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Interchromosomal Interactions and Olfactory Receptor Choice

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SUMMARY

The expression of a single odorant receptor (OR) gene from a large gene family in individual sensory neurons is an essential feature of the organization and function of the olfactory system. We have used chromosome conformation capture to demonstrate the specific association of an enhancer element, *H*, on chromosome 14 with multiple OR gene promoters on different chromosomes. DNA and RNA fluorescence in situ hybridization (FISH) experiments allow us to visualize the colocalization of the *H* enhancer with the single OR allele that is transcribed in a sensory neuron. In transgenic mice bearing additional *H* elements, sensory neurons that express OR pseudogenes also express a second functional receptor. These data suggest a model of receptor choice in which a single *trans*-acting enhancer element may allow the stochastic activation of only one OR allele in an olfactory sensory neuron.

INTRODUCTION

Individual olfactory sensory neurons in mice express only one of 1300 odorant receptor genes (Chess et al., 1994; Malnic et al., 1999). The choice of a specific odorant receptor defines the functional identity of a sensory neuron, and the receptor also provides an instructive cue that dictates the site of projection in the brain (Wang et al., 1998; Feinstein and Mombaerts, 2004; Barnea et al., 2004). Thus, the expression of a single receptor gene in a sensory neuron is an essential feature of olfactory perception.

One extreme model for the control of olfactory receptor expression invokes the existence of 1300 distinct populations of sensory neurons, each expressing a unique combination of regulatory factors that governs the choice of a different olfactory receptor gene. This deterministic model predicts that all olfactory receptor genes will contain different *cis*-regulatory sequences that are recognized by unique sets of transcription factors. An alternative, stochastic model of receptor gene choice suggests that all odorant receptor genes contain common *cis*-regulatory elements that are recognized by the same set of transcription factors (Shykind, 2005). Superimposed upon these shared elements must be different regulatory sites that define the zone of expression in the epithelium. In this model a special mechanism must exist in order to assure that only one receptor gene is expressed from only one of the two alleles in a given neuron. Recent experiments support the stochastic choice of receptor gene expression within a given topological zone (Qasba and Reed, 1998; Serizawa et al., 2000, 2003; Vassalli et al., 2002; Lewcock and Reed, 2004; Shykind et al., 2004).

What transcriptional mechanism can assure the random choice of only one of the multiple OR genes in a given neuron? One attractive model invokes the existence of a single expression site in the genome. A unique regulatory DNA sequence might function to control the transcription of all 1300 OR genes, such that an individual neuron would only express one randomly chosen receptor. For example, DNA recombination at a single active expression site, as observed for trypanosome surface antigens and B and T cell antigen receptors, provides a mechanism to stochastically express one member of a set of genes that mediate cellular interactions with the environment (Van der Ploeg, 1991; Navarro and Gull, 2001; Hozumi and Tonegawa, 1976). We have asked whether DNA recombination is involved in odorant receptor gene choice by generating cloned mice that derive from the transfer of nuclei of mature olfactory sensory neurons into oocytes. Cloned mice express the full repertoire of odorant receptors, indicating that irreversible alterations in DNA do not accompany odorant receptor gene choice (Eggan et al., 2004; Li et al., 2004).

It remains possible, however, that within the genome there exists a single regulatory sequence that might be capable of associating with the promoters of all odorant receptor genes without the requirement for recombination. This model invokes a single enhancer that may act in *trans* to activate only one of the multiple OR promoters. The expression of one cluster of OR genes (including *MOR28*, *MOR10*, *MOR83*, and *MOR29*) in transgenic mice requires an enhancer element, *H*, that resides 75 kb upstream of the *MOR28* gene (Serizawa et al.,

2003). This is the only example of an OR gene cluster that requires a long-range *cis*-enhancer element for transgene expression. Moreover, for some genes, faithful expression can be obtained with as little as 161 bp of 5′-flanking DNA sequences (Rothman et al., 2005). We therefore considered the possibility that the *H* enhancer, which functions in *cis* on its adjacent OR cluster, might also function in *trans* on other OR promoters on different chromosomes to provide the essential transcriptional regulatory machinery necessary to activate OR gene expression. Since the *H* element is a single copy DNA sequence, this would provide a mechanism to explain the singularity of OR gene expression in each sensory neuron.

In this study, we demonstrate that the *H*-enhancer element on chromosome 14 associates with multiple OR promoters on different chromosomes. DNA and RNA fluorescence in situ hybridization (FISH) experiments reveal the colocalization of the *H* enhancer with the single OR allele that is transcribed in a sensory neuron. In olfactory sensory neurons, one of the two *H* alleles is methylated, suggesting that the *H* enhancer is functionally monoallelic. Finally, in transgenic mice bearing additional *H* elements, sensory neurons that express OR pseudogenes also express a second functional receptor. These data suggest a model of receptor gene choice in which a single *trans*-acting enhancer element, *H*, may allow the stochastic activation of only one OR allele in an olfactory sensory neuron.

RESULTS

Initial experiments to test the model of *trans*-enhancement employed chromosome conformation capture, 3C (Dekker et al., 2002), to determine whether OR genes located on multiple different chromosomes can be found in association with the *H* enhancer. In this experimental approach, nuclei from sensory neurons were treated with paraformaldehyde to crosslink proteins that may mediate the association of the *H* enhancer with an OR promoter. The crosslinked chromatin was digested with a restriction enzyme, and DNA ligase was added to covalently link DNA sequences that colocalize in the nucleus independent of their chromosomal location. After removing the protein, DNA sequences that associate with the *H* enhancer were identified by polymerase chain reaction (PCR) (Figure 1A).

PCR reactions were initially performed with a DNA primer from the *H* enhancer and primers complementary to 5′ sequences flanking DNA from eight OR genes that reside on six different chromosomes (Figure 1B). PCR products were observed with primer pairs of *H* and each of the eight OR genes. In each instance, the size of the PCR product was that predicted for the ligation of *H* with 5′-flanking OR DNA. Moreover, the identity of the *H*-OR gene product was confirmed by DNA sequence analysis. The eight OR genes that associate with *H* include genes that are expressed in the different zones in the epithelium. The most abundant PCR products were obtained with primers to *H* and the *MOR28* and *MOR10* genes. These two genes are linked to the *H* enhancer and are the most prevalent OR genes expressed in the sensory epithelium (Young et al., 2003). No PCR products resulting from an *H*-OR gene association are observed if 3C is performed with chromatin from spleen nuclei that do not transcribe odorant receptor genes. As a control for the specific association of *H* with OR promoter sequences in chromatin from sensory neurons, 3C was performed with naked DNA. No evidence for linkage of *H* with OR DNA was observed by PCR analysis. Moreover, the association of *H* with OR genes is specific and is not observed for other highly expressed genes in olfactory neurons. No PCR products are obtained that reflect the linkage of *H* to 5′ DNA flanking either the olfactory marker protein gene (*OMP*) or the cyclic nucleotide-gated ion channel gene, two genes expressed at high levels in olfactory sensory neurons.

