RESEARCH ARTICLE

Negative Feedback Regulation Ensures the One Receptor–One Olfactory Neuron Rule in Mouse

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In the mouse olfactory system, each olfactory sensory neuron (OSN) expresses only one odorant receptor (OR) gene in a monoallelic and mutually exclusive manner. Such expression forms the genetic basis for OR-instructed axonal projection of OSNs to the olfactory bulb of the brain during development. Here, we identify an upstream cis-acting DNA region that activates the OR gene cluster in mouse and allows the expression of only one OR gene within the cluster. Deletion of the coding region of the expressed OR gene or a naturally occurring frame-shift mutation allows a second OR gene to be expressed. We propose that stochastic activation of only one OR gene within the cluster and negative feedback regulation by that OR gene product are necessary to ensure the one receptor–one neuron rule.

The mouse has more than 1000 OR genes clustered at multiple loci on many different chromosomes (*1*). The OR genes are expressed in a mutually exclusive, monoallelic manner by OSNs in the nasal olfactory epithelium (OE) (*2*–*5*). OSNs expressing a specific OR gene are distributed in one of four zones within the OE (*6*–*8*), and their axons converge on a specific set of glomeruli in the olfactory bulb (*9*–*11*). The one receptor–one neuron rule is essential for the conversion of olfactory signals into an accurate topographical map in the olfactory bulb. Using transgenic mice carrying a large DNA region of an OR gene cluster in which the *MOR28* gene resides, we previously showed that the transgenic and endogenous alleles of *MOR28* are expressed in a mutually exclusive manner (*4*). The exclusion was also observed for separately tagged *MOR28* transgenes that were integrated in tandem into the same chromosomal location. Although these *MOR28* genes have the same coding and regulatory sequences, no more than one allele of *MOR28* is expressed under any circumstance. Such an unusual mode of gene regulation has previously been reported only for the antigen receptor genes of the immune system.

A cis-acting locus control region activates the OR gene cluster. Using transgenic constructs in yeast artificial chromosomes (YACs), we have studied the expression of the OR gene cluster containing the *MOR28* gene (*4*). By comparing the DNA structures of various YAC constructs and their expression patterns, we found that the DNA region 40 to 150 kb upstream of *MOR28* appears to contain a cis-acting regulatory element that is necessary for the expression of transgenic OR genes. Sequence comparison of the mouse and human genomes revealed that a 2-kb sequence 75 kb upstream of the murine *MOR28* gene is highly homologous to the human sequence 32 kb upstream of the human *HOR28* gene (Fig. 1A). Percent identity plotting between the mouse and human sequences (*12, 13*) revealed that this 2-kb homology (H) region is the only conserved sequence in the upstream region of *MOR28* (Fig. 1B).

To determine whether the H region is necessary for the expression of *MOR28* and other downstream OR genes in the cluster, we deleted the H region from the YAC-290 construct in which the three OR genes *MOR28*, *MOR10*, and *MOR83* had been separately tagged with *lacZ* (expressing *Escherichia coli* β-galactosidase), *EGFP* (expressing enhanced green fluorescent protein), and *WGA* (expressing wheat germ agglutinin), respectively (Fig. 1C) (*14*). The control YAC-290, without the H deletion, expressed all three OR genes in zone 4 of the OE. When the H region was deleted (YAC-290dH), none of these transgenes were expressed. It was later found that the YAC-290 construct contained another OR gene, *MOR29*, that is expressed in zone 1 of the OE (*15*). We separately tagged *MOR28* and *MOR29* with *lacZ* and *EGFP*, respectively. When the H region was deleted, the expression of both *MOR28* (*lacZ*-tagged) and *MOR29* (*EGFP*-tagged) was abolished (Fig. 1D). These results indicate that the zone 4–specific *MOR28*, *MOR10*, and *MOR83* genes, as well as the zone 1–specific *MOR29* gene, are under the control of the H region.

Our previous transgenic studies showed that the shorter YAC constructs lacking the upstream region of YAC-290 do not express the *MOR28* transgene (*4*). We examined whether the expression of these constructs could be restored by adding the H region. All of the OR transgenes in the YAC-H180 construct—*MOR28* (*lacZ*-tagged), *MOR10* (*EGFP*-tagged), *MOR83* (*WGA*-tagged), and *MOR29* (*DsRed*-tagged, expressing red fluorescent protein from the coral *Discosoma*)—were reactivated when the H region was attached to the upstream ends of YAC-180 (Fig. 2A). Interestingly, comparison of YAC-H140 to YAC-290 showed that the number of OSNs expressing *MOR28* was greatly increased, whereas those for the downstream genes were substantially reduced (Fig. 1, C and D, and Fig. 2B). This phenomenon is likely attributed to the relocation of the H region immediately upstream of the *MOR28* in the YAC-140 construct. We then examined whether the H region can activate the *MOR28* minigene tagged with *EYFP* (expressing enhanced yellow fluorescent protein), which contains exons 1 and 2 flanked by 3-kb regions of genomic DNA (Fig. 2C). Unlike the minigenes examined by Vassalli *et al*. (*16*), our minigene could not be expressed on its own. When the H region was attached (*H-MOR28*), a large number of OSNs expressed the minigene in 5 of 10 mouse lines, whereas the control minigene without H was not expressed in any of the 8 lines examined. Because the transgene-positive cells were particularly dense in zone 4 of the OE, we were curious as to whether the transgene-expressing cells still showed mutually exclusive expression of ORs. In situ hybridization with the RNA probes for the zone 4–specific OR genes revealed that cells expressing both endogenous and transgenic OR genes were rarely found (less than 1%) among several hundred OSNs expressing the endogenous OR genes (*17*).

