

# The Characterization of the Olf-1/EBF-Like HLH Transcription Factor Family: Implications in Olfactory Gene Regulation and Neuronal Development

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The Olf-1/EBF helix-loop-helix (HLH) transcription factor has been implicated in olfactory gene regulation and in B-cell development. Using homology screening methods, we identified two additional Olf-1/EBF-like cDNAs from a mouse embryonic cDNA library. The Olf-1/EBF-like (O/E) proteins O/E-1, O/E-2, and O/E-3 define a family of transcription factors that share structural similarities and biochemical activities. Although these O/E genes are expressed within olfactory epithelium in an identical pattern, they exhibit different patterns of expression in the developing nervous system. Although O/E-1 mRNA is present in several tissues in addition to olfactory neurons and

developing B-cells, O/E-2 and O/E-3 are expressed at high levels only in olfactory tissue. In O/E-1 knock-out animals, the presence of two additional O/E family members in olfactory neurons may provide redundancy and allow normal olfactory neurodevelopment. Further, the identification of the O/E family of HLH transcription factors and their embryonic expression patterns suggest that the O/E proteins may have a more general function in neuronal development.

**Key words:** Olf-1/EBF; O/E; olfactory gene regulation; neurodevelopment; sensory neurons; differentiation and maturation; transcription factors

Intrinsic and extrinsic cues contribute to the differentiation and maturation of cells in the mammalian nervous system (Calof, 1995). Considerable evidence suggests that the genetic programs necessary for neuronal development are governed by the spatial and temporal patterns of transcription factor expression. For example, *En-1* and *Pax-6* play direct roles in midhindbrain and eye development, respectively (Wurst et al., 1994; Grindley et al., 1995). The vertebrate basic helix-loop-helix (bHLH) proteins MASH, NeuroD, and Neurogenin also play a role in neural development and can induce neurogenesis when ectopically expressed in *Xenopus* embryos (Chitnis and Kintner, 1996; Ma et al., 1996). Additional factors are likely to play subsequent roles in directing the terminal differentiation of the neuronal phenotype.

Olfactory receptor neurons (ORNs) undergo continuous replacement throughout the lifespan of the animal and thus serve as a useful model for neuronal differentiation and maturation. During ORN replacement new neurons arise from neuroblast-like cells that migrate apically through the epithelium (Mackay-Sim and Kittel, 1991). The components of the odorant transduction pathway and other markers of mature ORNs are expressed as these cells achieve the differentiated state.

Analysis of the promoter regions of the odorant transduction pathway components and other mature ORN markers revealed a consensus sequence, the Olf-1 site, that bound a factor present in olfactory nuclear extracts (Kudrycki et al., 1993; Wang et al.,

1993). Transgenic studies suggested that this site is important in directing olfactory-specific olfactory marker protein (OMP) expression *in vivo* (Walters et al., 1996). The factor that bound to this site, the Olf-1 protein, was identified by using a yeast genetic selection scheme, and its expression in the neuronal lineage within olfactory epithelium was confirmed by immunocytochemistry (Wang and Reed, 1993). Olf-1 encodes a novel form of HLH domain without the characteristic basic residues and can function as a homodimer specifically to bind the Olf-1 site and activate transcription. Olf-1 was identified independently and cloned as early B-cell factor (EBF) that regulates the *mb-1* gene (Hagman et al., 1993). Mice with an Olf-1/EBF null mutation display a profound B-cell deficit, but the olfactory epithelium is morphologically normal and expresses OMP and  $G_{olf}$  (Lin and Grosschedl, 1995). The presence of additional Olf-1/EBF-like proteins, analogous to the functional redundancy suggested for *Engrailed* and *MyoD* family members (Hanks et al., 1995; Wang et al., 1996), was hypothesized to account for the absence of an olfactory phenotype in the knock-out animals.

Recent studies of mammalian Olf-1/EBF expression during mouse embryogenesis revealed immunoreactivity in postmitotic cells of the developing central and peripheral nervous systems of the mouse (Davis and Reed, 1996). Additionally, a *Drosophila* gene, *collier*, encoding an Olf-1/EBF-like protein has been implicated in development. Within the developing nervous system *collier* is expressed in a segmentally reiterated pattern in the ventral nerve cord, in cells of the peripheral nervous system, and in patches of brain cells (Crozatier et al., 1996). These studies suggest that, besides regulating the differentiation of olfactory neurons and B-cells, Olf-1/EBF performs a more general function in neuronal development.

Here we report the identification of two new Olf-1/EBF-like transcription factors, named O/E-2 and O/E-3 (Olf-1/EBF-like), from a mouse embryonic day (E) 12.5 cDNA library. The bio-

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chemical characterization of these proteins revealed that O/E-1, O/E-2, and O/E-3 possess similar DNA binding and transcriptional activation properties. High expression of O/E-2 and O/E-3 is restricted to the olfactory epithelium among the 11 tissues examined in adult mice. Although the expression patterns of the three O/E genes are identical in adult olfactory epithelium by *in situ* hybridization, they are expressed differentially in the neuronal tissues of developing mouse embryo. These results suggest that the O/E proteins together regulate olfactory gene expression and that they have a more general function during neuronal differentiation and maturation.

## MATERIALS AND METHODS

**Cloning methods.** Degenerate RT-PCR was performed with two degenerate primers (5'-GGCCGATCCTTYYTNAARTTYTCT-3' and 5'-GGCCGAATTCGTNCCYTRCARAAAYTGYTT-3') containing *Bam*HI and *Eco*RI restriction sites, respectively. These primers corresponded to aa 186–aa 192 (FFLKFFL, sense) and aa 333–aa 339 (KQFCKGT, antisense), two highly conserved regions between O/E-1 and *collier* (Crozatier et al., 1996). PCR was conducted for 35 cycles at 50°C annealing temperature by using 1 μM of each primer and 1% of reverse-transcribed product from 2 μg of total E14.5 RNA. The PCR products were digested with *Bam*HI and *Eco*RI and subcloned into pBluescript KS (Stratagene, La Jolla, CA) for sequencing.

Degenerate PCR was performed with an oligo-dT-primed λZAP II E12.5 mouse cDNA library (Dr. Se-Jin Lee, Johns Hopkins University) as described above, and the PCR product was random prime-labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP and used to screen the library. The first round of screening gave full coding sequences of O/E-1 and O/E-3 and a partial sequence of O/E-2. An E14.5 mouse brain cDNA library (Dr. Paul Worley, Johns Hopkins University) was screened with the cloned O/E-2 RT-PCR product. Two independent clones were obtained, but neither contained the full coding region of O/E-2. Therefore, 5' rapid amplification of cDNA ends (RACE) was performed with a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's instruction. The first-strand cDNA was synthesized with SW37 oligonucleotide (5'-TTGCTCTGTCTGACGCCATTG-3'). The PCR reaction was performed with SW37 and AP1, and the PCR product was digested with *Pst*I/*Not*I and subcloned into pBluescript KSII. This RACE experiment yielded a 320 bp sequence corresponding to O/E-2, but it did not contain the initiating methionine as predicted by sequence comparison with O/E-1 and O/E-3. The E12.5 mouse cDNA library therefore was re-screened with the RACE product. One clone was identified, and it contained the entire coding sequence predicted by comparison with O/E-1 and O/E-3 sequences.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assay (EMSA) was performed as described (Wang et al., 1993), with the following modifications. Protein extract was mixed with 0.05 ng of probe in 20 μl of binding reaction and incubated on ice for 20–30 min. For competition experiments, labeled probe and unlabeled competitor were mixed before the addition of protein extracts. The oligonucleotides used for labeling and for competition have been described (Wang et al., 1993).