In a second set of experiments, we performed 3C analysis on sensory neuron nuclei using inverse PCR primers from within 5′ flanking DNA from the *M71* gene (Figure 1C). This approach does not bias the nature of the PCR products and allows us to identify all DNA sequences that associate with the *M71* promoter. The association of the *M71* promoter with the *H* enhancer should result in the amplification of a 2.3 kb fragment that includes *H* sequences. We observe several specific PCR products by gel analysis. The most abundant 2.3 kb DNA product reveals *M71* sequences linked with *H* DNA as determined by DNA sequence (Figure 1C). The *M71-H* product obtained by 3C in nuclei from olfactory epithelia is not observed in the absence of DNA ligation, nor can it be obtained from spleen nuclei. A second intense band at 1 kb was also sequenced and revealed a unique nontranscribed sequence from chromosome 13. This sequence is not found in association with the other olfactory receptor promoters analyzed and therefore the significance of its association with the *M71* promoter is unclear at present.

A final set of experiments involving 3C analysis was performed using reverse primers from within the *H* enhancer to identify the full complement of DNA sequences that associate with *H* in sensory neurons that can be captured by this experimental approach (Figure 1D). Unlike the two previous PCR experiments that generate a single DNA product linking *H* with a specific receptor gene, this approach should capture all of the receptor genes that associate with the *H* element and should therefore generate multiple PCR products. In accord with this prediction, we observe a large array of PCR bands upon 3C analysis (Figure 1D). These products were cloned into a plasmid vector, and individual clones were then selected and identified by DNA sequence.

Three hundred and twenty cloned PCR products were sequenced, and eighty-four reveal the *H* element linked to sequences immediately upstream of OR genes. The remaining 236 sequences show ligation of *H* to repeat sequences, largely LINE elements, scattered throughout the genome, and that do not reside adjacent to expressed

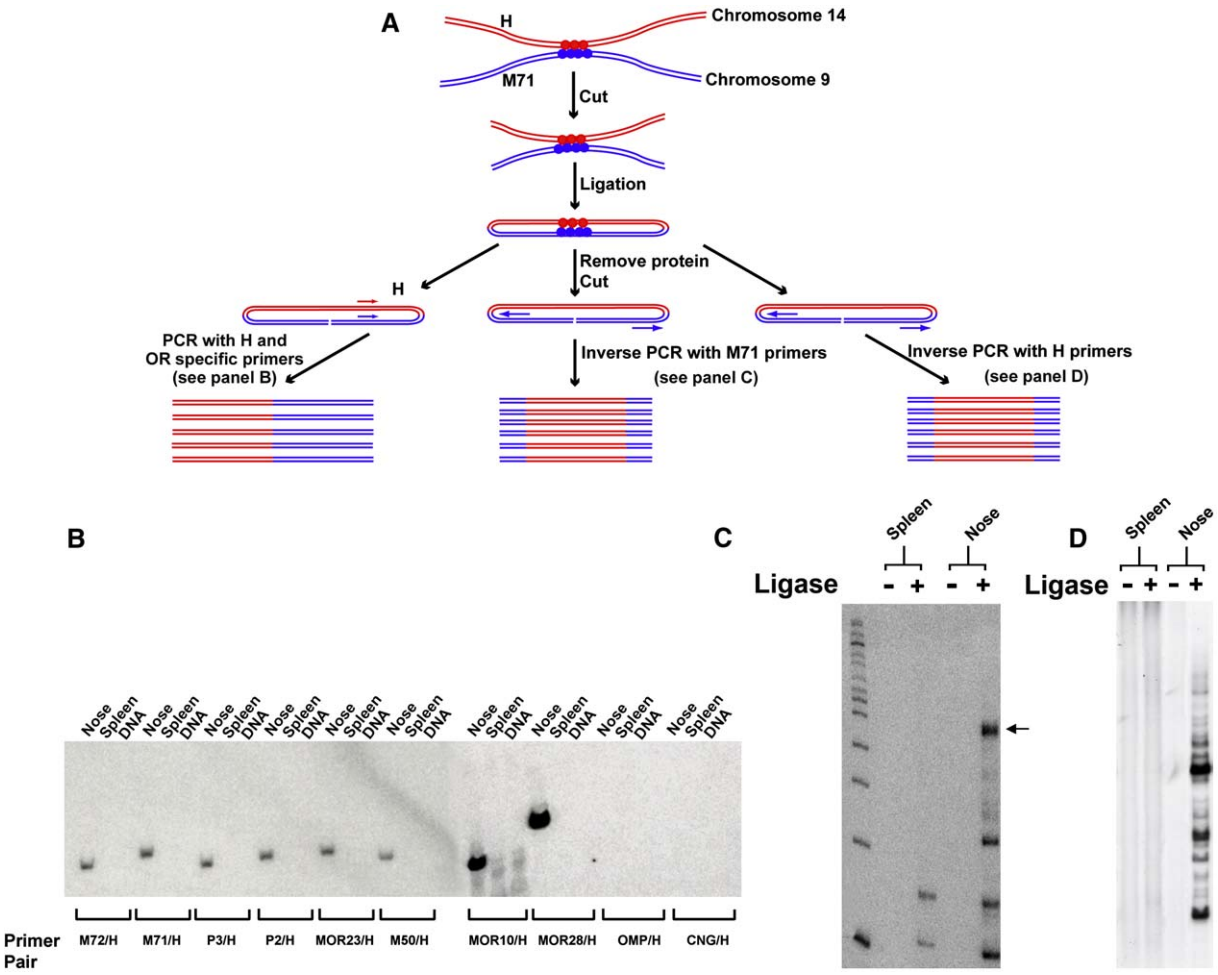

Figure 1. Chromosome Conformation Capture Reveals the Association of the *H* Enhancer with Olfactory Receptor Genes

(A) A schematic representation of three different 3C strategies to reveal the association of the *H* element with OR promoters. On the left, a PCR primer specific to *H* is used with the second primer specific to OR gene promoters. In the center, a pair of inverse primers specific to *M71* is used to isolate sequences associated with the *M71* gene. On the right, a pair of inverse PCR primers specific to *H* is used to detect DNA sequences that colocalize with the *H* element.