Deleting the coding region permits expression of other OR genes. To achieve the mutually exclusive expression, we assumed that negative regulation was taking place. We postulated that once a particular OR gene is stochastically chosen, the gene product prevents further activation of the remaining genes. To test this hypothesis, we deleted the entire coding sequence of the *MOR28* gene in YAC-290G (Fig. 3A). To visualize the transgene-expressing OSNs, we tagged *MOR28* with *IRES-tau-EGFP* in the 3′-untranslated region. We analyzed two sets of transgenic

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mice: one carrying the deletion-type *MOR28* (*del-MOR28*) and the other carrying the wild-type *MOR28* allele.

The OE sections were analyzed by fluorescence microscopy for transgene expression (Fig. 3B). Although the OSNs with activated *del-MOR28* expressed only the EGFP protein, their densities and distribution in the OE were indistinguishable from those for the endogenous *MOR28* and for the wild-type transgene (Fig. 3B). We then examined whether mutually exclusive expression of OR genes is violated in those OSNs expressing *del-MOR28*. If the expressed OR gene products inhibit further activation of other OR genes, deletion of the coding sequence in the *MOR28* should allow for the expression of the second allele of *MOR28* or other OR genes. In contrast, if the activation process itself is responsible for the monoallelic and mutually exclusive expression of OR genes, then secondary activation of other OR genes should not be observed even in the OSNs with an activated *del-MOR28* locus. We first examined the exclusion between the endogenous *MOR28* and the transgenic *del-MOR28* genes (Fig. 3C). The OE sections were hybridized with an RNA probe for the *MOR28* coding sequence and then treated with antibodies to EGFP (anti-EGFP). Because *del-MOR28* does not contain the *MOR28* coding region, the RNA probe only detects OSNs that express the endogenous *MOR28* (shown in red), whereas immunostaining detects cells expressing the transgene (shown in green). Using confocal microscopy, we carefully analyzed the stained OSNs for coexpression. Yellow-colored cells were frequently found (Fig. 3C). Note that the detection of double-stained cells is not due to the overlap of red and green cells in different optical planes.

We also examined the coexpression of the transgenic *del-MOR28* gene with other endogenous OR genes whose expression areas in the OE overlap those of *MOR28*. Among more than 200 OR genes analyzed by in situ hybridization, about 40 OR genes were found to share expression areas with *MOR28*. For these endogenous OR genes, coexpression was examined with *del-MOR28* (Fig. 4A); wild-type

