{Mash1} and \textit{Ngn1} during OE neurogenesis.

The genes of the \textit{Hes} family encode bHLH transcription factors that are most closely related to two groups of negative regulators of neurogenesis in \textit{Drosophila}, hairy and the products of the \textit{Enhancer of Split} complex (\textit{Espl}). Both \textit{hairy} and \textit{Espl} products have been shown to directly repress transcription of the proneural gene \textit{achaete} (Ohsako et al., 1994; Van Doren et al., 1994), but their activity is required in different contexts. \textit{hairy} is a prepattern gene (Skeath and Carroll, 1991, 1994; Fisher and Caudy, 1998). It is required in large areas of the wing and leg imaginal discs to prevent ectopic expression of the proneural gene \textit{achaete} and the formation of ectopic bristles (Skeath and Carroll, 1991; Orenic et al., 1993; Fischer and Caudy, 1998). The genes of the \textit{Espl} complex are neurogenic genes that are activated by Notch signalling in a process of lateral inhibition during embryonic and adult neurogenesis (Jennings et al., 1994). Activation of the \textit{Espl} genes blocks the accumulation of high amounts of proneural protein in most cells of the proneural clusters, thereby preventing them from adopting a neural fate (Bray, 1997). In this report, we show that \textit{Hes1} regulation and mutant phenotype in the developing OE suggest that \textit{Hes1} has a dual role, acting as a prepattern (\textit{hairy}-like) gene at the onset of neurogenesis in the olfactory placode and subsequently as a neurogenic (\textit{Espl}-like) gene regulating \textit{Mash1} expression in OE progenitors. In addition, the regulation of \textit{Hes5} expression in the olfactory placode and the placodal phenotype of \textit{Hes1:Hes5} double mutants support a role for \textit{Hes5} as a neurogenic gene acting at a later step in the OSN lineage.

\textbf{\textit{Hes1} has characteristics of a prepattern gene in the olfactory placode}

\textit{Hes1} is expressed in a broad domain of the olfactory placode before the onset of \textit{Mash1} expression and of neurogenesis. Once \textit{Mash1} expression has been initiated (at the 24- to 26-somite stage), \textit{Hes1} expression is maintained at high level...
throughout the periphery of the placode and is reduced in more central areas, where expression of *Mash1* and other neural bHLH genes has been induced (Fig. 1; Cau et al., 1997). This early expression pattern is independent of the emergence of neural precursors, and thus of lateral inhibition, as it is unaffected in *Mash1* mutant embryos in which most OSN progenitors are missing. Therefore, earlier signals involved in olfactory placode patterning must be responsible for the induction of *Hes1* expression. The phenotype of the *Hes1* mutant olfactory placodes, which present a vast enlargement of *Mash1*-expressing areas, provide further evidence that the early function of *Hes1* is unrelated to Notch-mediated lateral inhibition. Together, these results support the notion that *Hes1* is involved in delimiting the domains of *Mash1* expression in the placode, and thereby in repressing its expression in carcinoma cells (Chen et al., 1997), suggesting that a direct interaction between these two genes could be a conserved mechanism regulating neurogenesis in vertebrates.

**Hes1 has also characteristics of a neurogenic gene**

Several observations indicate that, after this early function as a prepattern gene, *Hes1* might be transcribed in response to Notch signalling and be involved in repression of neurogenesis in a process of lateral inhibition. Activation of Notch1 has been shown to induce *Hes1* expression in a variety of cell lines and in retinal explants (Jarriault et al., 1995, 1998; Ohtsuka et al., 1999). This induction of *Hes1* expression requires the binding of the mouse homolog of suppressor of Hairless, RBP-J to a consensus site in the *Hes1* promoter (Jarriault et al., 1995, Fig. 4).
1998) and thus appears to involve the same mechanism as for transcription of E(spl) genes in Drosophila and Xenopus (Artavanis-Tsakonas et al., 1995; Wettstein et al., 1997). In the OE at E12.5, we have shown that Hes1 is expressed in apical cells and that this expression is lost in Mash1 mutants. These results suggest that Hes1 participates in a regulatory loop between Mash1 and a Notch-mediated lateral signalling pathway in apical cells of the OE, similar to the loop which is involved in the selection of bristle precursors in the Drosophila PNS (Heitzler et al., 1996).

The analysis of the Hes1 mutant phenotype also supports the notion that Hes1 participates in a process of lateral signalling in the OE. Loss of Hes1 results in an increase in Mash1 transcripts levels in areas of the placode that normally express the gene, in addition to an expansion of these areas mentioned earlier. Moreover, we have observed with an antibody that MASH1-positive precursors are found grouped in clusters of adjacent cells in Hes1 mutant placodes, whereas they remain scattered in wild-type placodes, suggesting that Hes1 is required to prevent adjacent placodal cells from expressing MASH1. Finally, the density of neurons in the placode is greatly increased in embryos double mutant for Hes1 and Hes5. These alterations in proneural gene expression and in distribution of neurons appear similar to the phenotypes observed in the nervous system of Drosophila or vertebrate embryos in which Notch signalling has been disrupted (Chitnis et al., 1995; Henrique et al., 1997; Simpson, 1997).

