Cloning and Characterization of Putative Vomeronasal Receptor Genes in Mouse

by

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Abstract

Vertebrate olfaction comprises two anatomically distinct systems: the main olfactory system, which responds generally to environmental odorants, and the vomeronasal system, which transduces species-specific cues into stereotyped behavior. Although molecular characterization of the vomeronasal system has lagged a few years behind that of the main olfactory system, putative phermone receptor genes were recently cloned in rat, and their expression pattern in the vomeronasal organ (VNO) characterized by in situ hybridization (Dulac and Axel, 1995). Here, several murine vomeronasal receptor genes are cloned by homology with rat and confirmed to be expressed in the VNO; in addition, progress is made towards determining whether these genes are allelically inactivated, as has been found for the family of seven transmembrane domain proteins expressed in the main olfactory epithelium (MOE) (Chess et al., 1994).

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Introduction

Sensory systems of all varieties have a common overall goal: transduction of stimuli in the external world into an internal representation. Olfaction, or the identification of "odorant" molecules present outside the body, is a facility of remarkable acuity and range, and is of essential importance to the survival and propagation of individuals and the perpetuation of species. Odorants elicit stereotyped behaviors which are undeniably adaptive: whereas olfaction appears to have become an accessory, aesthetic sense in humans, from an evolutionary perspective it is the primal sense. Interestingly, in terrestrial vertebrates olfaction is accomplished by not one, but two anatomically distinct systems (Eisthen, 1992), whose sensory organs are the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), and whose domains of function seem equally separate.[1]

The MOE, though secluded physically within the nasal cavity, is nonetheless exposed to incoming air, and hence volatile odorants. The VNO, which lies anterior to the MOE, along the ventral edge of the nasal septum, is a closed, cigar-shaped organ accessed by liquids--and thus, nonvolatile odorants such as steroids and fatty acids--through a duct-like opening towards the front of the nose (Halpern, 1987) (Figure 1). The epithelia of both organs undergo turnover approximately every two months (Graziadei and Monti Graziadei, 1979) and possess bipolar sensory neurons; however, these epithelial neurons differ morphologically, the MOE possessing cilia at their dendritic termini, the VNO neurons microvilli (Halpern, 1987).

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[1] The function of the vomeronasal organ in humans, contrary to popularization, is unsubstantiated. While the VNO may indeed function in utero (Boehm and Gasser, 1993), in adult humans its epithelium is poorly developed; in addition, no accessory olfactory bulb (see below) has been found. Informal observations show that women living in close contact often experience a synchronization of menstrual cycles; however, suggestive phenomena such as these might ultimately prove to have no basis in the vomeronasal system.

Perhaps the most telling anatomical difference between the MOE and VNO is found in their connectivity. Sensory neurons of the MOE, which presumably bind to incoming odorants, project their axons to the main olfactory bulb, which then sends axons primarily to the olfactory cortex, from which higher cortical areas receive input. In contrast, the VNO projects to a distinct relay station, the accessory olfactory bulb, which projects directly to the amygdala and hypothalamus. Whereas sensory input to the MOE is transduced into interpretations which are involved in innumerable varieties of associational learning, the VNO instead mediates the temporally appropriate execution of stereotyped (unmodifiable, pre-programmed) behaviors and neuroendocrine responses. Thus, the VNO is more aptly described as an organ of chemical communication than of sensation: it receives chemical signals, termed "pheromones," released by other members of the same species, and hence is involved in such intraspecies responses as reproduction, dominance, and parental care (Halpern, 1987).

Yet despite the critically different domains of the olfactory and vomeronasal systems, and the consequent likelihood that the odorant repertoires they respond to are non-overlapping, enough anatomical similarities exist to suggest a conceivable evolutionary relationship between them. At least two ongoing lines of work are addressing this overarching question indirectly, and an extension of one of them is initiated by the present study. In addition, it is important to note that, even if the similarities between the systems prove merely the result of convergent evolution, comparisons are likely to shed light upon the concomitant elucidation of the molecular and cellular mechanisms by which they succeed so remarkably well as sensory systems.