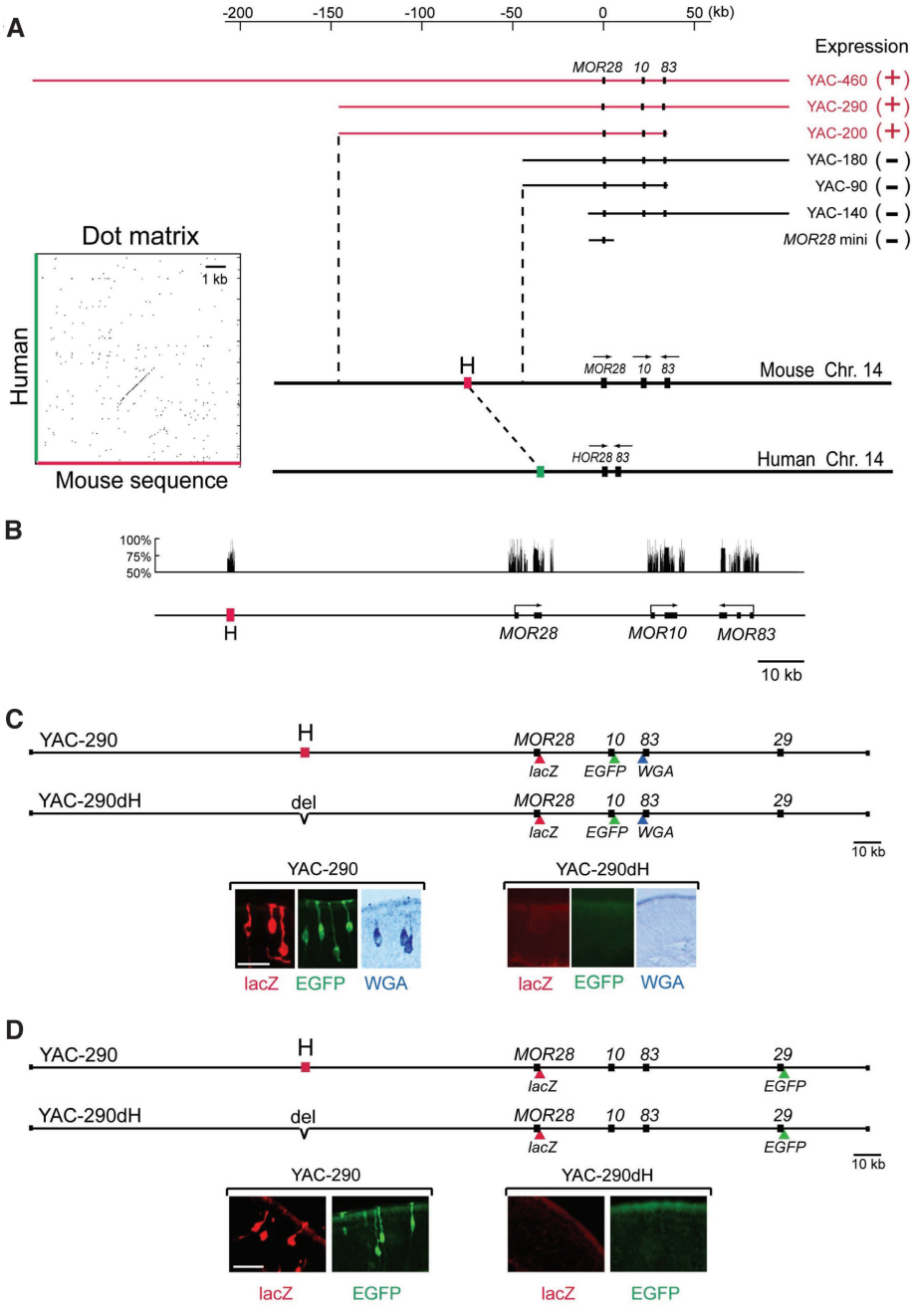

Fig. 1. The homology (H) region that activates the *MOR28* cluster. (**A**) Structures of seven transgenic constructs are compared. *MOR28* in each construct is shown as expressed (+) or not (–). Nucleotide sequences were compared between mouse and human for the upstream regulatory region. A 2-kb homology (H) was identified (dot matrix) 75 kb upstream of *MOR28*. (**B**) Percent identity plot for the mouse *MOR28* cluster against the human homolog. Repetitive sequences were masked with RepeatMasker (*12, 13*). Similarities higher than 50% are shown as vertical bars. (**C**) Deletion of the H region abolishes the expression of the *MOR28* cluster. The H region was deleted from the YAC-290 construct (*4*), generating YAC-290dH. Three zone 4–specific OR genes in the constructs were separately tagged. OE sections of the transgenic mice were examined for the expression of the transgenes by fluorescence (*EGFP*) or by immunostaining (*lacZ* and *WGA*). The same results were obtained from six transgenic lines for YAC-290 and five lines for YAC-290dH. (**D**) Expression of the zone 1–specific *MOR29*. OE sections were analyzed by fluorescence (*EGFP* for *MOR29*) and by immunostaining (*lacZ* for *MOR28*). The same results were obtained from four transgenic lines for YAC-290 and five lines for YAC-290dH. Scale bars, 20 μm.

transgenic *MOR28* without deletion was used as a control (Fig. 4B). OE sections of the transgenic mice were first hybridized with RNA probes of the endogenous OR genes and immunostained with anti-EGFP to detect transgene expression. In Fig. 4, the OSNs expressing the transgene are shown in green, whereas those expressing the endogenous OR genes are in red. Most OR genes that share expression areas with the *MOR28* gene yielded yellow-stained OSNs in the merged pictures, indicating their coexpression with *del-MOR28*. Examples for *MOR230-1*, *MOR244-2*, *MOR248-2*, and *MOR246-2* are shown in Fig. 4A. In all transgenic lines examined, more than 75% of the OSNs with activated *del-MOR28* coexpressed one of the 40 tested endogenous OR genes. We expect this fraction to be higher with an expanded repertoire of OR gene probes. No yellow-stained cells were detected with the control transgene, the *EGFP*-tagged wild-type *MOR28*, for all three lines examined (Fig. 4B).

We then examined the projection patterns of OSNs expressing *del-MOR28*. Whole-mount views of the olfactory bulbs were compared for the wild-type *MOR28* and *del-MOR28* transgenes (Fig. 5, A and B). Projecting axons were tagged by EGFP. OSNs expressing the endogenous *MOR28* gene or the wild-type transgene projected their axons to a specific site in the posteroventral portion of the olfactory bulb (Fig. 5A). In contrast, the axons of OSNs that expressed *del-MOR28* did not converge, but appeared to wander and target to multiple glomeruli near the *MOR28* projection site (Fig. 5B). To examine the projection more closely, we immunostained serial olfactory bulb sections with anti-EGFP. Multiple glomerular structures were targeted by the *del-MOR28*–expressing OSNs (Fig. 5D). It is possible that these glomeruli represent the target sites for the secondarily chosen OR genes in the *del-MOR28*–expressing OSNs. In the control mouse, whose endogenous *MOR28* gene had been tagged with *EGFP*, only one distinct glomerular structure was detected in the sections from the posteroventral portion of the olfactory bulb (Fig. 5C).

Frame-shifted pseudogenes enable concurrent OR gene expression. A substantial number of pseudogenes are present in the mouse OR gene family (*18*–*20*). Because our transgenic studies indicated an inhibitory role for OR gene products, we expected that some of the pseudogenes would allow the secondary activation of other OR genes, even after the pseudogenes had been chosen for expression. To examine this possibility, we searched for