Protein extracts were made by transiently transfecting HEK293 cells with mammalian expression vectors (pCIS) directing expression from a CMV promoter (Wang and Reed, 1993) and preparing whole-cell extracts with cell culture lysis reagent (Promega, Madison, WI). The pCIS-O/E-1(0), pCIS-O/E-1(8), and pCIS-O/E-3 plasmids contained the full-length O/E-1 and O/E-3 cDNAs. The pCIS-O/E-2(9L) and pCIS-O/E-2(0S) plasmids were cloned as *Not*I/*Hind*III fragments. The C-terminal-truncated O/E proteins were generated by subcloning fragments (*Eco*RI/*Ava*II for O/E-1, *Not*I/*Xmn*I for O/E-2, and *Eco*RI/*Bsa*BI for O/E-3) into a pBluescript KSII plasmid derivative in which stop codons in all three frames had been placed between the *Hind*III and *Xho*I sites. Then the O/E cDNAs were excised along with the stop codons and cloned into the pCIS vector.

**Luciferase activity assay.** The pGL-AC3R/B vector contained a 1.55 kb *Eco*RI/*Bam*HI DNA fragment of type III adenyl cyclase (ACIII) promoter, including 500 bp of 5'-UTR and an O/E binding site at position –270 cloned into the polylinker region of pGL2 basic vector (pGL-B, Promega). The pGL-OMP vector was composed of a 2.7 kb DNA fragment of OMP containing 59 bp of 5'-UTR and two O/E binding sites (–180 and –700) cloned into the *Xho*I site of the pGL-B vector (R. Tsai and R. Reed, 1997).

The vector containing 10 concatamerized O/E binding sites, pGL/EX10, was constructed by the following procedures. Two synthetic oligonucleotides, MW89 (5'-GATCCTCTCAGGATCCCCAGGGAGGGGACA-3') and MW90 (5'-GATCTGTCCCCTCCCCTGGGGAATCTGAGAG-3'), containing the distal O/E binding sequence in the 50.06 promoter region (Wang et al., 1993) flanked by *Bam*HI and *Bgl*II sites were annealed in buffer (100 mM KCl, 10 mM Tris, pH 8.0, and 1 mM EDTA) at 100°C for 5 min, at 68°C for 1 hr, and followed by 1 hr at room temperature. Annealed fragments were ligated, digested with *Bam*HI and *Bgl*II to remove head-to-head and tail-to-tail ligation events, and fractionated on a 4% low-melting-point gel. DNA fragments of 310 bp, corresponding to 10 concatamerized O/E binding sites, were isolated, subcloned into the *Bam*HI site of pBluescript KSII, and confirmed by sequence analysis. A *Sac*I/*Bam*HI fragment containing 10 O/E binding sites was isolated and subcloned into the *Sac*I/*Bgl*II sites in pGL-2 promoter vector (pGL-P).

For expression experiments one 60 mm plate of HEK293 cells was transiently transfected with 1 μg of the indicated pGL-based reporter plasmid along with the indicated amount of pCIS-O/E plasmid. All transfections were adjusted to 5 μg of total DNA with pCIS vector DNA. The luciferase reporter activity was measured from an equivalent amount of protein lysate of each sample with a luciferase assay system (Promega) and Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The relative luciferase activity was calculated by the reference indicated in each experiment.

**RNAse protection assay.** RNAse protection assay (RPA) was performed with the RPA II kit (Ambion, Austin, TX) according to the manufacturer's instruction. Total RNA (25 μg), isolated from 11 mouse tissues with RNazol B (Tel-Test, Friendswood, TX), was hybridized with individual riboprobes (1 × 10<sup>6</sup> CPM) for 16 hr at 45°C. Digestion was performed with 0.5 U/ml of RNase A and 20 U/ml of RNase T1 at 37°C for 30 min. After inactivation of the RNases, samples were ethanol-precipitated, size-fractionated on 6% denaturing PAGE in 1× TBE buffer, and subjected to autoradiography. The probes were generated by subcloning fragments (*Apo*I/*Hind*III for O/E-1, *Bsa*AI/*Hind*III for O/E-2, and *Bsp*HI/*Xmn*I for O/E-3 corresponding to 275, 283, and 241 bp of 3'-untranslated region, respectively) into pBluescript KSII vector, digesting with *Not*I, and using the linearized fragments as templates for *in vitro* transcription reaction with T3 RNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]-UTP.

**In situ hybridization.** Olfactory tissue was fixed in Bouin's solution as described (Davis and Reed, 1996). Fresh-frozen mouse embryos and 4% paraformaldehyde-fixed adult mouse brains were used as indicated in the text. The RNA *in situ* hybridization was performed as described (Tsuchida et al., 1994), with the following modifications. Prehybridization treatment of the 20 μm tissue sections was performed without proteinase K treatment. Hybridization was performed with 0.5 μg/ml of digoxigenin-labeled riboprobes at 65–70°C overnight. Posthybridization wash was performed twice in 0.2× SSC at 70°C with 20 μg/ml intervening RNase A treatment, and antibody incubation was performed in the presence of 5% heat-inactivated normal goat serum.

Digoxigenin-labeled riboprobes for *in situ* hybridization (synthesized with DIG RNA Labeling Mix from Boehringer Mannheim, Indianapolis, IN) contained the divergent sequences from C-terminal coding regions and the 3'-untranslated regions of the O/E cDNAs. Southern hybridization analysis demonstrated that a high-stringency wash condition (0.2× SSC, 65°C) eliminated cross-hybridization among the three O/E genes even when the probes were made to the most conserved regions (data not shown). The O/E-1 (*Hind*III/*Ava*II), O/E-2 (*Xmn*I/*Hind*III), and the O/E-3 (*Pst*I/*Bsp*HI) probes were subcloned first into pBluescript KSII (Stratagene), and antisense and sense (control) probes were synthesized with T3 or T7 RNA polymerase (Stratagene), respectively.

## RESULTS

### Cloning of mammalian Olf-1/EBF-like proteins

The presence of the Olf-1 cis-acting element in proximity to six genes preferentially expressed in olfactory epithelium suggests a role for Olf-1/EBF (Kudrycki et al., 1993; Wang et al., 1993), and transgenic studies with the olfactory marker protein (OMP) promoter provide additional support for Olf-1/EBF as an important regulator of olfactory gene expression (Walters et al., 1996). However, the absence of phenotypic changes in the olfactory epithelium of Olf-1/EBF null mutant mice (Lin and Grosschedl, 1995) suggests that additional Olf-1 site binding proteins may

exist. The high degree of conservation between mammalian Olf-1/EBF and *Drosophila collier* (Crozatier et al., 1996) allowed us to design approaches for the identification of additional members in mouse. We used RT-PCR with degenerate oligonucleotides to identify Olf-1/EBF-like genes expressed in mouse E14.5 RNA. The PCR products of ~450 bp were analyzed, and three distinct sequences were identified corresponding to mouse Olf-1/EBF (renamed O/E-1) and two novel sequences, O/E-2 and O/E-3. Independently, a mixed probe derived from PCR on an E12.5 whole-embryo cDNA library was labeled and used to screen the same library at low stringency. We isolated cDNA clones predicted to encode full-length O/E-1 and O/E-3 proteins and a partial clone corresponding to a truncated O/E-2 protein. A full-length O/E-2 clone was isolated by a combination of 5' RACE and additional cDNA library screens.