Our results therefore support the idea that Hes1 regulates neurogenesis in the OE in two clearly distinct contexts. Apparently conflicting results have previously been reported on whether Hes1 expression and function depend on Notch signalling. Hes1 has been shown to be transcribed in response to Notch activation and Hes1 activity to be important for Notch signalling in embryonic neural tissues (Ohtsuka et al., 1999). In contrast, Hes1 transcript levels are not detectably affected in the neural tube and other tissues of embryos mutant for RBP-J or Notch1, suggesting on the contrary that the transcription of Hes1 is largely independent of Notch signalling (de la Pompa et al., 1997). Therefore Hes1 may also have both Notch-dependent and Notch-independent activities in embryonic tissues other than the OE and particularly in the neural tube.

**Hes5 is a neurogenic gene**

In contrast to the complexity of Hes1 expression patterns and functions, our results and published data provide strong evidence that Hes5 is transcribed in the embryonic nervous system solely in response to Notch signalling and that Hes5 functions only in lateral inhibition. Firstly, as for Hes1, the Hes5 gene contains consensus binding sites for RBP-J, and Notch signalling activates the Hes5 promoter in cell lines, and induces endogenous Hes5 expression in retinal explants (Kageyama and Nakaniishi, 1997; Ohtsuka et al., 1999). Secondly, Hes5 transcripts normally present in the brain and spinal cord are almost completely eliminated in RBP-J mutant embryos and severely reduced in Notch1 mutant embryos, supporting the view that Hes5 transcription requires Notch signalling (de la Pompa et al., 1997). Similarly, Hes5 expression in olfactory placodes is greatly reduced in Mash1 mutant embryos and is thus likely controlled by Notch activity in this tissue as well.

Although Hes5 mutant animals do not show any gross abnormalities, the sharp increase in the frequency of neurulation defects in double Hes1:Hes5 mutant embryos, and the neurogenic phenotype of their olfactory placodes (increased size and number of Ngn1-positive cell clusters and increased neuronal density), argue that Hes5 is a negative regulator of neurogenesis in the mouse embryo, and that Hes1 and Hes5 can to some extent functionally compensate for the loss of one another. This conclusion is strongly supported by results of experiments in which forced expression of an activated form of Notch1 was able to accelerate the differentiation of neuroepithelial cells from wild-type embryos as well as Hes1 and Hes5 single mutants, but not from Hes1:Hes5 double mutant embryos (Ohtsuka et al., 1999). Therefore, either Hes1 or Hes5 appear to be required in the neural tube of mouse embryos to mediate Notch signalling. However, the neurogenic phenotype that we observe in the OE of double Hes mutants appears to be only partial, since the majority of MASH1-positive cells remain isolated and only a subset of them are grouped in clusters, suggesting that other genes mediating lateral inhibition must exist in this tissue.

**Regulation of the OSN lineage by Notch signalling at several steps**

Distinct stages of OSN progenitor maturation have been defined by the sequential expression of the three neural bHLH genes Mash1, Ngn1 and NeuroD (Cau et al., 1997). Mash1 is involved in the generation of basal OSN progenitors (Cau et al., 1997 and our unpublished data), whereas Ngn1 is required for their differentiation (E. C., Q. Ma, D. J. Anderson and F. G., unpublished data), and the function of NeuroD in this lineage has not yet been characterized. Analysis of the Hes1 mutant phenotype demonstrates that Hes1 regulates OSN development at the level of Mash1 expression. Despite the enlargement of the Mash1-positive cell population in Hes1 mutant olfactory placodes, there is only a relatively small increase in expression of Ngn1, suggesting that the step of Ngn1 expression is also subject to negative regulation. Indeed, the phenotype of Hes1:Hes5 double mutant placodes shows a further increase in number of Ngn1-positive cells and of SCG10-positive neurons without change in expression of Mash1, indicating that Hes5 is likely to regulate OSN development at the level of Ngn1 expression. Altogether, these data suggest that Notch signalling, acting through different Hes genes, regulates the production of OSNs by targeting the expression of two bHLH genes which control the development of the OSN lineage at two levels, proneural gene transcription and downstream step, is not unprecedented. It has been proposed that Drosophila E(Spl) proteins antagonize proneural genes not only by binding to their promoter and repressing their transcription, but also by dimerizing with proneural proteins, and also possibly by competing with them for binding to the promoters of common target genes (Nakao and Campos-Ortega, 1996; Jennings et al., 1999). Similarly, HES1 represses Mash1 transcription but also interferes with MASH1 activity.