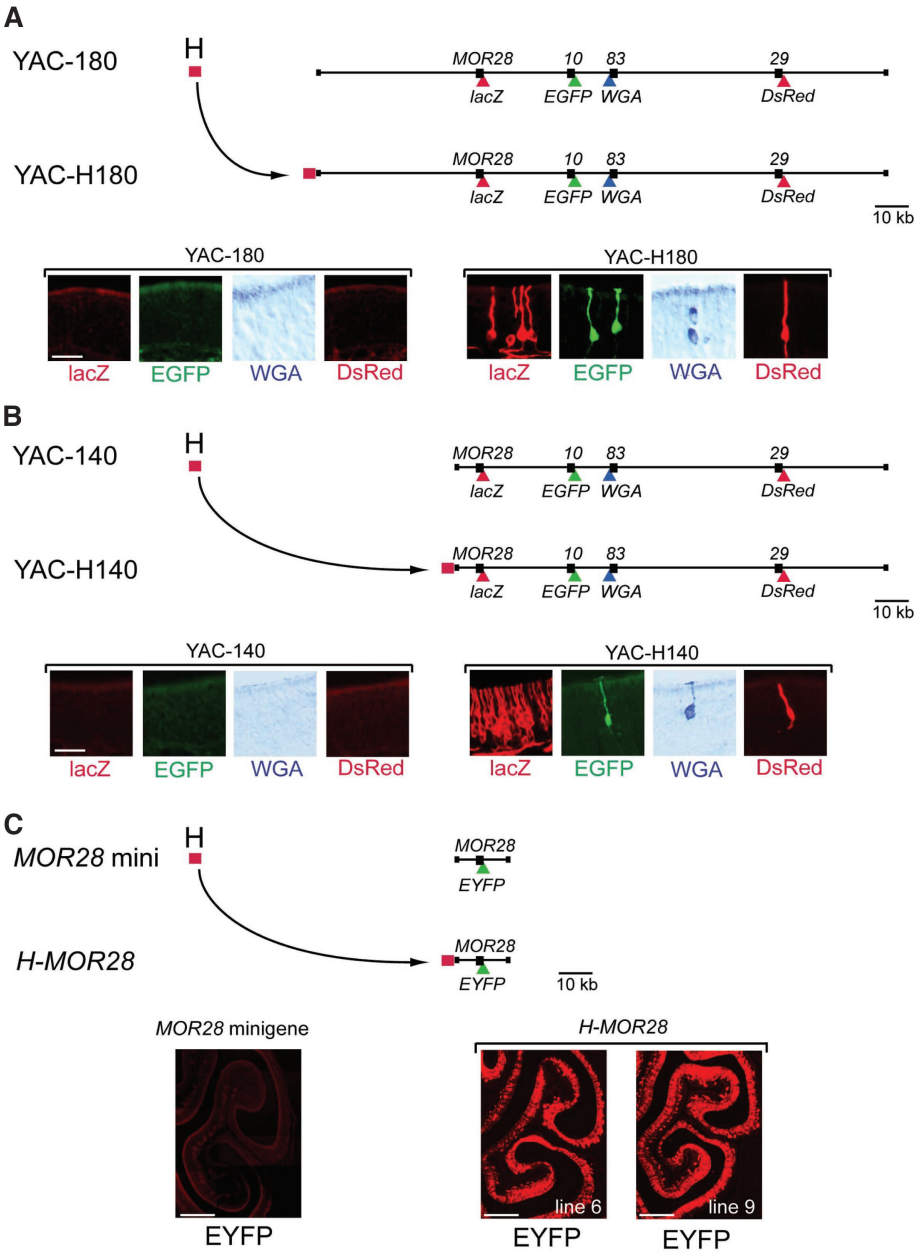

Fig. 2. Addition of the H region to the truncated transgenic constructs. The H region was joined at the upstream ends of the truncated transgenic constructs YAC-180 (**A**), YAC-140 (**B**), and the *MOR28* minigene (**C**). The constructs lacking the H region do not express the transgenes. The same results were obtained from three transgenic lines for YAC-180, eight lines for YAC-140, and eight lines for the *MOR28* minigene. All OR genes in the constructs were separately tagged as illustrated. When the H region was added, expression of the *MOR28* as well as other transgenes was restored. The same results were obtained from five lines for YAC-H180 and two lines for YAC-H140. The *H-MOR28* minigene was expressed in 5 of 10 lines. Copy numbers of the *H-MOR28* minigene in line 6 and line 9 were 2 and 1, respectively. OE sections of the transgenic mice were stained to detect the expression of the transgenes by fluorescence (*EGFP* and *DsRed*) or by immunostaining (*lacZ*, *EYFP*, and *WGA*). Scale bars, 20 μm [(A) and (B)], 200 μm (C).

OR pseudogenes in the C57BL/6J mouse genome database from the Mouse Genome Sequencing Consortium. At least 100 OR genes were confirmed to be pseudogenes; some lack the start codon, some contain a frame shift, and others have stop codons in their coding sequences. Among 33 genes examined by in situ hybridization, 3 were found to give sufficient signals for transcription in the OE.

To test for coexpression, we used the transgenic minigene *H-MOR28* tagged with *ECFP* (expressing enhanced cyan fluorescent protein) as a partner, because of the enhanced frequency of the *H-MOR28* activation and mutually exclusive mode of expression. OE sections of the *H-MOR28* transgenic mouse were hybridized with the pseudogene probes and then immunostained for ECFP. Three pseudogenes, *MOR171-43*, *MOR135-18*, and *MOR248-12*, were sequenced and found to contain frame shifts and premature stops (Fig. 6A). Hybridization of OE sections with these probes yielded double-stained OSNs in the superimposed pictures (Fig. 6B). For example, the *MOR171-43* probe gave 35 coexpressing cells (shown in yellow) among 635 cells expressing *MOR171-43* (stained red). As a positive control for double staining, the OE section of the *H-MOR28* transgenic mouse was hybridized with the *MOR28* probe and treated with anti-EGFP. All of the transgene-expressing OSNs turned yellow (Fig. 6C). As negative controls, probes for functional OR genes, *MOR171-1* and *I7*, were used for in situ hybridization. Although the cells expressing the partner gene (shown in green) were very crowded, the OSNs that hybridized with the OR gene probes (shown in red) were not yellow in the merged pictures (Fig. 6C). These results with the pseudogenes further confirm the notion that the OR gene products act as inhibitors to prevent the further activation of other OR genes.