The sequences of the O/E cDNAs revealed a family of highly conserved proteins (Fig. 1). The O/E-2 and O/E-3 cDNAs predicted proteins of 596 and 575 amino acids, respectively. Analysis of the predicted O/E proteins revealed >75% overall identity and >90% identity over a stretch of 370 residues suggested to be essential for dimerization and DNA binding (Hagman et al., 1995). The HLH dimerization motif of these proteins is located at the carboxyl end of this highly conserved region, and each displays a high degree of identity between helix 1 and helix 2 that was noted previously in O/E-1 and described as the repeat helix-loop-helix (rHLH) (Wang and Reed, 1993). Each of the O/E proteins has a C-terminal domain that is rich in serine (>20%) and proline (>10%). These domains display less amino acid identity (~50%) among the members of the O/E family. Previous studies have shown that this region of O/E-1 is sufficient to activate transcription when it is fused to a heterologous DNA binding motif (Hagman et al., 1995).

The Olf-1 and EBF sequences differ by an eight-amino acid insertion apparently arising by alternative splicing of an optionally included exon. Sequences corresponding to this exon are present in O/E-2 cDNAs, but with one additional amino acid in the exon (Fig. 1). RT-PCR results indicate that both splicing forms of the O/E-1 and O/E-2 mRNA are present in the olfactory tissue (data not shown). No O/E-3 cDNAs containing the additional exon were found in the initial RT-PCR reactions from library screening or when O/E-3 specific oligonucleotides across this region were used for RT-PCR analysis (data not shown). The O/E-1 cDNAs with and without the eight-amino acid insert are designated as O/E-1(8) and O/E-1(0), respectively. A second alternatively spliced variant was found in the O/E-2 cDNA at a site 40 amino acids from the C terminus. Two of the four independent O/E-2 cDNA clones have a 36-amino acid truncation at this site and lack the nine-amino acid insertion at the first alternative splice site; thus they are designated as the 0S forms (0 amino acid at the first site, short form/with the 36 amino acids truncation). The cDNAs that have the inserts at both sites are designated as the 9L forms (9-amino acid insertion, long form). O/E-1(0) and O/E-2(9L) were used for the biochemical analysis of O/E proteins unless indicated otherwise.

### All O/E proteins bind the same DNA sequences *in vitro* as dimers

The specific recognition of DNA sequence by the O/E proteins was investigated by expressing each of the cDNAs in HEK293 cells, preparing extracts, and performing an electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotide containing the olfactory cyclic nucleotide-gated channel (OcNC) O/E

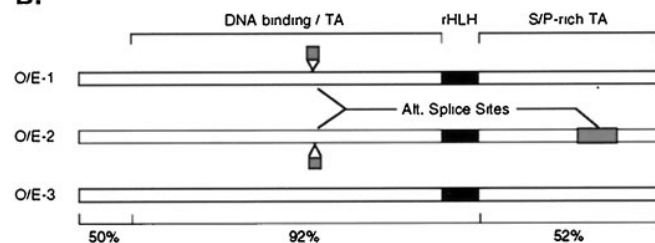
### A.

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1  MFGIQESDQRSQSSMKEEPLGSGMNAVRITWQAGVLDANTAAGSGVGLARAHFEKQPPS O/E-1
1  MFGIQESDPRGGTTMKEEPLGSGMNPVRSWHTAGVVDANTAAGSGVGLARAHFEKQPPS O/E-2
1  MFGIQDTLGRG-PALNDKSLGAEMDSRSVSRVNVGVDDANAAQSGVALSRAHFEKQPPS O/E-3
61  NLRKSNFFHFVFLALYDRQGQPVVEIERTAFVGFVEKEKEANSKRTNNGIHYRLQLLYSNGI O/E-1
61  NLRKSNFFHFVFLALYDRQGQPVVEIERTAFVDFVVEKEKEPNNEKNTNNGIHYKQLLYSNGV O/E-2
60  NLRKSNFFHFVFLALYDRQGQPVVEIERTAFVDFVNDKEQQNGEKNKNGTHYKQLLYSNGV O/E-3
121  RTEQDLVYRLIDSMTKQAIYVEGQDKNPEMCRVLLTHEIMCSRCCDKKSCGNRNETPSPD O/E-1
121  RTEQDLVYRLIDSMTKQAIYVEGQDKNPEMCRVLLTHEIMCSRCCDKKSCGNRNETPSPD O/E-2
120  RTEQDLVYRLIDSVTKQPIAYVEGQDKNPEMCRVLLTHEVMCSRCCEKKSCGNRNETPSPD O/E-3
181  VIIDRFLLKFFLLKCNQCLKNAGNPRDMRRFQVVVSTTVNVGDHVLAVSDNMFVHNSKH O/E-1
181  VIIDRFLLKFFLLKCNQCLKNAGNPRDMRRFQVVVSTTVNVGDHVLAVSDNMFVHNSKH O/E-2
180  VIIDRFLLKFFLLKCNQCLKNAGNPRDMRRFQVVLESTTVNVGDHVLAVSDNMFVHNSKH O/E-3
241  GRRARRLDPSGETSPSYLAHAAATPCIKAIISPSEGWTTGGATVIIIGDNFDFGLQVIFGTM O/E-1
241  GRRARRLDPSGETSPSYLAAVATPCIKAIISPSEGWTTGGATVIIIGDNFDFGLQVVFSTGM O/E-2
240  GRRARRLDPSGE-----ATPCIKAIISPSEGWTTGGATVIIIGDNFDFGLQVVFSTGM O/E-3
300  LVNSELITPHAIRVQTPPRHIPGVVEVTLSYKSKQFCCKGTFGRFIYALNEPTIDYGFQR O/E-1
300  LVNSELITPHAIRVQTPPRHIPGVVEVTLSYKSKQFCGAPGRFIYALNEPTIDYGFQR O/E-2
290  LVNSELITPHAIRVQTPPRHIPGVVEVTLSYKSKQFCCKGAPGRFIYALNEPTIDYGFQR O/E-3
360  LQKVIPRHPGPERLPKEVLLKRAADLVEALYGMPHNQEILKRAADIAEALYSVPRNH O/E-1
360  LQKVIPRHPGPERLPKEVLLKRAADLVEALYGMPHNQEILKRAADIAEALYSVPRNH O/E-2
350  LQKVIPRHPGPERLAKEMLLKRAADLVEALYGTPHNQQEILKRAADIAEALYSVPRNP O/E-3
420  NQLFALANTSVHAGMMGVNSFSGLAVNVSEASQATNG-QFTRNSSSVSHGYVPSTTPQ O/E-1
420  NQIPDLGNTPAHTGMMGVNSFSGLAVNVSETSQANDRVGRNTSSVSPRGYVPSTTPQ O/E-2
410  SDIPALSSPAHSGMMGTNSYGSGLQMSTSTSGTGNG-GYTRNTSSTSPRGSVPSTTPQ O/E-3
479  QTNYSMTISSMNGYGSAANSNLQ--GSPTFLNGSANSPYATVPSSPTMASST-SLPNSQ O/E-1
480  QSNYNTVSTSMNGYGSGAMANLGVPGSPGLNGSSANSPYGVPSSPTAASVTVPSNSQ O/E-2
469  QSNYSTISSMNGYSNVPMANLGVPGSPGLNGSPTGSPYGSIMSPTVGSSS- O/E-3
536  SSSSGFFSSPANVMSAVKQSSAFAPVVRPQTSPPTCTSNGSLQALEQMIVPPM O/E-1
540  SSTHTFFSSPANVMSAVKQSSAFAPVVRPQASPPSCTSNANGNGLQAMSGLVVPPM O/E-2
523  SS---MLPES-SSMFPAVKQSSAFAPMTRPQGSESHACSEGNGNGFRAMTGLVVPPM O/E-3