The observation that Notch signalling regulates neurogenesis at several levels, proneural gene transcription and a downstream step, is not unprecedented. It has been proposed that Drosophila E(Spl) proteins antagonize proneural genes not only by binding to their promoter and repressing their transcription, but also by dimerizing with proneural proteins, and also possibly by competing with them for binding to the promoters of common target genes (Nakao and Campos-Ortega, 1996; Jennings et al., 1999). Similarly, HES1 represses Mash1 transcription but also interferes with MASH1 activity.
Double mutant placodes also suggests that Ngn1/Mash1/Hes1 between two populations of progenitors are not yet segregated. The synergy of these genes interact, because it focused on placodes at E10.5, when the stage in the OSN lineage at which positive and negative regulators act and their expression pattern in the apical-basal axis of the OE. The early activity of Mash1 in OSN precursor determination (Cau et al., 1997) and of Hes1 in Mash1 regulation (this paper) correlate with the apical expression of these two genes, while the later function of Ngn1 in OSN differentiation (our unpublished data) and of Hes5 in Ngn1 regulation (this paper) correlate with the basal expression of these two genes. This suggests a model whereby Mash1-positive apical cells would represent an early progenitor stage in the OSN lineage, regulated by Hes1. These cells would give rise to more mature progenitors which migrate basally, express Ngn1, and are regulated by Hes5. To test this model will require studying the lineal relationships between apical and basal precursors, and between apical precursors and OSNs. Also, our analysis of the Hes1 mutant and Hes5 mutant phenotypes has not addressed whether these genes were acting in apical cells and basal cells, respectively, because it focused on placodes at E10.5, when the two populations of progenitors are not yet segregated. The synergy between Hes1 and Hes5 in Ngn1 regulation (Fig. 4; Table 3) could be due to Hes5 directly repressing Ngn1, and Hes1 repressing the positive regulator Mash1. Alternatively, Hes1 could also act directly on Ngn1 regulation (shown in the diagram by a dashed line). The comparison between the modest increase in Ngn1 expression and the strong increase in number of SCG10-positive neurons in Hes1/Hes5 double mutant placodes also suggests that Hes5 could regulate a step in OSN differentiation downstream of Ngn1 expression (dashed line).

in hippocampal neuron cultures, possibly by titration of a dimerization partner of MASH1 (Castella et al., 1999). The exact mechanisms by which Hes1 and Hes5 regulate the transcription of Mash1 and Ngn1 and the activity of their products in the OSN lineage remain to be examined.

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Fig. 5. A model of the interactions between the negative regulators Hes1 and Hes5 and the positive regulators Mash1 and Ngn1 during OSN lineage development. The arrows represent regulations at the transcriptional level. Mash1 controls the expression of both Hes1 and Hes5 by a mechanism which, by analogy with other systems where achaete-scute and enhancer of split genes interact, is likely to involve the Notch pathway. The arrow with a question mark refers to the earlier, Mash1-independent, regulation of Hes1. There is an interesting correlation between the stage in the OSN lineage at which positive and negative regulators act and their expression pattern in the apical-basal axis of the OE. The early activity of Mash1 in OSN precursor determination (Cau et al., 1997) and of Hes1 in Mash1 regulation (this paper) correlate with the apical expression of these two genes, while the later function of Ngn1 in OSN differentiation (our unpublished data) and of Hes5 in Ngn1 regulation (this paper) correlate with the basal expression of these two genes. This suggests a model whereby Mash1-positive apical cells would represent an early progenitor stage in the OSN lineage, regulated by Hes1. These cells would give rise to more mature progenitors which migrate basally, express Ngn1, and are regulated by Hes5. To test this model will require studying the lineal relationships between apical and basal precursors, and between apical precursors and OSNs. Also, our analysis of the Hes1 mutant and Hes5 mutant phenotypes has not addressed whether these genes were acting in apical cells and basal cells, respectively, because it focused on placodes at E10.5, when the two populations of progenitors are not yet segregated. The synergy between Hes1 and Hes5 in Ngn1 regulation (Fig. 4; Table 3) could be due to Hes5 directly repressing Ngn1, and Hes1 repressing the positive regulator Mash1. Alternatively, Hes1 could also act directly on Ngn1 regulation (shown in the diagram by a dashed line). The comparison between the modest increase in Ngn1 expression and the strong increase in number of SCG10-positive neurons in Hes1/Hes5 double mutant placodes also suggests that Hes5 could regulate a step in OSN differentiation downstream of Ngn1 expression (dashed line).