(B) Acrylamide gel electrophoresis of the PCR products using a primer specific to *H* together with primers specific to 5′ flanking DNA from genes that are expressed in the olfactory epithelium. PCR was performed on chromatin from olfactory epithelium (Nose), spleen (Spleen), or naked DNA from olfactory epithelium (DNA).

(C) Agarose gel electrophoresis of the PCR products using inverse primers specific to the *M71* promoter following 3C. Either spleen or olfactory epithelium chromatin were subjected to 3C in the presence or absence of ligase. The arrow points to the 2.3 kb band that was amplified from nose chromatin and corresponds to the ligation product of *H* with *M71* sequences.

(D) Acrylamide gel electrophoresis of PCR products using inverse primers specific to the *H* enhancer. 3C was performed in the presence or absence of ligase and subjected to PCR with inverse primers specific to the *H* element. The products were then cloned and analyzed by DNA sequencing.

genes. Forty-eight of the eighty-four sequences derive from the *MOR28* promoter and sixteen from the *MOR10* promoter, two genes linked to the *H* enhancer on chromosome 14. The 20 remaining clones are different and contain sequences that flank OR genes on 12 different chromosomes. These 20 OR genes appear to be a random subset of the OR gene family (see Experimental Procedures). The frequency of ligation of *H* to the linked *MOR28* and *MOR10* genes following 3C is far greater than any other receptor gene. This difference may in part reflect the observation that these genes are expressed about ten times more frequently than other olfactory receptor genes (Young et al., 2003). However, given the frequency of *H-MOR28* clones, it is likely that the 3C procedures exhibit a strong bias toward short-range *cis* interactions. Despite this bias, we continue to observe the specific ligation of *H* to multiple unlinked OR genes. Thus, three independent experimental approaches to chromosome conformation capture reveal the specific association of OR promoters on multiple chromosomes with a single regulatory element, the *H* enhancer on chromosome 14.

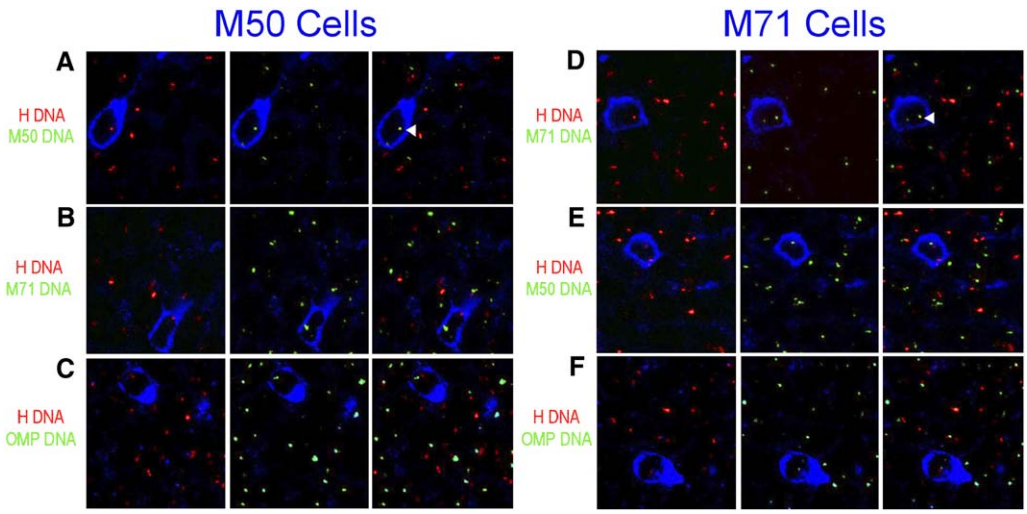

Figure 2. DNA FISH Reveals the Colocalization of the *H* Element and OR Genes

(A) Cells expressing M50 were identified with an M50-specific antibody directed against the C terminus of the receptor (blue). A digoxygenin-labeled (DiG) DNA probe was used to detect the *M50* locus and was visualized with FITC-conjugated anti-DIG antibody (green). A biotin-labeled DNA probe was used to detect the *H* enhancer locus and was visualized with rhodamine-conjugated neutravidin (red). The arrowhead points to the colocalization of the *H* and *M50* signals in the nucleus.

(B) In cells expressing M50 (blue), the *M71* locus (green) does not colocalize with the *H* enhancer locus (red).

(C) In cells expressing M50, the *OMP* locus (green) does not colocalize with the *H* enhancer locus (red).

(D) Cells expressing M71 were identified with an M71-specific antibody directed against the C terminus of the receptor. A DIG-labeled DNA probe was used to detect the *M71* locus and was visualized with FITC-conjugated anti-DIG antibody (green). A biotin-labeled DNA probe was used to detect the *H* enhancer locus and was visualized with rhodamine-conjugated neutravidin (red). The arrowhead points to the colocalization of the *M71* and *H* signals.

(E) In cells expressing M71, the *M50* locus (green) does not colocalize with the *H* enhancer locus (red).

(F) In cells expressing M71, the *OMP* locus (green) does not colocalize with the *H* enhancer locus (red).

Colocalization of the *H* Enhancer with OR Promoters

We next performed in situ hybridization experiments coupled with immunohistochemistry to visualize the association of *H* with OR genes in sections through the olfactory epithelium. Tissue sections were stained with an antibody to M71 to identify the subpopulation of sensory neurons that express the *M71* gene. *M71* and *H* DNA probes were labeled with digoxygenin and biotin-substituted nucleotides, respectively. After in situ hybridization, the relative positions of the probes were visualized with differentially labeled fluorescent antibodies (Figure 2). If the *H* enhancer is involved in the activation of unlinked OR genes, this would predict the colocalization of *H* DNA with *M71* DNA only in those cells expressing the M71 receptor.

Probes for the *M71* gene reveal two hybridizing loci in 98% of the nuclei. Probes for *H* reveal two distinct loci in 67% of the cells, suggesting that this probe is hybridizing less efficiently. Cells expressing M71 were identified by immunohistochemistry with an antibody to the C terminus of the receptor, an epitope that is conserved in M71 and M72. Moreover, the BAC probe we have used for hybridization includes both *M71* and *M72* sequences. Therefore our FISH and immunohistochemical experiments will recognize both M71- and M72-expressing cells. In 28% of the cells expressing M71, the *M71* gene colocalizes with *H* DNA despite the fact that these two sequences reside on different chromosomes (Figure 2; Table 1). In M50-expressing cells, *M50* DNA colocalizes with *H*-enhancer DNA in 29% of the cells. We define colocalization as 25% overlap of pixels from the *H* and OR signals on sections from a Z series. The inability to detect colocalization of *H* with OR genes in all cells is likely due to our inability to retain these interchromosomal interactions stoichiometrically throughout the fixation and denaturation procedures.