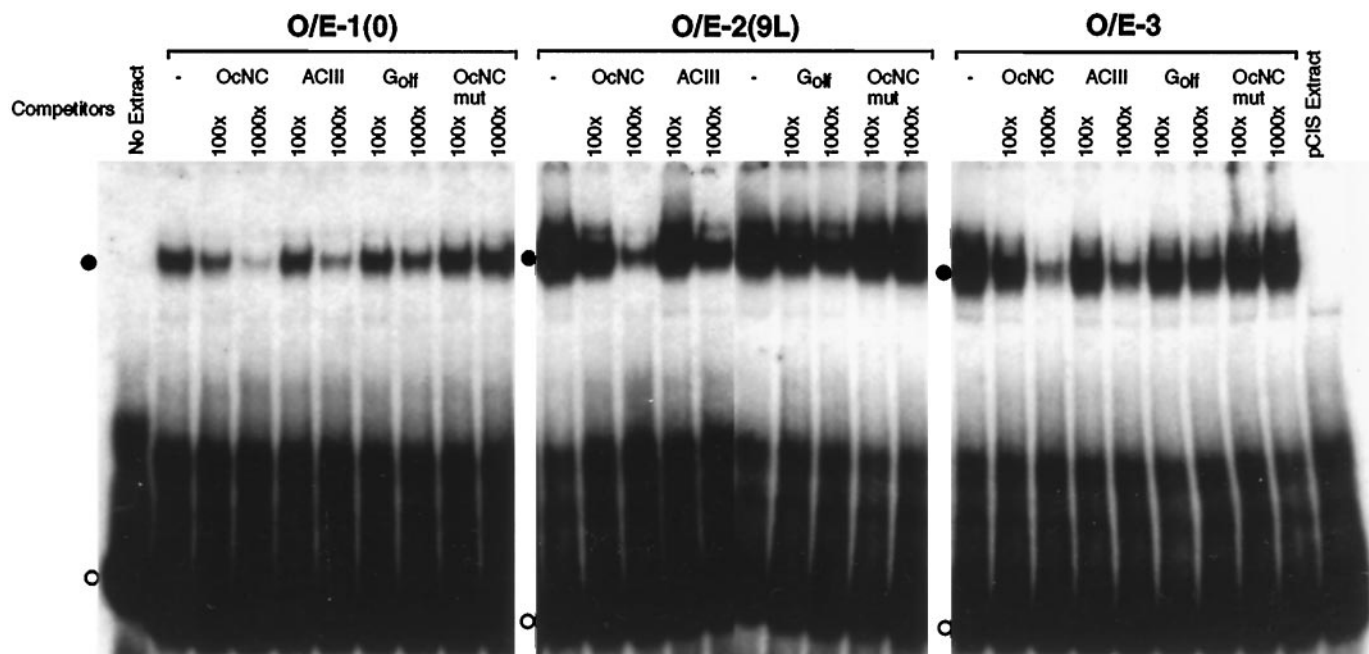
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### B.



**Figure 1.** Structure and sequences of O/E proteins. *A*, Protein sequence alignment of O/E-1(8), O/E-2(9L), and O/E-3. Sequences are shown with single-letter amino acid code, and amino acids corresponding to alternative exons are represented in *italics*. The O/E-1(0) sequence lacks the first alternative exon. The O/E-2(0S) sequence lacks both alternative exons and has a methionine in the place of the second alternative exon. Identical amino acids are indicated by boxed regions. The nucleotide sequences have been deposited into GenBank, and the accession numbers for O/E-2(9L), O/E-2(0S), and O/E-3 are U92703, U92704, and U92702, respectively. *B*, Schematic drawing of O/E-1, O/E-2, and O/E-3. The shaded boxes represent the alternative exons, and black boxes represent the rHLH dimerization domains. The DNA binding and the putative transactivation (TA) domains are as labeled. The O/E proteins share >75% overall identity, and the regional identities are given in the schematic drawing.

site was shifted by O/E-1-containing, O/E-2-containing, and O/E-3-containing extracts, but not by extracts of cells transfected with pCIS empty vector (Fig. 2). The specificity of the observed protein–DNA interactions was demonstrated by using an unlabeled oligonucleotide containing the OcNC O/E site to compete for complex formation. A similar oligonucleotide containing a mutation in the binding site failed to reduce the intensity of the specific band. The relative affinities of O/E-1(0), O/E-2(9L), and O/E-3 for different O/E sites were determined in a similar manner by using the O/E sites found in the promoters of OcNC, type III adenylyl cyclase (ACIII), and the olfactory-enriched G-protein  $\alpha$ -subunit ( $G_{olf}$ ) as competitors. The OcNC O/E site was most



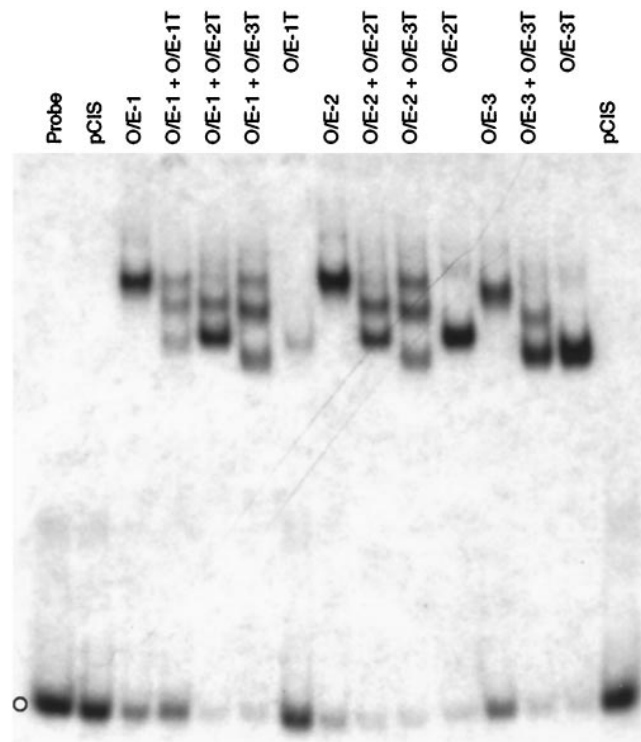
**Figure 2.** Electrophoretic mobility shift assay. The ability of the O/E proteins to bind the OcNC O/E site and their relative affinities for different O/E sites are shown. Unlabeled OcNC, ACIII, or G<sub>off</sub> O/E binding sites were added in molar excess, as indicated. The positions of O/E–DNA complexes are indicated by closed circles, and the positions of free probes are indicated by open circles. *mut* refers to the mutant competitor oligonucleotides.

effective in competing for binding, the ACIII O/E site was less effective, and the G<sub>off</sub> O/E site showed the weakest competition. O/E-1(8) and O/E-2(0S) also exhibited similar properties (data not shown). Although different O/E sites exhibited different efficacies of binding to the O/E proteins, the EMSA results indicate that each O/E protein displays a similar order of preference for these O/E sites.