The first thread of evidence addresses the possibility of common molecular components in the intracellular signal transduction pathways held responsible for the transduction of odorant binding into electrical signals. Evidence in the main olfactory system for a mechanism involving G-protein-coupled receptors was bolstered in 1991 by Buck and Axel, who cloned numerous members of a family of about 1000 seven transmembrane domain proteins expressed exclusively in the rat olfactory epithelium. A smaller, 100-member family of VNO-specific receptors was eventually identified in rat, but only after failed attempts at cloning by homology with the MOE

receptors; in fact, the VNO set was found to have a weak homology instead with the family of prostaglandin receptors (Dulac and Axel, 1995). Hence, the VNO receptors appear evolutionarily more closely related to the internal endocrine system than to the main olfactory system. Furthermore, the G proteins and second messengers involved in the two chemical sensory systems are apparently distinct. G_{olf} was identified as the key G-protein in the MOE (Jones and Reed, 1989), and is not expressed in the VNO (Berghard et al., 1996); instead, $G_{alpha\text{-}o}$ and $G_{alpha\text{-}i2}$ are found in the VNO epithelium, in nonoverlapping "zones" (Berghard and Buck, 1996; Jia and Halpern, 1996).[2] Knockout experiments have demonstrated that cyclic AMP is the principal second messenger in the MOE (Brunet et al., 1996). Although a current can be induced in vomeronasal neurons by intracellular cAMP injection (Taniguchi et al., 1996), the adenylyl cyclase expressed in the VNO is a different isoform than that found in the MOE (Berghard et al, 1996); surprisingly, evidence from work with the hamster mounting pheromone aphrodesin implicates a role for IP_3, as opposed to cAMP (Kroner et al., 1996). While the details are not yet clear, the absence of common signal transduction components and lack of homology between the receptor classes [also see footnote 2] certainly mitigates against the model proposed by Dulac and Axel (1995), in which the evolution of terrestrial vertebrates was accompanied by a segregation of the two types of neurons into separate organs, to accommodate the disparate modes of delivery of the two classes of odorants on land.

However, a second trail of evidence, similar patterns of gene expression, promises comparison of the modes of function of the two systems--even if they are indeed unrelated evolutionarily. Most of the research in gene expression to date has been performed in the main

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[2] Intriguingly, the zone of $G_{alpha\text{-}i2}$ expression corresponds exactly with the restricted expression pattern observed by Dulac and Axel (1995) for the VNO receptors, i.e., the apical two-thirds of the region of olfactory marker protein (OMP) expression. Corroboration of the coexistence of two molecular subsystems within the VNO is surfacing, as a second family of VNO-specific receptors has been identified which is confined to the "zone" of Go-positive cells (Herrada and Dulac, 1997; Matsunami and Buck, 1997). This family displays homology to neither previous family of olfactory receptors, but instead to the parathyroid hormone receptor (40%) and the metabotropic glutamate receptors (about 15%)--thus, the molecular data continues to hint that the vomeronasal system has a common ancestor with the endocrine system.

The heritage of the main olfactory system, however, may also be remotely linked with the endocrine system. Recent work has demonstrated that a specific repertoire of main olfactory receptors is expressed in mammalian spermatozoa, there of conceivable utility in chemotaxis (Vanderhaeghen et al., 1997).

olfactory system. In situ hybridization experiments indicate that any given MOE receptor is expressed in a stochastic distribution of cells across the epithelium in catfish (Ngai et al., 1993b). Expression patterns are similar in rat, yet each receptor is found only in cells of one of four discrete (but overlapping) topographic zones; in addition, the sparseness of cells expressing each receptor suggests that each cell in the epithelium expresses only one of the 1000 receptors (Vassar et al., 1993; Ressler et al., 1993). The genes encoding these receptors are found in numerous clusters dispersed throughout the mammalian genome, yet members of a cluster are expressed in cells of various epithelial zones (Sullivan et al., 1996). The clusters appear to have been generated by repeated duplication of an ancestral repertoire, enabling divergence within several subfamilies of receptor genes (Ben-Arie et al., 1994).