In control experiments, FISH was performed with *H* DNA and either *M50* or *OMP* DNA in cells expressing the M71 receptor. In situ hybridization with *M50* DNA shows colocalization with *H* in only 3.6% of the M71-expressing cells. Moreover, *H* does not colocalize with the *OMP* gene (3.2% colocalization) that is expressed at high levels in these cells (Figure 2; Table 1). Colocalization of either *M71* or *OMP* in M50-expressing cells is observed at

Table 1. Frequency of Colocalization of *H* with Different Loci in *M50*- or *M71*-Expressing Cells

	M50 Cells	*M71* Cells
H/M50	103/356 (29%)	10/275 (3.6%)
H/M71	5/203 (2.4%)	90/312 (28.8%)
H/OMP	5/305 (1.6%)	9/278 (3.2%)

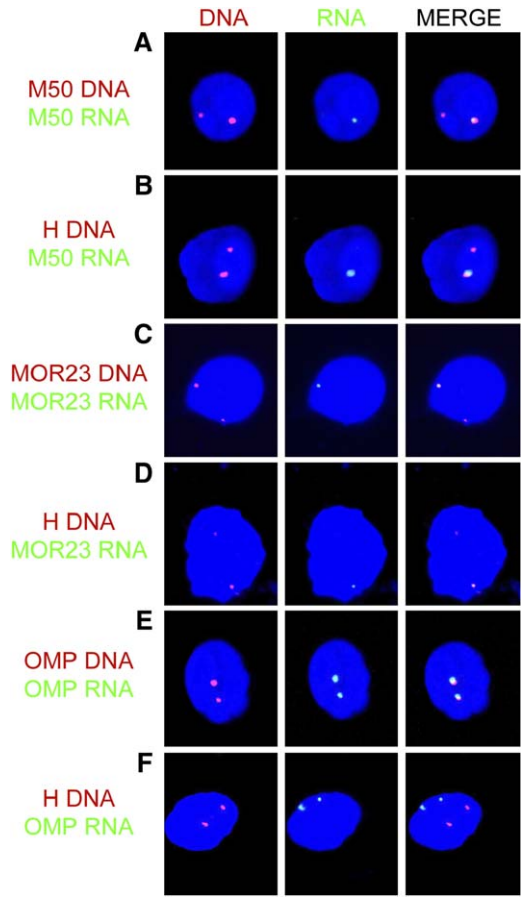

Figure 3. RNA and DNA FISH on Sensory Neuron Nuclei Reveal Colocalization of *H* Element with the Transcriptionally Active OR Allele

(A) Combined RNA and DNA FISH on nuclei from olfactory epithelium. DIG-labeled oligo-nucleotides complementary to the RNA of the olfactory receptor M50 were used for the detection of the nuclear transcripts of *M50*. The signal was visualized with FITC-conjugated anti-DIG antibody (green). A biotin-labeled DNA probe was used for the detection of the *M50* locus and was visualized with rhodamine-conjugated neutravidin (red). The nuclei were counterstained with TOTO-3 (blue). RNA FISH detects the single transcriptionally active *M50* allele whereas DNA FISH detects both *M50* alleles.

(B) RNA FISH to detect *M50* nuclear RNA (green) combined with DNA FISH detecting the *H* enhancer (red). The nuclei were counterstained with TOTO-3 (blue). The transcriptionally active *M50* allele colocalizes with one *H* allele.

(C) RNA FISH to detect nuclear transcripts of the olfactory receptor MOR23 (green) combined with DNA FISH to detect the *MOR23* locus (red). The nuclei were counterstained with TOTO-3 (blue). The RNA signal colocalizes with only one *MOR23* allele.

(D) RNA FISH to detect nuclear RNA of the olfactory receptor MOR23 (green) combined with DNA FISH to detect the *H* enhancer (red). The nuclei were counterstained with TOTO-3 (blue). One *H* allele colocalizes with the *MOR23* RNA signal.

(E) RNA FISH to detect *OMP* nuclear RNA (green) combined with DNA FISH to reveal the two *OMP* loci (red). The nuclei were counterstained with TOTO-3 (blue). RNA transcripts are observed at both the *OMP* alleles.

(F) RNA FISH to detect *OMP* nuclear RNA (green) combined with DNA FISH to detect the *H* enhancer (red). The nuclei were counterstained with TOTO-3 (blue). The *H* loci do not colocalize with the transcriptionally active *OMP* alleles.

frequencies of 2.4% and 1.6%, respectively (Figure 2; Table 1). These experiments are consistent with observations made upon chromosome conformation capture and provide visual evidence for the colocalization of the *H* enhancer with the single OR gene that is expressed in a given sensory neuron.

H DNA Colocalizes with OR Receptor RNA

An olfactory sensory neuron expresses a single receptor gene from only one of the two alleles. We have combined DNA FISH with RNA FISH to determine whether the *H* element colocalizes with the receptor allele that is actively transcribed. We found it difficult to simultaneously perform nuclear RNA and DNA FISH on sections through the epithelium. We therefore dissociated olfactory sensory neurons and performed RNA FISH on nuclei to identify the subpopulation of neurons expressing the *M50* gene (Figure 3). We observe intense staining, presumably reflecting hybridization to nascent RNA at a single nuclear site in about 0.1% of the neurons. A similar frequency of staining is observed for the *MOR23* gene that also reveals a single locus of RNA hybridization. To verify that this locus represents nascent RNA, we combined RNA and DNA FISH with probes specific for the *M50* sequence. We observe colocalization of one of the two *M50* DNA alleles with *M50* RNA in over 98% of the cells that express *M50* RNA. RNA FISH with the control *OMP* gene reveals staining at two discrete sites in the nucleus reflecting the transcription of *OMP* RNA from both alleles.

DNA FISH using *H*-enhancer DNA reveals the colocalization of *H* DNA with nascent *M50*-receptor RNA in 85% of the cells expressing *M50* RNA (Figure 3). Colocalization of *H* DNA is also observed with *MOR23* RNA transcripts at similar frequencies in cells expressing this gene. In control experiments, the colocalization of *H*-enhancer DNA with nascent *OMP* RNA transcripts is observed in less than 1% of the sensory neurons (Figure 3). These experiments demonstrate the association of the *H* enhancer with the OR allele expressed in a given sensory neuron.