Activation of one OR gene within the cluster. It is now well established that each OSN expresses only one member of the OR gene family in the mouse olfactory system (*2*–*5*). However, it is still not clear how one OR gene is chosen for expression, and how the remaining OR genes are kept silent. We have shown that the H region far upstream of the *MOR28* OR gene cluster is responsible for the activation of all OR genes in the cluster. How is it that transgenic *MOR28* is chosen so frequently when the H region is relocated closer to the promoter? Why does selection of downstream genes in the YAC-H140 construct become down-regulated? We

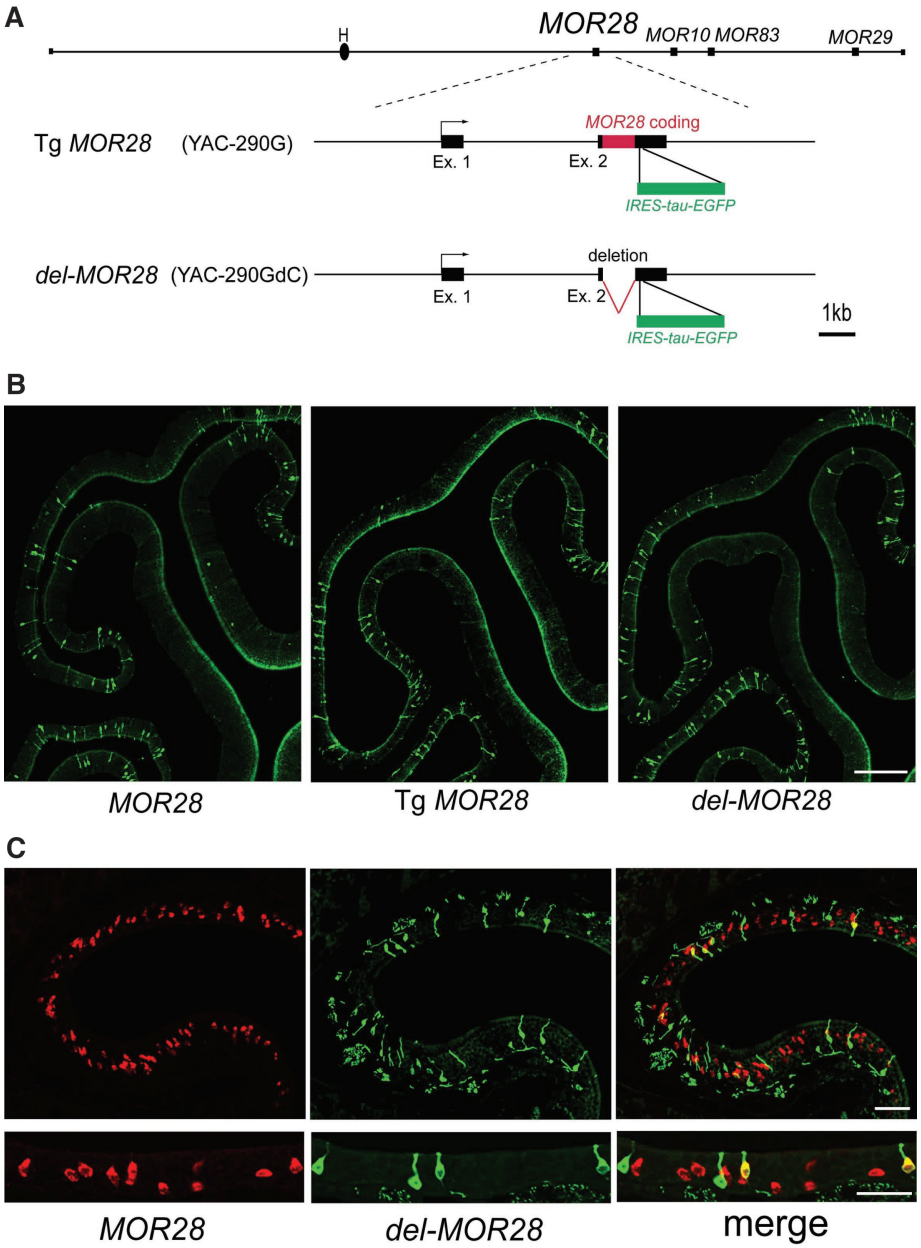

Fig. 3. A coding-deletion mutant of the *MOR28* transgene. (**A**) Structure of *del-MOR28*. The coding sequence was deleted from exon 2 of *MOR28* in the YAC-290G construct (*4*), generating YAC-290GdC. *MOR28* was tagged with *IRES-tau-EGFP*. (**B**) Detection of EGFP fluorescence in the OE sections. Three different *EGFP*-tagged *MOR28* genes were analyzed for their expression patterns in the OE: the endogenous *MOR28* gene in the knock-in mouse (*MOR28*) (*4*), the wild-type *MOR28* transgene (Tg *MOR28*), and the deletion-type transgene (*del-MOR28*). (**C**) Coexpression of the transgenic *del-MOR28* with the endogenous *MOR28* gene. A section of OE from the YAC-290GdC mouse was hybridized with the *MOR28*-coding probe to detect the expression of the endogenous *MOR28* without the *EGFP* tag (stained red). The same section was immunostained with anti-EGFP to detect the expression of *del-MOR28*, which lacks the coding sequence (stained green). The two pictures were superimposed (merge) to detect the coexpressed cells. Enlarged photos are shown at the bottom. The *MOR28* coding probe is cross-hybridizable with the *MOR10* mRNA. Scale bars, 100 μm (B), 50 μm (C).

suggest that a transcription-activating complex is formed in the H region that possibly interacts with a promoter site, activating the OR gene (Fig. 6D). It is possible that the smaller the distance between the H region and promoter, the more efficiently the OR gene is activated and expressed. This probably explains why the H region is located far from the OR gene cluster. If the H region were located too close to the cluster, the proximal OR gene would be activated quite frequently and the downstream genes would rarely be chosen for expression. It is important to ask how the expression of one particular OR gene within the activated cluster is ensured. We propose that the activation complex formed in the H region interacts with only one promoter site in the cluster.