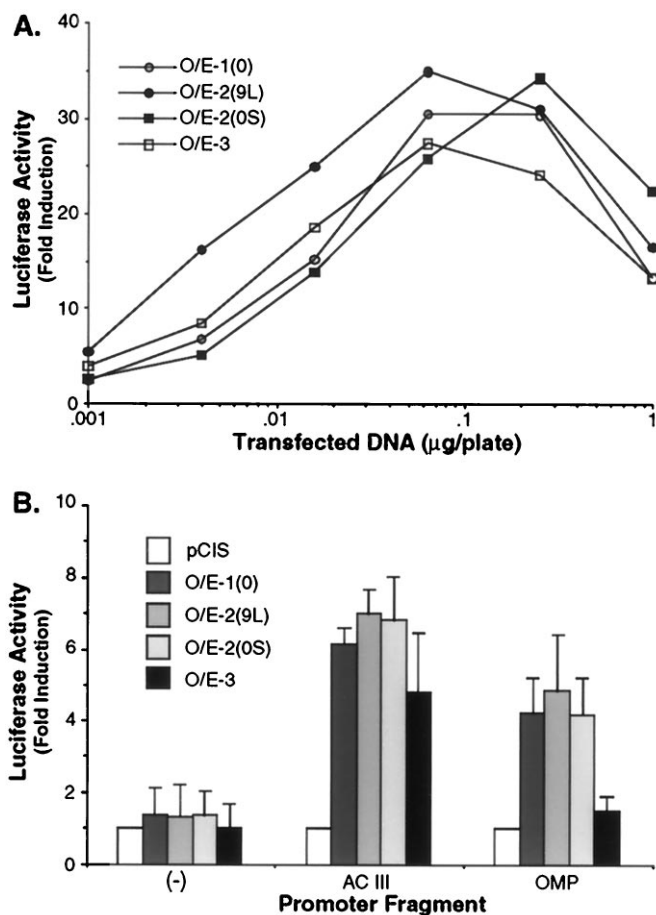
Previous studies demonstrated that O/E-1 forms a homodimer that binds to DNA and activates transcription (Hagman et al., 1993; Wang and Reed, 1993). The presence of nearly identical rHLH domains of the O/E proteins suggests that they might form heterodimers as well as homodimers. A C-terminal-truncated form of O/E-1 lacking the transactivation domain retained its ability to bind DNA and resulted in a complex with faster mobility than was observed with the full-length protein (Wang and Reed, 1993). The coexpression of full-length and truncated forms of O/E-1 resulted in the formation of an additional protein–DNA complex of intermediate mobility representing a dimeric protein complex containing a full-length and a truncated O/E-1 (Wang and Reed, 1993). On the basis of this observation, we constructed truncated forms of O/E-1(0), O/E-2(9L), and O/E-3; coexpressed them with the full-length proteins in HEK293 cells; and prepared extracts. Protein–DNA complexes of intermediate mobility in EMSA were observed for all combinations and confirmed that all O/E proteins are able to dimerize with themselves and with other members of the O/E family (Fig. 3). Taken together with the data from EMSA competition experiments, we suggest that the O/E proteins are very similar in their interaction with DNA and with members of the O/E family.

#### All O/E proteins can activate reporter gene expression *in vitro*

The ability of O/E-1 to activate transcription of a reporter gene and the high degree of conservation among the O/E family members imply that O/E-2 and O/E-3 exhibit a similar transac-



**Figure 3.** O/E dimer formation. Coexpression of full-length and truncated (T) forms of O/E-1(0), O/E-2(9L), or O/E-3 results in protein–DNA complexes of three distinct mobilities representing homodimers of full-length (slow) and truncated (fast) O/E and a heterodimer of both forms of O/E (intermediate mobility). The position of the free probe containing the OcNC O/E binding site is indicated by an open circle.



**Figure 4.** Luciferase assays. *A*, The ability of O/E proteins to activate transcription was demonstrated with a reporter construct containing a luciferase gene and 10 O/E sites adjacent to an SV40 minimal promoter. The data represent the average values from six to eight experiments. Baseline activity was determined with the extract of cells cotransfected with pCIS empty expression vector and the luciferase reporter construct. *B*, The ability of O/E proteins to activate transcription from native promoters was demonstrated with reporter constructs containing the *ACIII*, *OMP*, or no (–) promoter. Extracts from HEK293 cells cotransfected with 500 ng of the pCIS expression construct and the indicated reporter constructs were assayed for luciferase activity. The results represent the average of three experiments, and the error bars reflect the SD.

tivational capacity. However, the differences in their C-terminal transactivation domains might alter significantly their ability to activate transcription. To address this question, we cotransfected HEK293 cells with O/E expression vector and a reporter construct containing the luciferase gene driven by 10 O/E binding sites adjacent to an SV40 minimal promoter. An increase in luciferase activity was observed when each of the O/E expression constructs was introduced, and the luciferase activity reached a maximum of 25- to 35-fold induction when between 64 and 250 ng of O/E expression vector DNA was used per plate (Fig. 4*A*). These results demonstrate that all O/E proteins positively regulate transcription. When three O/E sites instead of 10 sites were present in the reporter vector, O/E expression constructs gave lower but otherwise similar results (data not shown).

The transactivation activity of O/E proteins on native promoters also was examined. The ability of O/E proteins to activate transcription of their target genes was investigated with reporter constructs consisting of a luciferase gene driven by 1.55 kb of the

*ACIII* promoter or 2.7 kb of the *OMP* promoter containing their native transcription start sites. The results demonstrate that O/E proteins are able to activate transcription of the luciferase gene driven by *ACIII* and *OMP* promoters (Fig. 4*B*) and support a functional role for the O/E proteins in the regulation of olfactory gene expression. Although the activation of both promoters with O/E-3 was consistently lower than that observed for the other O/E proteins, different amounts of O/E-3 expression vector might produce greater activation of reporter expression (Fig. 4*A*).

### Expression of the O/E genes in adult

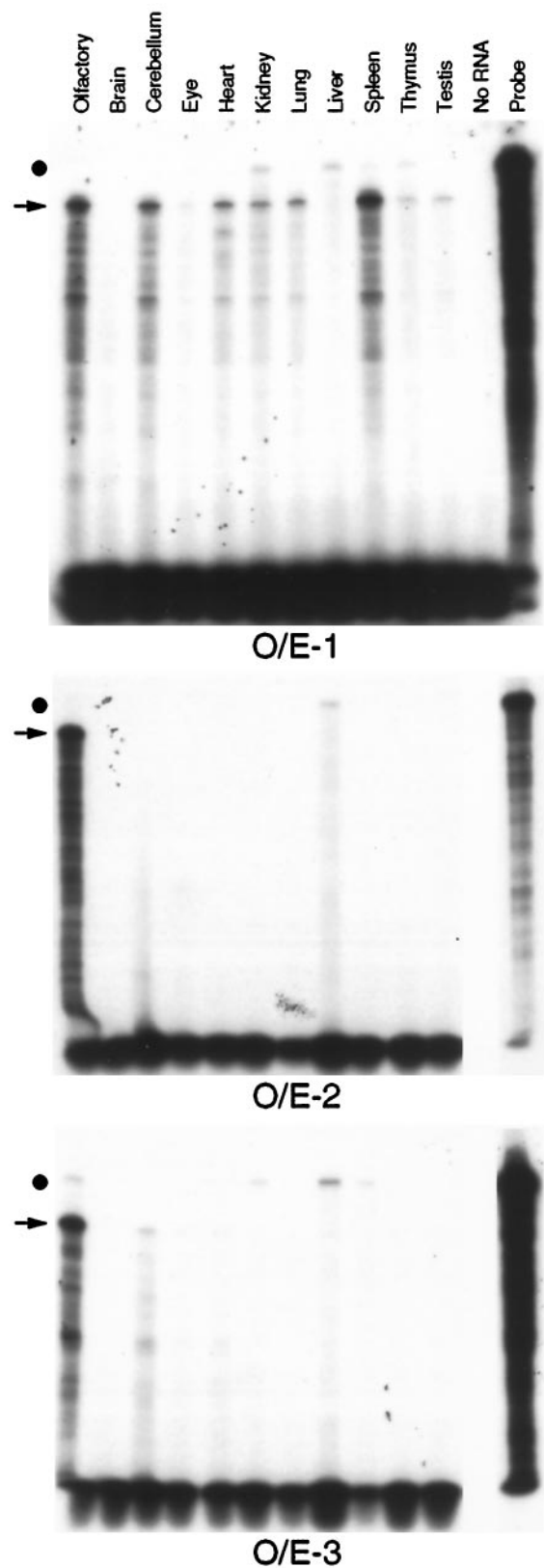
The distribution of O/E mRNAs in 11 adult tissues was determined by RNase protection assays (RPA) with probes corresponding to the 3'-untranslated regions (3'-UTR) of the O/E messages that share no homology (Fig. 5). Although O/E-1 is expressed at highest levels in olfactory epithelium and spleen as previously reported (Hagman et al., 1993; Wang and Reed, 1993), significant levels of expression were observed in several other tissues. In contrast, the O/E-2 and O/E-3 genes display highly restricted expression patterns and were readily detectable only in olfactory epithelium. The O/E-3 message was observed in one other tissue, cerebellum, where it is present at a much lower level than in olfactory tissue.