One *H* Allele Is Methylated in Sensory Neurons

If the localization of the *H* enhancer activates an OR gene transcription, how is the expression of a single receptor gene assured in the presence of two *H* alleles in the cell? It is possible that one *H* allele is methylated, leaving a single functional *H* enhancer in each sensory neuron. We have therefore employed bisulfite modification of DNA to examine the pattern of cytosine methylation of the *H*-enhancer sequences. Exposure of genomic DNA to bisulfite results in the conversion of cytosine residues to thymidine, whereas methylation on cytosine prevents this modification. PCR amplification of the products of

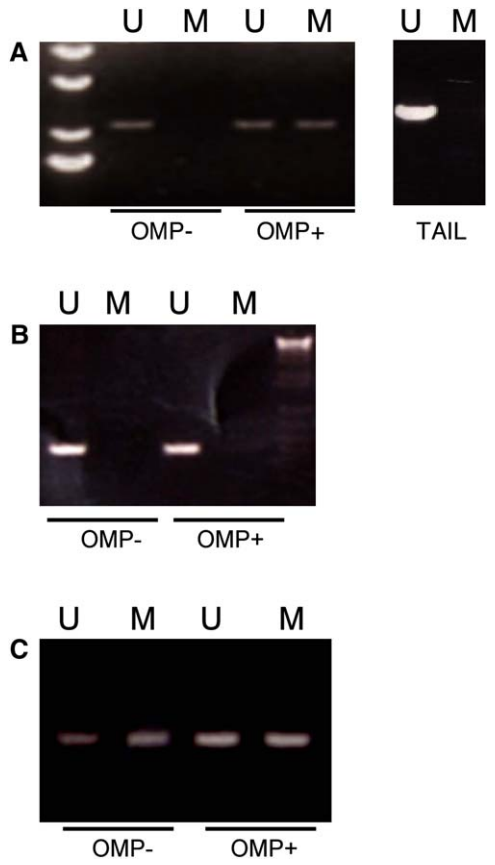

Figure 4. Methylation of One Allele of *H* in Sensory Neurons

Cells from the olfactory epithelium were dissociated and sorted into *OMP*-positive and *OMP*-negative populations. The DNA from each population was treated with bisulfite and analyzed by PCR with primers specific for the DNA products of bisulfite modification of methylated (M) and unmethylated (U) DNA.

(A) PCR with primers specific to methylated and unmethylated products of bisulfite modification of *H* reveals DNA methylation of one allele only in the OMP^+ population.

(B) Methylation-sensitive primers specific to *OMP* reveal that both *OMP* alleles are unmethylated in both cell populations.

(C) PCR with methylation-sensitive primers specific to the parentally imprinted gene, Igf2 reveals that one of the two alleles is methylated in both populations.

bisulfite modification followed by DNA sequence analysis can be used to determine levels of cytosine methylation along specific sequences.

Cells from the olfactory epithelium of mice expressing an *OMP-IRES-GFP* allele were sorted to obtain pure populations of GFP^+ sensory neurons that express OR (Shykind et al., 2004). GFP^- cells were obtained as controls. DNA from the two populations was then treated with bisulfite and analyzed by PCR. Primers from within the *H* element were designed to distinguish the converted unmethylated cytosines from the protected methylated cytosines in DNA. In GFP^+ sensory neurons, we observe methylation of a subset of *H* alleles, whereas no evidence of methylation is observed in the GFP^- OMP^- cell population (Figure 4). Similarly, methylation is not observed on *H* sequences in DNA prepared from tail (Figure 4). The observation that *H* alleles are modified in sensory neurons is not a consequence of incomplete bisulfite modification since complete conversion is observed under the same conditions with the unmethylated DNA preparations from GFP^- or tail cells (Figure 4). Although the PCR reveals methylated and unmethylated products of equal intensity, we cannot accurately quantitate the ratio of modified and unmodified alleles since the primer pairs differ for the two PCR reactions.

The PCR products following bisulfite treatment were cloned and sequenced, and this revealed that the majority of the protected cytosines in the *H* element are located in CpA dinucleotides. Thirty-one of 154 CpA dinucleotides are methylated in each of 10 DNA samples analyzed from sensory neurons. CpA methylation has been reported previously only in ES cells, and the enzyme capable of this modification is a de novo DNA methyltransferase, Dnmt3A, an enzyme expressed in high levels in olfactory epithelium (MacDonald et al., 2005; Ramsahoye et al., 2000). Although the consequence of this modification has not been determined, we suggest that the CpA methylation marks one of the two *H* alleles and results in the inactivation of *H*, leaving only one functional enhancer in sensory neurons.

Transgenic Mice with Additional *H* Sequences Express Two Receptor Genes

The presence of one functional *H* allele in the nucleus could assure the expression of only a single OR gene. The introduction of additional *H* enhancers into the genome via transgenesis, however, should result in the expression of multiple receptor genes in a single sensory neuron. To test this, we generated multiple lines of transgenic mice bearing random insertions of the *H* enhancer in the mouse genome. In one line, a tandem array of six *H* sequences was integrated at a single locus on chromosome 7. In initial experiments, we asked whether cells stably expressing two receptors could be observed in these transgenic mice. Immunohistochemistry with antibodies specific for two receptors, MOR28 and MOR50, fails to reveal cells that simultaneously express these two genes in either wild-type or transgenic mice. In a second experiment, we combined antibody staining for MOR28 and M50 with RNA in situ hybridization using a mixture of RNA probes that detect ten different olfactory receptor genes. We did not observe coexpression of any of the ten OR genes with either M50 or MOR28 in either wild-type or transgenic mice (data not shown).

Previous studies have suggested that the expression of a functional odorant receptor elicits a feedback signal that suppresses the activation of additional olfactory receptor genes. In transgenic mice with two functional *H* loci, we would predict that a single receptor gene might be activated either by the endogenous or transgenic *H* enhancer. This could elicit a feedback signal that would precede and

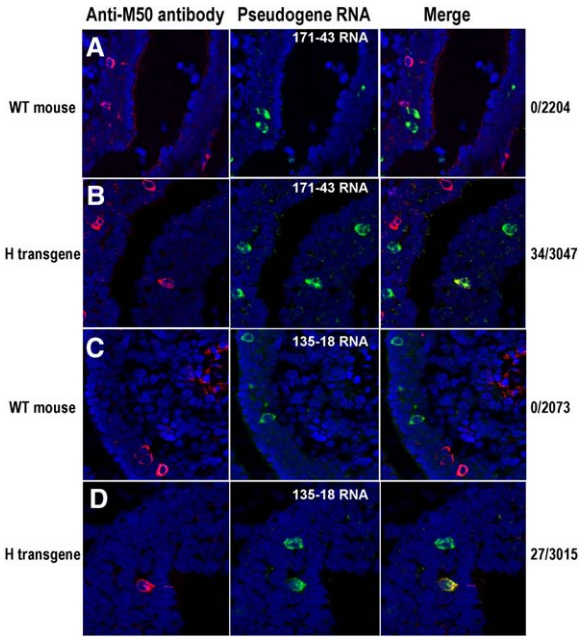

Figure 5. Coexpression of M50 and OR Pseudogenes in *H* Transgenic Mice

Cells expressing the M50 receptor were identified with an M50-specific antibody (red). Digoxigenin-labeled (Dig) antisense RNA probe was used to detect cells expressing the pseudogene RNA (green). The frequency of cells coexpressing pseudogene OR and M50 is indicated on the right of the merged panel.