Negative feedback regulation by the OR gene product. We have shown that a coding-region deletion of the transgenic *MOR28* gene permitted the activation of secondary OR genes in the transgene-expressing OSNs. Similar observations were also made with natural frame-shift mutants. These observations indicate that the OR gene product, most likely the protein, has a regulatory role in the expression of the OR genes. This model explains some curious observations previously made by other investigators. For example, Qasba and Reed (*21*) reported that the transgenic *lacZ* gene carrying the OR gene *M4* promoter was coexpressed with the endoge-

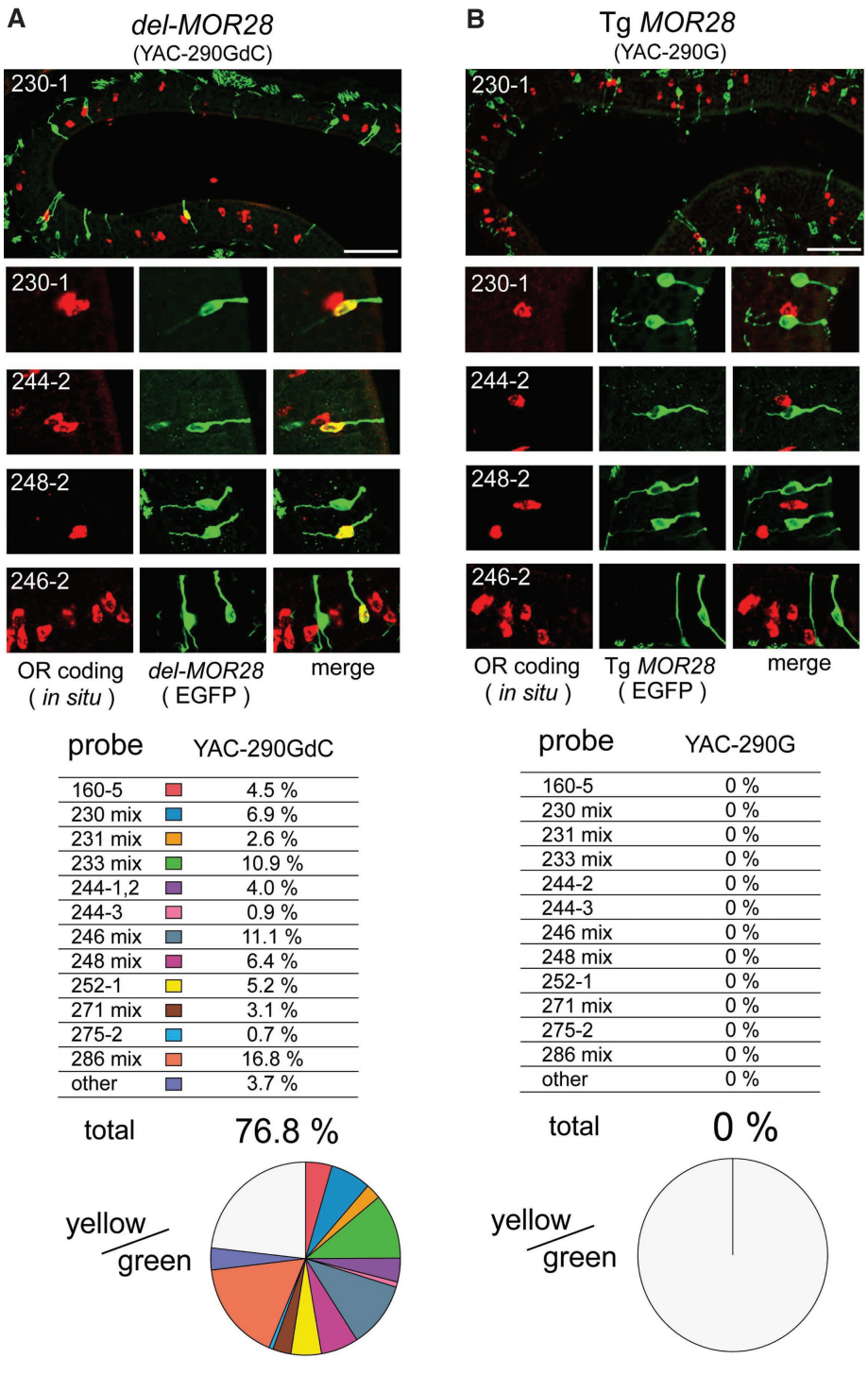

Fig. 4. Coexpression of the *del-MOR28* transgene with the endogenous OR genes. (**A**) Coronal OE sections of the YAC-290GdC mice were hybridized with various RNA probes of endogenous OR genes (stained red). The same sections were then immunostained with anti-EGFP to detect the expression of *del-MOR28* (stained green). Examples of the four endogenous OR genes are shown (*MOR230-1*, *MOR244-2*, *MOR248-2*, and *MOR246-2*). Coexpression rates of *del-MOR28* with various OR genes were calculated for the YAC-290GdC line 1 mouse. The percentage of the probe-positive cells among ~500 *del-MOR28*–expressing cells is shown. Because the coding-region probes are cross-hybridizable with related genes, they detect multiple OR genes belonging to the same subfamily. The total coexpression rate with 13 different probes was 76.8%. (**B**) The OE of the YAC-290G mouse was analyzed in parallel as a negative control. No yellow-colored cells were detected with any OR gene probes tested thus far. Scale bars, 50 μm.