The cellular localization of the O/E messages was determined by RNA *in situ* hybridization with probes derived from 3'-coding regions and 3'-UTR that share no extensive homology. In olfactory epithelium the patterns of O/E-expressing cells are identical for O/E-1, O/E-2, and O/E-3, and each is restricted to the neuronal and basal layers (Fig. 6). The vast majority of cells within the neuronal and basal layers that hybridize with each of these probes suggests that individual cells are likely to express all three members of the O/E family. In particular, the olfactory neurons of O/E-1 null mutant mice are likely to express the other members of the O/E family. In the adult cerebellum O/E-1 and O/E-3 messages are present in the Purkinje cells (Fig. 6). In agreement with the RPA results, O/E-1 exhibits a higher level of expression than O/E-3.

### Expression of the O/E genes during development

The expression of O/E-1 in mouse embryos had been examined by using antiserum directed against a C-terminal peptide of that protein (Davis and Reed, 1996). This antiserum also reacts with O/E-2 and O/E-3 by Western blot and immunocytochemistry when those proteins were expressed in HEK293 cells (data not shown). To distinguish the expression of each O/E member, we used RNA *in situ* hybridization on E14 mouse embryos at a time when O/E immunoreactivity was present in the nervous system. No additional structures were detected by immunostaining in older embryos (Davis and Reed, 1996).

The pattern of O/E expression throughout the developing animal was assessed in sagittal sections of E14 embryos (Fig. 7). Hybridization signals were observed in diencephalon, mesencephalon, and rhombencephalon. Within the diencephalon O/E-1 message was observed in dorsal thalamus and epithalamus, O/E-2 was detected in epithalamus and hypothalamus, and a low level of O/E-3 was found in epithalamus. Within the mesencephalon and rhombencephalon, O/E-2 exhibits widespread expression whereas O/E-1 and O/E-3 are more restricted. The cerebellar primordium expresses similar levels of all three O/E genes. O/E-2 is expressed throughout the brainstem and enriched near the ventricular zones of mesencephalon and in rostral rhombencephalon. O/E-1 is expressed at lower levels in the mesencephalon and enriched near



**Figure 5.** Adult tissue distribution of O/E messages. The distribution of O/E messages in 11 adult tissues was determined by RNase protection assay. The arrows mark the size of protected probes, and the black dots mark the size of undigested probes. The quality of RNA was confirmed by agarose gel or by RPA with an actin probe (data not shown).

the ventricular zone. Stripes of cells within the caudal rhombencephalon also express a low level of O/E-1. O/E-3 expression was found only in cells near the ventricular zone of mesencephalon and on the ventral surface of rhombencephalon. In addition to signals observed in the developing brain, prominent hybridization signals were observed in the olfactory epithelium, spinal cord, and dorsal root ganglia. The O/E-expressing cells were highly concentrated in the nervous system, and the only non-neuronal O/E positive cells were found in the developing digits.

The transverse sections of E14 embryos revealed that the O/E expression was enriched in several structures that mediated sensory processes in the adult (Fig. 8). The O/E mRNAs were found in the developing olfactory epithelium and vomeronasal organ (VNO), and their levels of expression were similar, based on the *in situ* hybridization signals. In the developing retina a high level of O/E-2 and a lower level of O/E-1 were found in the postmitotic cells; O/E-3 was completely absent. Abundant expression of O/E-1 and O/E-2 and lower expression of O/E-3 were observed within the developing spinal cord in the superficial layer of the dorsal horn. Additional subpopulations of cells in the intermediate region between dorsal and ventral horns of the developing spinal cord express O/E-2 but not O/E-1 and O/E-3. The developing dorsal root ganglia (DRG), trigeminal (V) ganglia, and glossopharyngeal (IX) nerve ganglia have similar O/E expression; high levels of O/E-1, low levels of O/E-2, and no O/E-3 were detected in these structures. The developing inner ear expresses O/E-1, but not O/E-2 or O/E-3. The presence of hybridization signals along the rostrocaudal axis of the spinal cord (Fig. 7) suggests that the patterns of O/E expression observed in the transverse sections of E14 embryos at the cervical level continue through the rest of the spinal cord.

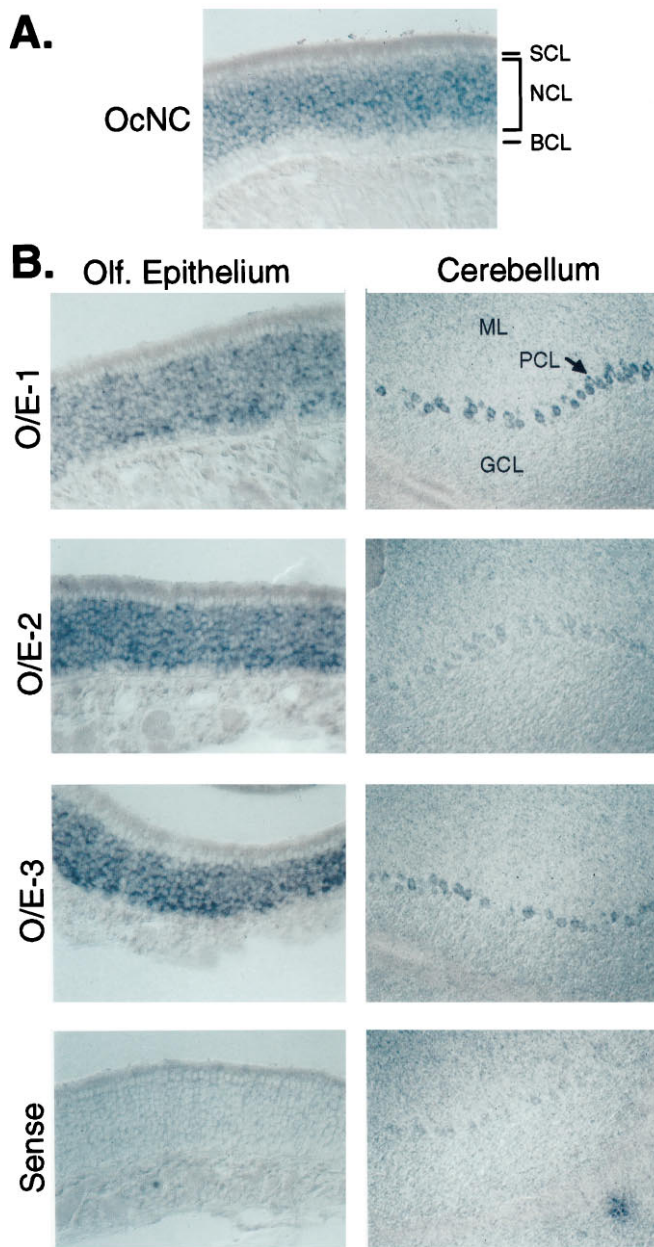
## DISCUSSION

### The O/E family of HLH transcription factors

We have identified two Olf-1/EBF-like transcription factors, O/E-2 and O/E-3, by degenerate RT-PCR. Together with O/E-1, they define a family of highly conserved rHLH transcription factors. Although the O/E rHLH motif shares modest similarity with a related structure in bHLH proteins, it lacks the characteristic upstream basic residues found in the bHLH motif (Wang and Reed, 1993). Therefore, O/E proteins define a novel class of HLH transcription factors that is distinct from the bHLH proteins. Deletion analysis of O/E-1 revealed that the rHLH domain is important for protein–protein interaction, and O/E-1/estrogen receptor chimeras demonstrated that it is sufficient to mediate dimerization (Hagman et al., 1995). Interestingly, the *Drosophila* O/E-like gene *collier* does not encode an rHLH domain. Instead, an alternative HLH motif was proposed (Croizatier et al., 1996).