(A) Sections through the olfactory epithelium of a wild-type mouse hybridized with a riboprobe to the pseudogene OR*171-43* reveal that no cells coexpress the pseudogene and M50.

(B) Sections through the olfactory epithelium of a mouse bearing the *H* transgene reveal that cells coexpress M50 and OR*171-43* RNA (yellow in the merged lane).

(C) Sections through the olfactory epithelium of a wild-type mouse hybridized with a riboprobe to the pseudogene OR*135-18* reveal that no cells coexpress the pseudogene and M50.

(D) Sections through the olfactory epithelium of a mouse bearing the *H* transgene reveal that cells coexpress M50 and OR135-18 RNA (yellow in the merged lane).

suppress the choice of a second OR gene by the additional *H* enhancers. If, however, one of the two functional *H* alleles activates a pseudogene, this will fail to elicit a feedback signal allowing the second *H* enhancer to choose a functional OR gene. This would result in the stable expression of both a pseudogene and a wild-type OR gene in a single neuron. In neurons from wild-type mice, however, we would not predict the coexpression of two genes since these cells maintain only one functional *H* element.

We therefore asked whether we could detect the simultaneous expression of OR pseudogenes with M50 in wild-type and transgenic mice. The expression of the M50 receptor was detected with specific antibodies whereas pseudogene expression was probed by RNA in situ hybridization. In wild-type mice, we identified 4000 cells expressing either pseudogene *171-43* or *135-18* and none of these cells coexpressed M50. In the transgenic line, however, about 1% of the cells expressing either pseudogene also express the functional M50 receptor (Figure 5). Similarly, about 0.3% of the 6000 cells expressing pseudogenes also express the MOR28 receptor. In the zone of pseudogene expression, about 1% of the neurons express M50 whereas about 0.3% of the cells have chosen *MOR28*. These observations suggest that all cells expressing a pseudogene will coexpress a second functional OR receptor. The introduction of an additional *H* locus allows for the expression of two receptor genes, but feedback repression dictates that one of these genes is a nonfunctional OR pseudogene. These data provide additional support for a model in which the active *H* enhancer stably interacts with a single OR promoter to assure the expression of only one functional OR in a sensory neuron.

DISCUSSION

H Association and OR Gene Expression

An olfactory neuron in the mouse expresses one of 1300 odorant receptor genes. The receptor is found in the dendrite, where it recognizes odorant, as well as in the axon, where it plays a role in guiding axons to spatially invariant loci in the olfactory bulb (Barnea et al., 2004; Wang et al., 1998; Mombaerts et al., 1996). Thus, the choice of a receptor dictates both the functional identity of the sensory neurons and its wiring pattern in the brain. The expression of a single receptor gene in a sensory neuron is therefore an essential feature of the organization and function of the olfactory system. One attractive mechanism to assure the stochastic choice of a single receptor employs a regulatory DNA sequence present as a single copy in the genome that is required for the expression of all odorant receptor genes. The stable engagement of such a singular DNA element with an OR promoter could result in transcriptional activation of only one receptor in a given neuron. A candidate DNA regulatory element emerged upon the identification of the *H* enhancer (Serizawa et al., 2003). Initial studies demonstrated that *H* is required for the expression of a linked cluster of OR transgenes. Long-range enhancer elements have not been found for other receptor genes, and faithful expression of OR transgenes has been observed with as few as 161 bp of 5′ flanking DNA (Rothman et al., 2005). In this study we present evidence for a model in which the *H* element acts more globally in the nucleus to transactivate multiple OR genes independent of chromosomal location.

This model predicts that *H* DNA associates with a different OR gene in different neurons, resulting in the activation of only one OR gene in each cell. Three approaches to chromosome conformation capture provide evidence for the specific association of *H* with a large number of OR gene promoters. The physical association of these unlinked DNA sequences is only observed in nuclei from olfactory epithelium. Further evidence for colocalization of the *H* enhancer with an expressed OR gene emerges from DNA and RNA FISH experiments. These experiments allow us to visualize the colocalization of the *H* enhancer

with the single OR allele that is transcribed in a sensory neuron.

If the association of *H* with an OR promoter indeed activates OR gene transcription, this is likely to be mediated by protein-protein interactions that recruit *H* to the OR promoter. This implies that a common protein must reside at all OR promoters capable of interacting with proteins on the *H* element. Such a model does not exclude the existence of other enhancer elements that may act in *cis* or *trans* and participate in receptor gene choice. An alternative model postulates that OR gene transcription is restricted to a localized structure in the nucleus (West and Fraser, 2005) that contains the factors necessary for OR gene expression. All OR genes might cluster at this nuclear location along with their *cis*-linked regulatory elements such as the *H* enhancer. Only a single OR gene, however, is chosen from among the 1300 localized genes. In this model, *H* may colocalize with unlinked OR genes but would play no role in regulating their expression. In cells expressing the *M71* gene, however, the *H* element is found in association with *M71* DNA but not *M50* DNA, a finding inconsistent with this model.

Genetic Evidence that *H* Functions as a *trans* Enhancer

Transgene experiments that introduce additional *H* elements into the chromosome provide further support for a model of *H* as a *trans* enhancer capable of activating multiple OR genes. We have generated transgenic animals that maintain a third *H* locus consisting of six tandemly linked *H* elements inserted at a single site on chromosome 7. In this strain, cells that express transcribed pseudogenes also express a second functional receptor gene at high frequency. For example, 1% of the cells expressing the pseudogene *171-43* also express a functional M50 receptor. If we assume that 100 different OR genes can be expressed in the zone of this pseudogene, then all cells that express a pseudogene are likely to also express a functional receptor gene. In wild-type mice, cells expressing this pseudogene do not express the MOR50 receptor. These observations support a model in which a wild-type cell contains one functional *H* element capable of activating a single OR gene. The expression of a functional receptor will result in a feedback signal that stabilizes receptor gene choice. Neurons that initially express a pseudogene fail to receive this signal and switch until a functional receptor is chosen. In cells from transgenic animals with a second functional *H* locus, the selection of a wild-type receptor by one active *H* will suppress subsequent choice of a second OR gene. The initial choice of a pseudogene in these transgenic animals will fail to elicit feedback, and the second active *H* is free to select a functional receptor, stabilizing the coexpression of both a pseudogene and a functional OR gene.