Furthermore, it has been demonstrated that "random allelic inactivation" leads each olfactory receptor neuron to express only one allele--either maternal or paternal--of the particular receptor it has selected (Chess et al., 1994). The evidence is thus consistent with a hierarchical regulatory model which ensures that only receptor gene is expressed per cell: allelic inactivation renders silent one entire allelic array of genes, and subsequently a gene on the active array is stochastically selected by a cis-regulatory element which requires the concurrent binding of an epithelial zone-specific transcription factor (Chess et al., 1994). (The problem of selecting a genomic locus from among the dozen or so distributed throughout the genome adds a layer of complexity, but does not fundamentally alter the problem, nor its proposed solution.)

The expression in each epithelial cell of only one or a small number of the thousand or so MOE receptor genes provides the raw materials for specificity within the system, but how is sensory transduction ultimately achieved? While each receptor is expressed in a stochastic array of epithelial cells, axons from those cells converge upon a small number of common glomeruli in the olfactory bulb; this suggests that the cortex associates each odorant with a specific pattern of activity in the bulb (Vassar et al., 1994). Crucial unanswered questions in this system thus include: How does a cell decide which olfactory receptor gene to express? I.e., what are the mechanisms underlying allelic inactivation and the cis stochastic process by which a gene is

presumably selected from the remaining array? And, how are these mechanisms involved in the convergence in the bulb of axons from cells expressing the same receptor?

Progress towards an understanding of sensory coding in the vomeronasal organ is in a slightly earlier phase; however, available data suggest that comparisons with the main olfactory system are pertinent. For example, the pheromone receptors have similar expression patterns, in terms of both randomness of disposition and consistency with a one-receptor-per-cell model (Dulac and Axel, 1995). Glomeruli are found in the accessory olfactory bulb, and preliminary data suggest that convergence of axons from cells expressing the same receptor occurs in this system as well, although with less refinement than observed in the main olfactory system (Catherine Dulac, personal communication). The order-of-magnitude difference in receptor repertoire between the VNO and the MOE, as well as the possibility that all 100 or so pheromone receptor genes reside at one genomic locus (L. Belluscio and R. Axel, personal communication), suggests that the mechanism of receptor gene choice in the VNO may differ; however, the essential mechanistic problem is clearly the same.

Interestingly, the details of sensory integration in the vomeronasal system also differ slightly from the olfactory system. In the MO system, the mitral cells, the output cells from the olfactory bulb, each receive input from only one glomerulus--implying that pattern recognition must be performed by networks in the cortex. In the VN system, on the other hand, multiple glomeruli of the accessory bulb synapse onto each mitral cell; hence, stimulus recognition may occur in the bulb itself, which is not surprising given the absence of cortical output. Nevertheless, the tasks faced by the vomeronasal system, specificity of expression and faithful integration of inputs, appear fundamentally the same as in the main olfactory system.

Hence, ensuring that one (or a very small number) of receptor genes is expressed per cell may be as important in the VNO as in the MOE. Evolutionary heritage notwithstanding, are the mechanisms underlying the expression of pheromone receptor genes similar to those operative upon the odorant receptor genes? The present work, in which pheromone receptor genes are cloned in mouse, opens up the possibility of studying the genomic elements required for

expression of a receptor gene. Progress is also made towards determining whether pheromone receptor genes are allelically inactivated. If they prove to be allelically inactivated, studying the gene regulation of these two large families of related genes in parallel will likely prove synergistic. If they prove not to be allelically inactivated, studying the gene regulation of the VNO genes may uncover strategies for specification of gene expression and axonal targeting not utilized in the main olfactory system, or any other sensory system.