Structure–function analysis of O/E-1 has revealed that it contains a zinc coordination motif as well as multiple dimerization and transactivation domains (Hagman et al., 1995). The region of O/E-1 upstream of the rHLH domain is implicated in DNA binding, dimerization (in the presence of optimized and correctly spaced half-sites), and transactivation. This region is highly conserved (>90% identity) among O/E-1, O/E-2, and O/E-3, and the zinc-binding residues of the three O/E proteins are identical. The extraordinary conservation shared among the O/E proteins suggests that they have very similar functional properties. This was confirmed *in vitro* by EMSA and luciferase reporter assays. The presence of multiple O/E proteins in ORNs and their similar DNA binding and transactivational properties suggest that they work in a coordinated manner *in vivo*. The multiple forms of O/E



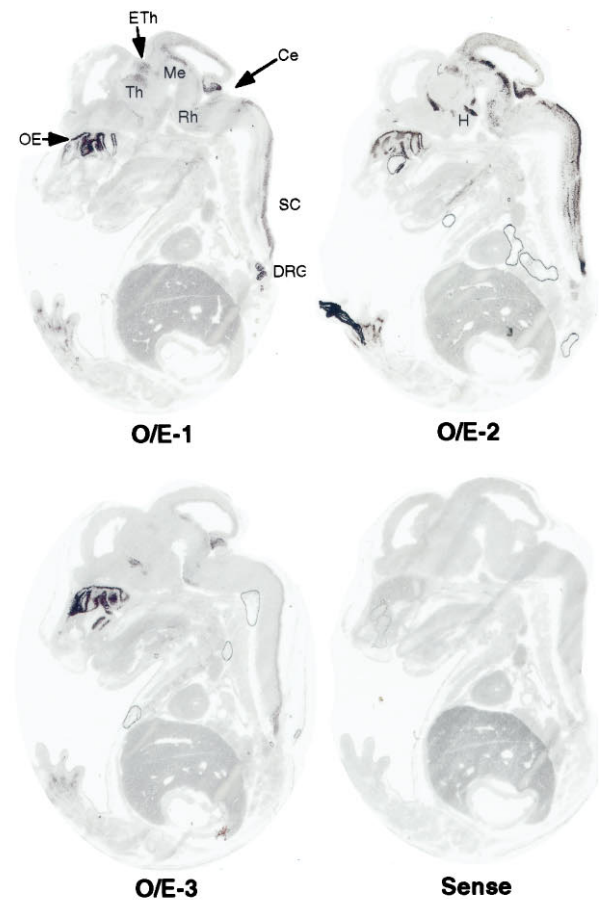


**Figure 6. Left.** *In situ* hybridization of adult olfactory epithelium and cerebellum. *A*, The pseudostratified layers of adult mouse olfactory epithelium. The expression of *OcNC*, detected with a digoxigenin-labeled probe, is restricted to the neuronal cell layer (*NCL*) and is absent in the sustentacular cell layer (*SCL*) and the basal cell layer (*BCL*). *B*, The patterns of *O/E* messages in adult mouse olfactory epithelium and cerebellum observed by *in situ* hybridization. *O/E-1* and *O/E-3* are expressed in the Purkinje cells (*PCL*) in the cerebellum and are absent in the molecular layer (*ML*) and the granule cell layer (*GCL*). Sense controls were performed for each gene, and representative panels (*O/E-3* for olfactory epithelium and *O/E-2* for cerebellum) are shown.

dimers may exhibit context-dependent properties that were not observed in our *in vitro* assays and potentially could direct preferential regulation of target gene expression by a small variation in the abundance of individual *O/E* proteins.

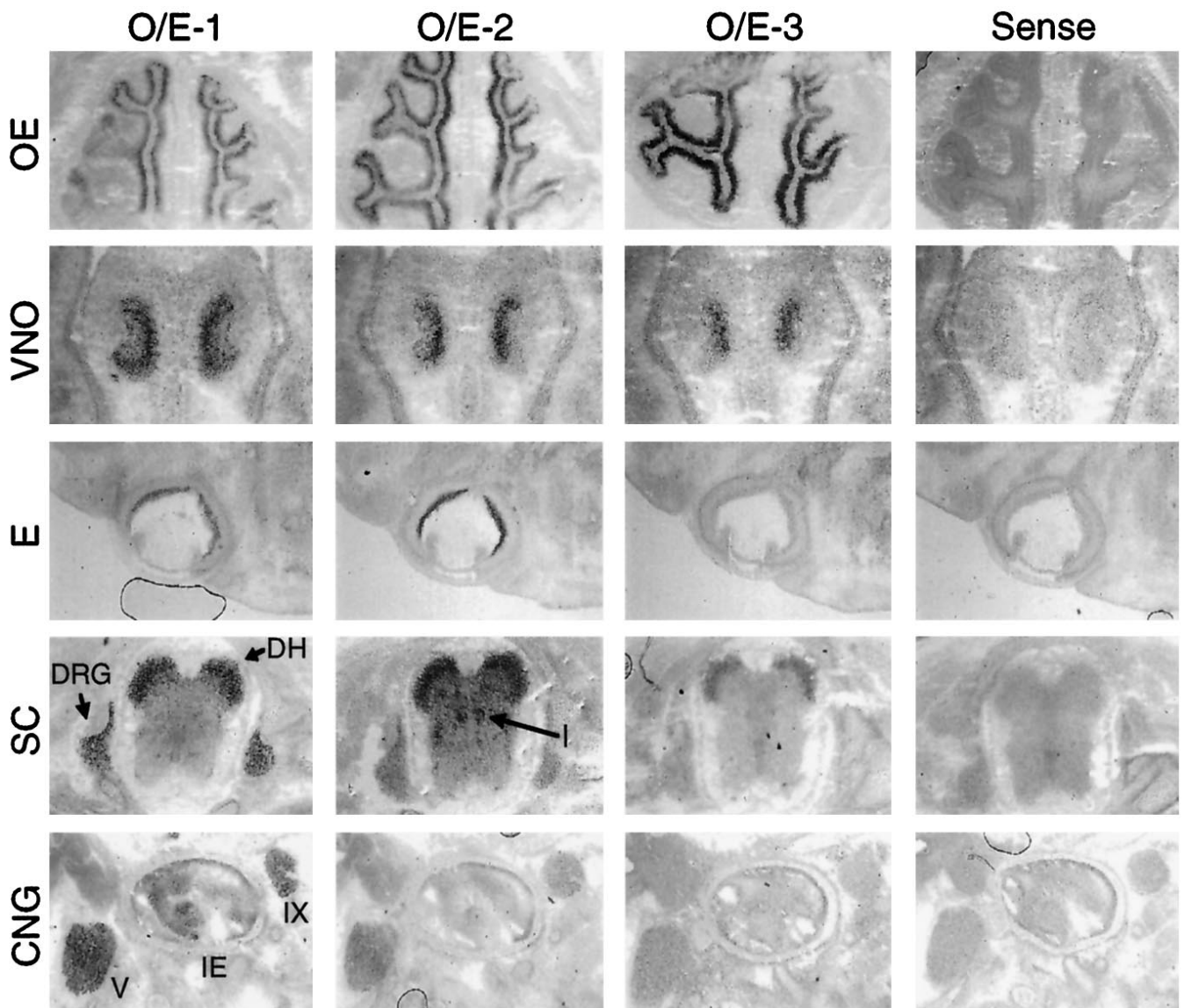
#### The role of the *O/E* proteins in adult olfactory tissue

*O/E-1* has been implicated in olfactory gene regulation and in B-cell development (Hagman et al., 1993; Wang et al., 1993).