Previous transgene experiments that introduce an extra copy of the *H* element (Serizawa et al., 2003) can also be interpreted in the context of a model of *H* as a *trans* enhancer. Mice that harbor a third *H* element as part of a YAC transgene encompassing *H* and the *MOR28* gene cluster reveal neurons that express the *MOR28* transgene. These cells do not express additional functional receptor genes but coexpress pseudogenes. However, if the *MOR28* gene is mutated in the YAC transgene and no longer encodes a functional receptor, the mutant *MOR28* is expressed along with a second receptor gene. Our interpretation of these results is that the addition of a second functional *H* locus allows the expression of a pseudogene along with the functional *MOR28* receptor. The expression of a mutant receptor from the YAC transgene fails to elicit a feedback signal allowing the formation of a second *H*-enhancer complex in the face of continued mutant *MOR28* expression also resulting in the coexpression of one functional gene with a pseudogene. Deletions of the *H* region could provide further evidence for a function for *H* as a global OR gene enhancer. However, *H* is imbedded in a 70 kb stretch of repetitive elements that have made it difficult to delete this region by homologous recombination in ES cells.

Two *H* Enhancers: One OR Gene

An olfactory neuron contains two alleles of the *H* enhancer but expresses only a single receptor gene. If the *H* enhancer serves a transactivation function, then this regulatory element must be functionally monoallelic. We have explored the possibility that one endogenous *H* domain undergoes methylation, leaving only one functional *H* enhancer. We observe DNA methylation on a subset of *H* sequences at 31 of 154 CpA sites. These sites are reproducibly methylated and determined from the sequence of 20 independent clones obtained following bisulfite modification. This nonclassical CpA methylation of *H* is only observed in sensory neurons. Methylation of CpA residues can be accomplished by the de novo methylase, Dnmt3a, and has been observed in DNA from embryonic stem cells. Although the functional consequences of CpA methylation remain unknown, we suggest that the modification of one *H* allele in neurons might render this sequence functionally monoallelic. A second mechanism to assure the expression of a single OR gene on a sensory neuron emerges from the observation that the expression of a functional odorant receptor elicits a feedback signal that stabilizes the initial OR gene choice and suppresses the expression of additional OR genes (Serizawa et al., 2003; Lewcock and Reed, 2004; Shykind et al., 2004). The formation of a transcription complex between one *H* enhancer and one OR gene may be a slow process that results in receptor expression. This will generate a feedback signal that suppresses switching and stabilizes OR gene choice for the life of the neuron.

Transvection and *trans* Enhancers

The model we propose, a single enhancer element in the genome that is required for transactivation of all odorant receptor promoters exploits the singularity of unique DNA sequences in the genome to assure the expression of a single receptor gene. The ability of a regulatory

element on one chromosome to influence gene expression on a second chromosome was first described for the bithorax complex of *Drosophila* and has subsequently been observed at several additional loci in the fly genome (Lewis, 1954; Gelbart, 1982; Geyer et al., 1990; Zimmerman et al., 2000). One common mechanism of transvection involves the transactivation of one allele by the enhancer on the second allele. Transvection effects most often involve somatic pairing between homologs such that the interchromosomal interactions engage the two alleles of the same gene. More rarely, experimental genomic manipulations reveal transvection between nonallelic sequences (Kravchenko et al., 2005).

The model of *trans* enhancement we suggest for the control of odorant receptor genes involves transvection between two nonallelic loci, the *H* enhancer, and an OR promoter. Recent data in mice reveal the association of regulatory sites within the interferon-γ locus on chromosome 10 with sites on chromosome 11 within a locus encoding the T_H2 cytokine genes interleukin 4, 5, and 13 (Spilianakis et al., 2005). This association of nonhomologous chromosomes regulates distinct patterns of gene expression in different T helper cell populations. Similarly, the imprinting control region in the Igf2/H19 locus on chromosome 7 colocalizes with the Wsb1/NF1 locus on chromosome 11, and this association regulates expression of the Wsb1/Nf1 gene (Ling et al., 2006). In olfactory sensory neurons, we observe the *H* enhancer in stable association with olfactory receptor gene promoters despite the fact that these DNA sequences are widely distributed along most chromosomes. These nonallelic, interchromosomal interactions may be relatively infrequent. However, the presence of 1300 functional odorant receptor genes capable of interacting with the *H* enhancer will significantly enhance the probability of a productive interaction. Thus, the presence of a large number of potential target genes might provide a mechanism to overcome a low frequency of nonallelic interchromosomal pairing events.

A single *H*-enhancer element that can transactivate all 1300 OR promoters provides a mechanism for the stochastic choice of a single receptor gene in an individual sensory neuron. The presence of a single receptor affords a neuron with recognitive specificity and also contributes to the precision of axon targeting to a single locus in the olfactory bulb. In systems in which multiple chemosensory receptors are expressed in a single neuron, as in *C. elegans* or mammalian taste cells (Wes and Bargmann, 2001; Zhao et al., 2003), the recognition capacity is maintained but the discriminatory power of the system is dramatically reduced. The mammalian nose contains millions of olfactory sensory neurons such that the stochastic choice of a single OR gene assures that every receptor will be expressed in a large population of cells. However, the frequency of receptor choice will be biased by the relative affinity of the *H* enhancer for each of the multiple different OR gene promoters.

The stochastic choice of one member of a set of genes has been described for other gene families that modulate cell interaction with a rapidly changing environment. Surface antigen variation in trypanosomes as well as antibodies and T cell receptors in lymphocytes involve systems in which the cell stochastically expresses a single surface receptor from very large gene families. In these systems, the regulated expression of a single gene is mediated by DNA rearrangements such that a given coding sequence is introduced into a single expression site that may reside on a different chromosome (Van der Ploeg, 1991; Navarro and Gull, 2001; Hozumi and Tonegawa, 1976). In other systems exemplified by the β-globin locus, a single regulatory element, the LCR, resides in *cis* to multiple different β-like genes such that only a single β-like gene can be expressed at a given time (Dillon et al., 1997; Tanimoto et al., 1999). Competition for a single expression site or for a single enhancer element, either in *cis* or in *trans*, is a property shared by each of these gene families to assure that only one member of the gene family is chosen. The olfactory system of vertebrates with over 1000 genes provides an extreme example of competition for a singular regulatory element without the requirement for DNA recombination or the restrictions of linkage. This may allow the evolution of adaptive responses to a rapidly changing environment.