Results

Cloning Mouse Pheromone Receptors

Murine homologs of the vomeronasal receptor family previously uncovered in rat (Dulac and Axel, 1995) were cloned by homology. Attempts to obtain probes for mouse genes by performing the polymerase chain reaction (PCR) on murine genomic DNA using primers to rat cDNAs were unsuccessful. Instead, primers for six of the seven rat cDNAs (all except VN3) were used upon rat genomic DNA to PCR-subclone coding region fragments of approximately 500 bp; these products were then used as a mixed probe, at low stringency, of a mouse genomic library in lambda phage. In a primary screen, 110 positive plaques were identified on duplicate filters; forty of these were replated at lower density, for twelve of which isolatable positive plaques were obtained. Plate supernatants were used to inoculate bacterial cultures at a high multiplicity of infection, and phage DNA was subsequently prepared from eight samples.

Phage DNA was digested with Not I, EcoRI, and Xba I. Not I digests in all cases left the inserts intact; these were subcloned into pBluescript in both orientations, and may prove of later use in identifying DNA elements involved in regulation of the VNO genes. EcoRI and Xba I fragments were shorter; a Southern blot was run, using the rat PCR-subclones (see above) as a mixed probe, to identify fragments containing VNO homology, and these too were subcloned (both members of a doublet were cloned from the Xba I digest of clone 61; these ultimately

appeared identical by sequencing). Orientation by PCR of these smaller subclones with respect to the polylinker, as well as PCR-subcloning of partial mouse coding regions (from phage DNA) using the rat-specific primers, facilitated an eventual complete sequencing of open reading frames of six distinct loci, here designated 10, 22, 25, 61, 103, and 109 (numbers refer to the positive plaques picked in the primary screen of the mouse genomic library; see Figure 3 for sequences aligned with rat genes from Dulac and Axel, 1995). Substantial amino acid identities to the rat genes (Figure 2c), comparable to their homologies with each other (cf. Figure 2a), leaves no doubt that these two sets of genes are subsets of interspecies homologous families.

In addition, significant homologies are observed between the mouse VNO receptors and several diverse G protein-coupled receptors: the Schizosaccharomyces pombe p-factor receptor, which binds a peptide mating pheromone (to gene 25, 20% identity over 79 residues); the human bombesin receptor subtype-3, which is involved in sperm cell function (to gene 10, 47% identity over 17 residues, 25% over 40, 19% over 56, and 34% over 23); and the rat adenosine A3 receptor, expressed during spermiogenesis (to gene 22, 26% identity over 61). These homologies are of comparable weight to that uncovered earlier between the rat VNO receptors and the prostaglandin receptor family (to VN2, 28% over 63); here, they further implicate an ancient evolutionary origin for the vomeronasal receptor family.

Towards an Assay for Allelic Inactivation

To facilitate an assay for monoallelic expression of these mouse vomeronasal receptor genes, the presumed 5'-untranslated regions (5' UTRs) were also sequenced wherever possible (see Discussion for analysis of homologies in this region), and introns were pursued in clones 10 and 22 by reverse transcription followed by PCR (RT-PCR). The 5' UTRs were targeted because many members of the main olfactory gene family have been found to possess an intron there (Chess et al., 1994), and preliminary evidence suggested vomeronasal genes might as well (Catherine Dulac, personal communication). Identifying the locations of splice junctions allows RT-PCR to be performed with primers spanning an intron, thereby eliminating the concern of

contamination from genomic templates; this tool will facilitate testing for monoallelic expression of receptor genes by individual cells, one of the signatures of allelic inactivation, as demonstrated by Chess and colleagues (1994) for the main olfactory system.[3]