**Figure 7. Right.** *In situ* hybridization of sagittal sections of E14 mouse embryos. The tissues that express *O/E* messages are indicated. *Ce*, Cerebellar primordium; *DRG*, dorsal root ganglia; *Eth*, epithalamus; *H*, hypothalamus; *Me*, mesencephalon; *OE*, olfactory epithelium; *Rh*, rhombencephalon; *SC*, spinal cord; *Th*, thalamus.

Mice with an *O/E-1* null mutation display a profound defect in their B-cell development, but their olfactory epithelium is morphologically normal and expresses mature olfactory neuronal markers (Lin and Grosschedl, 1995). The identification and cloning of *O/E-2* and *O/E-3* may account for the absence of an olfactory phenotype in the *O/E-1* knock-out mice. By RPA, *O/E-2* and *O/E-3* were readily detectable only in olfactory tissue;



**Figure 8.** *In situ* hybridization of transverse sections of E14 mouse embryos. The patterns of O/E messages were examined, and signals were found in the developing olfactory epithelium (OE), vomeronasal organ (VNO), eye (E), spinal cord (SC), and two cranial nerve ganglia (CNG). In the SC sections, dorsal root ganglia (DRG), the intermediate zone (I) between dorsal and ventral horns, and the position of dorsal horns (DH) are indicated (arrows). The CNG sections contain trigeminal (V) ganglia, glossopharyngeal (IX) nerve ganglia, and the inner ear (IE).

by *in situ* hybridization, they were expressed in a pattern identical to O/E-1 in the cells of neuronal lineage. In contrast to O/E-1 expression, O/E-2 and O/E-3 were not detected in spleen, suggesting that O/E-1 is sufficient to regulate *mb-1* expression in B-cells. On the basis of these observations, we propose that in the O/E-1 null mutant mice failure to express the single O/E protein present in spleen, O/E-1, accounts for the B-cell defect, whereas the presence of O/E-2 and O/E-3 compensates for the loss of O/E-1 in olfactory epithelium.

All three of the O/E messages were detected by RNA *in situ* hybridization in mature ORNs in the neuronal layer as well as in the cells of neuronal lineage in the basal layer of olfactory epithelium. The absence of mature ORN markers, the presumed targets of O/E proteins, from the basal layer despite O/E expression (Fig. 6) suggests a mechanism that negatively regulates O/E transactivational activity. Roaz, an inhibitor of O/E activity, is

expressed exclusively in the basal layer of olfactory epithelium and may account for the absence of target gene expression in these cells. A heteromultimeric complex of O/E proteins and Roaz also can activate transcription from distinct DNA sequences. The similar functional properties resulting from Roaz interaction with each of the O/E proteins (Tsai and Reed, 1997) suggest a common regulatory mechanism for O/E proteins earlier in olfactory neuronal differentiation.

Two distinct transactivation domains have been identified in O/E-1 (Hagman et al., 1995). Although the O/E proteins are less conserved in their C-terminal domain, the luciferase reporter assays, in conjunction with the above study, suggest that they share similar capacity for transactivation *in vitro*. There are conserved potential glycogen synthase kinase 3 (GSK3) phosphorylation sites in the C-terminal domain of the O/E proteins. This is intriguing particularly in light of the negative regulation of GSK3



by EGF via the MAP kinase pathway (Eldar-Finkelman et al., 1995) and the ability of EGF to promote basal cell proliferation and suppress neurogenesis in dissociated olfactory epithelium culture (Mahanthappa and Schwarting, 1993). In addition to Roaz-mediated inhibition, reduced phosphorylation by GSK3 provides a potential mechanism for the downregulation of O/E activity. The presence of multiple transactivation domains also suggests the existence of complex mechanisms that regulate O/E protein activity.

The expression of mature ORN markers in olfactory epithelium and *mb-1* in B-cells is mutually exclusive. Similarly, the vomeronasal organ (VNO), site of pheromone perception, and the main olfactory epithelium express different mature neuronal markers (Berghard et al., 1996; Wu et al., 1996). The presence of O/E proteins in all three structures (olfactory epithelium, spleen, and VNO) suggests that their presence alone is insufficient to activate their target genes in each of these tissues; there must exist additional factors that, in conjunction with activated O/E proteins, select the repertoire of target genes and are required for high levels of tissue-specific target gene expression *in vivo*.

### The role of the O/E proteins during development

Families of related transcription factors play important roles in mammalian development, and the O/E proteins share several features with other transcription protein families. The MyoD family (MyoD, Myf-5, MRF4, and myogenin) of myogenic bHLH proteins can homodimerize and heterodimerize with other bHLH proteins and regulate the expression of target genes (Murre et al., 1989; Buckingham, 1992; Molkenin et al., 1995). They have been shown to be functionally redundant and display overlapping patterns of expression (Wang et al., 1996). In summary, the MyoD family of transcription factors forms a molecular circuitry that governs muscle fate determination and differentiation by activating their target genes. Similar to the MyoD family, the vertebrate bHLH proteins MASH, NeuroD, and Neurogenin also play a role in neural development and can induce ectopic neurogenesis when expressed in *Xenopus* embryos (Chitnis and Kintner, 1996; Ma et al., 1996). Based on the pattern of O/E expression in the developing nervous system, this protein family is likely to play a role during the later stages of neuronal differentiation.

O/E genes are expressed in the nervous system during development and enriched in a number of structures that mediate sensory activities. The expression of O/E genes in postmitotic cells suggests a role for O/E proteins in neuronal differentiation (Davis and Reed, 1996). Although each O/E protein potentially could perform a different task during development, our *in vitro* characterization suggests that they are functionally redundant, analogous to the redundancy demonstrated for Engrailed and MyoD family members (Hanks et al., 1995; Wang et al., 1996), and their physiological roles in development are determined by their temporal and spatial patterns of expression. The product of the *Caenorhabditis elegans unc-3* gene is very similar to the mammalian O/E proteins (B. Prasad and R. Reed, unpublished data) and is required for proper axonal connectivity (Herman, 1987). By analogy, the O/E proteins may regulate the expression of factors essential for axonal pathfinding in developing neurons and in adult olfactory epithelium.

A number of transcription factors perform distinct functions during development and, subsequently, in adult animals. For instance, Pax-6 is essential for the normal development of the

nervous system. Pax-6 mutant mice exhibit defects in forebrain as well as in olfactory and eye development (Grindley et al., 1995; Stoykova et al., 1996). Pax-6 is implicated also in the continual expression of a lens protein, crystallin, in adult animals (Richardson et al., 1995). The O/E proteins may exhibit similar dual functionality like Pax-6: regulating the differentiation of developing neurons in embryos and maintaining the expression of mature ORN markers in adults. Alternatively, the continual replacement of ORNs throughout adult life may require the expression of factors that control neuronal differentiation and maturation in a process similar to the developing nervous system, and the O/E proteins participate in a common genetic program for sensory neuron differentiation in addition to the regulation of mature markers in adult olfactory receptor neurons.

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