EXPERIMENTAL PROCEDURES

Chromosome Conformation Capture, 3C, on Mouse Tissue

Adult mice were perfused with PBS followed by 1% paraformaldehyde. The crosslinking reaction was terminated by perfusion with a solution of 250 mM glycine, and olfactory epithelia and spleens were collected. The tissues were homogenized in PBS, and the cells were centrifuged at 350 g at 4°C for 8 min. The cell pellet was lysed in buffer containing 0.25% TritonX-100, 0.5% NP-40, 10 mM EGTA, 1 mM EDTA, and protease inhibitors (4°C for 10 min). Cell membranes were disrupted by 20 strokes of a dounce homogenizer. The nuclei were collected by centrifugation through a sucrose cushion for 5 min at 1000 g, resuspended in lysis buffer, and centrifuged for 5 min at 500 g without the sucrose cushion. The nuclei from five epithelia were washed twice in restriction enzyme buffer and then resuspended in 1 ml restriction buffer containing 0.3% SDS. After incubation for 1 hr at 37°C with constant agitation, TritonX-100 was added to final concentration of 1.8%, and the lysed nuclei were incubated for an additional hour. Five hundred units of restriction enzyme were then added, and the reactions were incubated overnight at 37°C with constant agitation. An aliquot of the reaction was removed and analyzed by gel electrophoresis and compared with a digest of naked DNA to demonstrate that the digest was complete. The cleaved chromatin was diluted to 0.5 ng/μl and was ligated for 2 days at 4°C with constant agitation followed by incubation at 16°C for 5 hr with additional enzyme and ATP. The reaction was terminated after another hour at room temperature, and the proteins were reverse crosslinked by incubation overnight at 65°C. The samples were then treated for 2 hr with proteinase K at 56°C and extracted with phenol-chloroform.

The purified DNA was digested with a restriction enzyme that cuts between the inverse PCR primers in order to eliminate products from self-ligated templates. A first round of PCR was performed using cold dNTPs for 15 cycles, and then the PCR products were purified using Qiagen PCR purification columns. A second round of PCR was performed using nested primers and radioactive dCTP. The products of these reactions were analyzed by acrylamide gel electrophoresis, and the gels were exposed to film overnight. The bands of interest were cut from the gel and eluted by agitation at 37°C overnight in

0.3 M NaAc. The purified DNA was amplified by PCR with a third set of nested primers, cloned into pGEM T-easy vector (Promega), and analyzed by DNA sequencing. The sequences that correspond to receptors that are not linked to chromosome 14 are from the following receptors: olfr1403,1411 (chromosome 1), olfr1340, olfr273 (chromosome 4), olfr536, olfr2 (chromosome 7), olfr807, olfr801 (chromosome 10), olfr262, olfr1453 (chromosome 19), olfr1506 (chromosome 2), olfr434 (chromosome 6), olfr921, olfr914 (chromosome 9), olfr382, olfr1378 (chromosome 11), olfr1361(chromosome 13), olfr282 (chromosome 15) and olfr113, olfr124 (chromosome 17).

Antibodies against the Olfactory Receptors *M50* and *M71*

We generated guinea-pig antibodies against a unique sequence in the C terminus of the mouse odorant receptor M50 (GenBank accession number AAG45190; amino acids 300–316). We also generated guinea-pig antibodies against a unique sequence in the C terminal of mouse odorant receptor M71 (Genebank accession number AAG29379; amino acids 294–309). A similar sequence with one amino acid substitution is present in mouse odorant receptor M72 (GenBank accession number AAG09780). This antibody recognizes both M71 and M72 receptors.

DNA FISH on Tissue Sections

Olfactory epithelia were dissected from adult mice, incubated in 10 mM EGS, and diluted in PBS for 1 hr at 37°C. The tissues were embedded and 12 μm sections were collected on polylysine-coated slides. The slides were incubated in a solution of 10 mM ethyl in 50% acetic acid for 30 min at 37°C, followed by two 10 min washes in a solution of 0.1% Triton X-100 in PBS. Immunohistochemistry was performed using standard procedures (Barnea et al., 2004). The secondary antibody was crosslinked to the tissue by incubation for 10 min in 10 mM EGS in PBS at room temperature. The slides were then treated with RNaseA for 2 hr at 37°C, followed by incubation in 0.1 M HCl for 5 min at room temperature. The sections were dried by an ethanol series and then denatured by incubation in a solution of 70% formamide in 2×SSC at 75°C for 10 min. The slides were immediately rinsed with ice-cold 2×SSC and dried by an ethanol series. The probes were applied to the slides, and the coverslips were immobilized with rubber cement. Washes and antibody staining were performed using standard techniques (Carter et al., 2002).

RNA FISH on Dissociated Nuclei

Olfactory epithelia were dissected from adult mice and incubated for 10 min in 0.01% trypsin in Ringer's solution containing 20 mM vanadyl-ribonucleoside complex. The cells were further dissociated by teasing with a forceps and a dounce homogenizer and subjected to cytospin for 5 min at 800 g. The slides were immediately incubated for 10 min in cytoskeleton buffer containing 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, 0.1% TritonX-100, and 20 mM vanadyl-ribonucleoside, and then they were fixed in a solution of 4% PFA in PBS containing 5% acetic acid for 15 min at room temperature. The slides were then incubated overnight in 70% ethanol at 4°C and dried by an ethanol series. The probes were applied directly to the dried tissue and incubated overnight at 37°C. As probes, we used sets of 30 digoxigenin-labeled oligo-nucleotides that span the transcripts of the genes *M50*, *MOR23*, and *OMP*. The slides were washed three times for 15 min in a solution of 55% formamide in 2 × SSC at 39°C. The signal was detected by incubation with anti-DIG antibody in a solution of 8% formamide, 0.1% BSA, and 20 mM vanadyl-ribonucleoside complex in 4×SSC. The antibody was crosslinked by 10 mM EGS, and the slides were incubated with RNaseA. DNA FISH was then performed as described above.

Methylation-Sensitive PCR

Cells from the olfactory epithelium of mice expressing an *OMP-IRES-GFP* allele were sorted to obtain pure populations of GFP^+ sensory neurons that express ORs (Shykind et al., 2004). GFP^- cells were obtained as controls. The DNA from each population was treated with bisulfite and analyzed by methylation-sensitive PCR according to the CpG WIZ kit (Chemicon).

Generation of Transgenic Mice with an Additional *H* Sequence

A NotI genomic DNA fragment containing 2.1 kb sequences flanking the *H* region was gel purified and microinjected into the pronuclei of fertilized eggs. Tail DNA from resulting mice was isolated using standard protocols and analyzed for the transgene by Southern blotting.

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