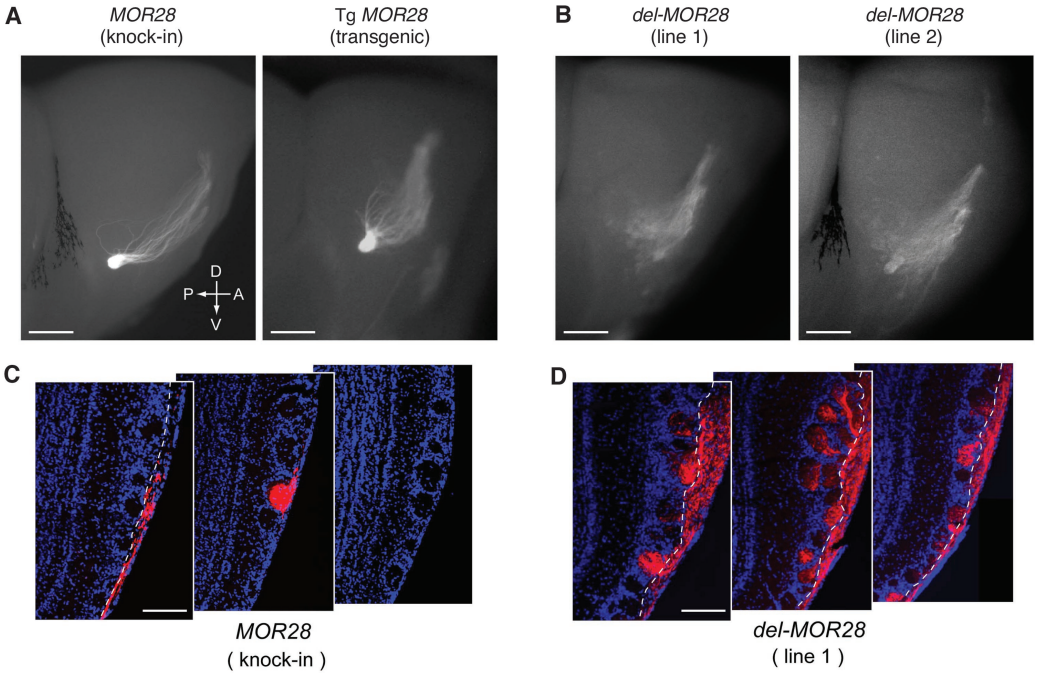

Fig. 5. Axonal projection of OSNs expressing the *EGFP*-tagged *MOR28*. (**A**) Whole-mount views of the lateral aspect of the olfactory bulbs visualized by the fluorescence of EGFP. Both the endogenous *MOR28* (knock-in) and the transgenic *MOR28* were analyzed as positive controls. Directions: A, anterior; P, posterior; D, dorsal; V, ventral. (**B**) Whole-mount staining of the olfactory bulbs isolated from the *del-MOR28* transgenic mice. Two independent lines were analyzed. Fluoresced axons did not converge to a specific site in both lines. (**C** and **D**) Coronal sections of the olfactory bulbs were immunostained with anti-EGFP (red) and counterstained by 4′,6′-diamidino-2-phenylindole (DAPI) (blue). Three consecutive sections (each separated by 96 μm) are shown. Broken lines demarcate the olfactory nerve from the glomerular layers. Scale bars, 500 μm [(A) and (B)], 200 μm [(C) and (D)].