In that earlier work, expression of main olfactory receptors was assayed to determine its allelic specificity, if any. In order to distinguish between maternal and paternal alleles, experiments were performed upon the progeny of crosses between two strains of mice, Mus musculus domesticus and Mus spretus (henceforth referred to as musculus and spretus), which possess numerous polymorphisms yet interbreed successfully. RT-PCR products specific to a particular receptor gene could therefore have their allele of origin determined by hybridization with allele-specific probes, as well as DNA sequencing. Limiting-dilution RT-PCR was performed upon single-cell suspensions from the olfactory epithelium: a dilution was found which yielded product from very few pools--rarely enough, in fact, that the majority of these positive pools statistically must have contained only one cell expressing the receptor. It was discovered that most of these positive pools were expressing only one of the two alleles, even if numerous aliquots from the same pool were assayed. Furthermore, the parental origin of the chosen allele varied stochastically, among mice as well as among pools drawn from the same mouse.

In the current study, since it was desired to determine whether the pheromone receptor genes are allelically inactivated as well, introns were sought in their 5' UTRs by performing RT-PCR (with nested primers in a secondary round of PCR) on a musculus mouse VNO RNA preparation. Complete sequencing of 1.4 kb upstream of the start codon of clone 22 revealed promising consensus splice sites; upon subcloning and sequencing an appropriate RT-PCR product, these putative splice sites proved to represent an intron of about one kilobase. Spretus genomic sequence of the region was obtained through PCR, yielding a polymorphic 19-bp deletion which eliminates a Pvu II restriction enzyme site present in musculus. In control trials using a new set of primers (see Experimental Procedures), all F1 RT-PCR product bands cut with Pvu II,

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[3] The second signature feature of allelic inactivation, and of other forms of monoallelic expression as well, is the asynchronous replication of DNA, and will be treated in the Discussion.

indicative of a musculus allele; no products were obtained from spretus RNA. PCR on genomic DNA of F1 mice assured that the alleles were equally amplifiable with the new primers. These results were thus highly suggestive of imprinting, an alternative mechanism by which the VNO receptor genes could be monoallelically expressed.[4] To investigate imprinting of a locus thoroughly, however, mice with reciprocal heritages (with respect to parental sex) must be tested. Successful interbreeding of musculus and spretus mice occurs only when the male is spretus, female musculus; an alternative means was needed to obtain the reciprocal cross at the VNO clone 22 locus. Hence, several progeny of an F1 female and a musculus male were genotyped by PCR (an Afl II site cuts in the coding region of musculus only, due to a polymorphism), yielding a mouse heterozygous for clone 22. However, further control RT-PCRs on all four RNA types (musculus, spretus, and both heterozygotes) suggested that at least two, presumably quite closely related, expressed genes were in fact being amplified. Increasing the stringency by lowering the magnesium chloride concentration and raising the annealing temperature did not clarify the results, nor did designing a new primer overlapping the spretus deletion in clone 22. Recognizing that simultaneous amplification of more than one receptor gene would permit experimentation so long as all the alleles were sequenced within the pertinent region, PCR products were subcloned, but this yielded only sequence from the gene already known. Ultimately, therefore, results were inconclusive with regard to allele specificity of expression. Nevertheless, there remains strong suggestive evidence (see above) that a pheromone receptor gene exists which is present and highly homologous in both strains of mice but transcribed only in musculus. Possible implications of this for speciation and molecular evolution of the gene family are explored in the Discussion.

A second clone, 10, was also investigated upon discovering a weak homology between its 5' UTR and the 5' UTR of a rat cDNA, displaced from alignment presumably by an intervening intron. A candidate RT-PCR product spanning the presumptive intron was cloned, but proved irrelevant sequence. However, it is conceivable, from examining putative splice sites, that the

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[4] Imprinting is essentially different from allelic inactivation in that it involves a germline marking of either the maternal or the paternal locus, causing all expression of a gene to be from one of the two alleles (cf. Bartolomei et al., 1991).

upstream primers used were contained within a second intron almost immediately upstream of the one being sought; PCR using a new upstream primer specific to the intervening sequence may yield success.