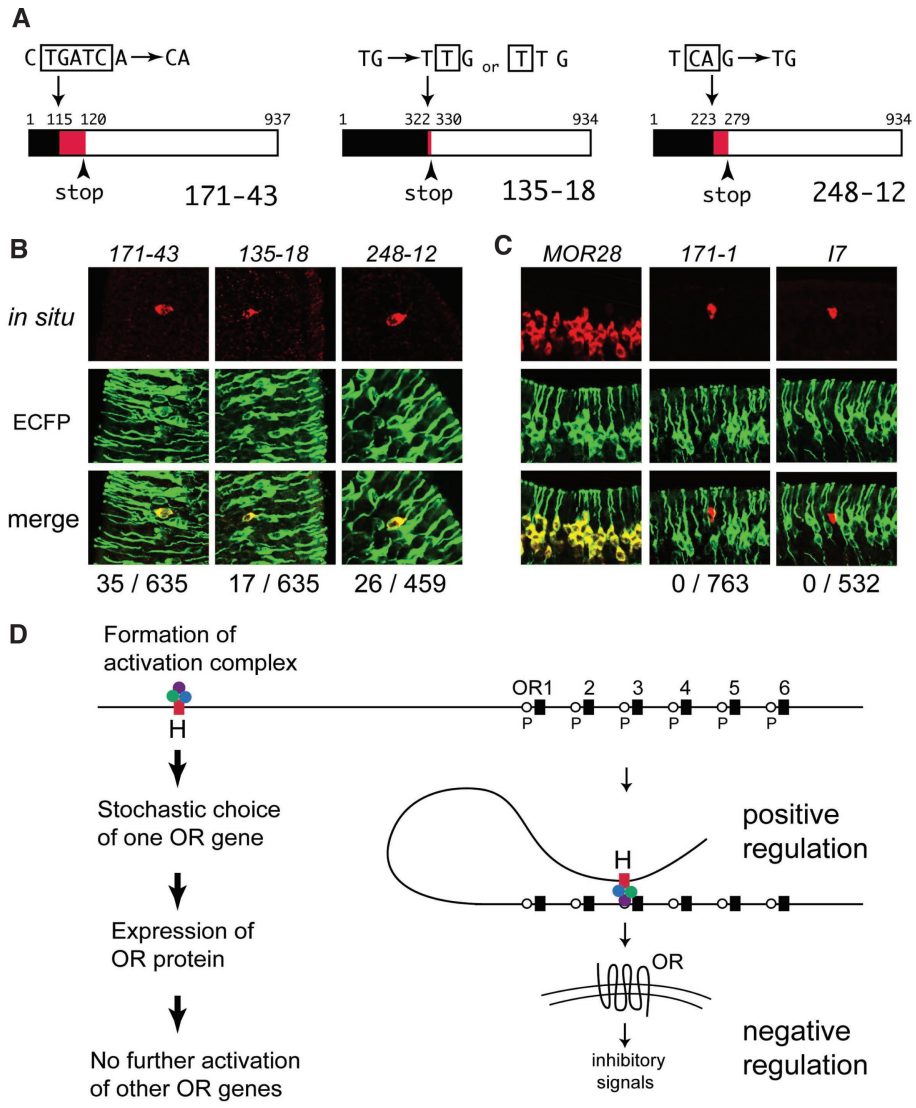

Fig. 6. Coexpression of the OR pseudogenes with the transgenic *H-MOR28* and a model for the OR gene expression. (**A**) Structures are shown for three pseudo OR genes. The frame-shift starting points are indicated by arrows. Residue numbers are for nucleotides. Nucleotide deletions or additions that have caused the frame shift are shown in boxes. Stop codons created by the frame shift are indicated by arrowheads. Frame-shifted peptide regions are in red. (**B**) OE sections of the *H-MOR28* transgenic mouse were subjected to in situ hybridization with pseudo OR gene probes of *MOR171-43*, *MOR135-18*, and *MOR248-12* (stained red). The same OE sections were then immunostained with anti-EGFP to detect ECFP for the transgene-expressing cells (stained green). Two pictures were superimposed (merge) for analysis of coexpression. The ratios of coexpressing cells (shown in yellow) to the total red-colored cells are shown at the bottom. (**C**) Functional OR genes, *MOR171-1* and *I7*, were examined as negative controls. (**D**) Schematic diagram of the mutually exclusive expression of the OR genes. We assume that the activation complex formed in the H region stochastically chooses one promoter (P) site by random collision, activating one particular OR gene member within the cluster. We propose that once the activated gene is expressed, the functional OR molecules transmit an inhibitory signal to block the further activation of additional OR genes or clusters.

nous *M4* gene in 1% of the *lacZ*-positive OSNs. The transgene-expressing cells probably allowed the activation of the endogenous *M4* promoter because of the absence of the functional receptor. A similar observation was made by Pyrski *et al*. (*22*) using the *lacZ* transgene with the *OMP* promoter that was integrated into the *OR-Z6* cluster. Interestingly, the transgene-expressing OSNs projected their axons to multiple glomeruli in the olfactory bulb, consistent with our observations of the *del-MOR28* gene. It is possible that the OSNs expressing truncated ORs allow further activation of other OR genes and project their axons to targets determined by the coexpressed ORs. Furthermore, Wang *et al*. (*23*) found that deletions and nonsense mutations in the *lacZ*-tagged P2 gene resulted in the inability of the OSNs to project their axons to a specific set of glomeruli.

These observations as well as our own results support the idea that the functional expression of an OR gene inhibits the activation of other OR genes. How is this negative regulation achieved? In the immune system, the pre-B cell receptor provides an inhibitory signal via Syk family tyrosine kinases (*24*) to prohibit the further activation of V_H (the variable region of immunoglobulin heavy chain genes), ensuring the allelic exclusion of these genes (*25*–*29*). We postulate a similar inhibitory role for the functional OR proteins. Stochastic activation of an OR gene and negative feedback regulation by the OR gene product, as shown in Fig. 6D, may ensure the maintenance of the one receptor–one neuron rule in the mammalian olfactory system.

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REPORTS

Biomolecular Interactions at Phospholipid-Decorated Surfaces of Liquid Crystals

Jeffrey M. Brake, Maren K. Daschner, Yan-Yeung Luk, Nicholas L. Abbott*

The spontaneous assembly of phospholipids at planar interfaces between thermotropic liquid crystals and aqueous phases gives rise to patterned orientations of the liquid crystals that reflect the spatial and temporal organization of the phospholipids. Strong and weak specific-binding events involving proteins at these interfaces drive the reorganization of the phospholipids and trigger orientational transitions in the liquid crystals. Because these interfaces are fluid, processes involving the lateral organization of proteins (such as the formation of protein- and phospholipid-rich domains) are also readily imaged by the orientational response of the liquid crystal, as are stereospecific enzymatic events. These results provide principles for label-free monitoring of aqueous streams for molecular and biomolecular species without the need for complex instrumentation.

Some of the most important biomolecular interactions occur at biological membranes, including the binding events that permit entry of protein toxins into cells (*1*), the binding and enzymatic events that trigger cell signaling pathways (*2*), the assembly of proteins, lipids, and cholesterol into rafts (*3*), the crystallization of proteins (*4*), and the binding events that are the first stage of viral infection (*5*). Past attempts to provide facile methods of reporting these biomolecular interactions (e.g., for biological sensing) have exploited the self-assembly of the constituents of biological membranes such as phospholipids and proteins at interfaces (*1*–*11*). These systems, however, have been difficult to analyze because they generally require the use of either labeled molecules (e.g., fluorescent labels) or complex instrumentation.

The work reported here was inspired by the observation that most biomolecular interactions at biological membranes are accompanied by a reorganization of the proteins, lipids, and other species that constitute the membranes (*1*–*11*). We report that fluid, phospholipid assemblies formed at interfaces between thermotropic liquid crystals (LCs) and aqueous phases are coupled to the orientations of the thermotropic LCs. The specific binding of proteins to these interfaces and their subsequent formation of organized lateral assemblies, as well as the activities of enzymes, are demonstrated to trigger spatially patterned orientational transitions in the LCs that are readily imaged with polarized light. This coupling permits label-free imaging of a range of dynamic molecular phenomena that occur at these interfaces (*12*, *13*). Because aqueous streams can also flow past the lipid-laden interface of the LC, these principles may also provide the basis of low-cost, passive (zero-power) indicators of the presence of targeted biological species.

Films of nematic LC [4′-pentyl-4-cyanobiphenyl (5CB)] were deposited into the pores (width ~ 283 μm; depth ~ 20 μm) of gold grids supported on octadecyltrichlo-

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