Expression Patterns of Murine Pheromone Receptor Genes

In order to confirm the expression patterns of the pheromone receptor genes cloned in mouse, and also to investigate the possibility of their occasional expression in the MOE, in situ hybridization was performed. Whole-mount hybridizations were performed with digoxigenin-labelled riboprobes (as in Vassar et al., 1993) on the anterior portion of adult mouse heads, cut in half medially; cutting a slit lengthwise in an exposed vomeronasal organ allowed both MOE and VNO epithelium to be probed simultaneously almost in their entirety. However, this approach was abandoned because clarity of results in the VNO was severely compromised due to the delicate nature of the epithelium inside the vomeronasal organ.

Trials on coronal sections from E16.5 mice were more successful, both with a coding region riboprobe from the olfactory receptor M12 (Mombaerts et al., 1996) and with probes transcribed from plasmids containing pheromone receptor coding regions as well as about 1.5 kb of adjacent sequence (this describes the probes which hybridized successfully, an example of which appears in Figure 4g/h; see Discussion). No hybridization of VNO probes to the MOE was observed, but data was not extensive. Sense probes were also tested, and in no cases exhibited signal. Finally, in order to visualize the pattern of expression in the VNO in greater detail, sections from dissected adult vomeronasal organs were used (Figure 4a-f). Coronal sections of the VNO reveal a crescent-shaped sensory epithelium medially; on the lateral side of the lumen is a vein (salient during dissection) surrounded by nonneuronal tissue. Hybridization results were comparable to those of Dulac and Axel (1995), though each receptor is perhaps expressed in a smaller percentage of cells in mouse than in rat. Positive signals are punctate (confined to single cells) and distributed randomly across the epithelium--although restricted to the apical two thirds of the region of olfactory marker protein expression, which is itself the apical two thirds of the

sensory epithelium. Additivity of percentages of labelled cells was not observed when a mixture of probes was assayed; thus, the results presented here do not corroborate the model wherein a single receptor gene is expressed by each cell--yet these results are preliminary and therefore do not offer evidence that multiple genes are coexpressed in individual cells.

Discussion

Sequence Analysis of the Pheromone Receptor Genes in Mouse and Rat

The probes used in the library screening which identified these mouse genes were from six rat genes (1, 2, 4, 5, 6, and 7; see Figure 2b), members of five different subfamilies as defined by cross-hybridization on a high-stringency Southern blot (Dulac and Axel, 1995). Thus, in theory the screen would yield mouse homologs from these five subfamilies, possibly including direct homologs--i.e., genes encoding receptors which respond to the same pheromone(s) in each species, differing presumably in a compensatory manner as those pheromones differ, while retaining similar functions for the organism. Though only six mouse lambda clones were analyzed here, it may be noted that four of them appear to be subfamily homologs with rat genes 1 and 2, while only two additional mouse genes group with three other rat genes (Figure 2c; rat gene 6 is an outlier from all rat and mouse genes here). To a first approximation, the amino acid identities (Figure 2c, d) suggest that subfamily members within a species are >85% conserved, whereas between rat and mouse they may be as little as 75% conserved.

Several interesting details emerge from an analysis of these genes, some with possible implications for the manner in which these multigene families have co-evolved, particularly since the divergence of the mouse and rat lineages. For example, while mouse proteins 61 and 109 are 94% identical overall, a closer look reveals complete identity at the nucleotide level for the first 420 bp of the coding region, as well as for at least 240 bp preceding the start codon. By any statistical

measure,[5] this implicates a recent gene conversion event between the upstream portions of these genes, preceded evidently by a gene duplication and subsequent divergence. Such clues begin to place the source of the diversity of the pheromone receptor gene family snugly alongside that of the main olfactory receptor genes, which immediately upon cloning and sequencing displayed evidence of duplications into tandem arrays and of motif mixing through gene conversion events, yet of none of the developmental recombination seen at the immunoglobulin loci (Buck and Axel, 1991; Tonegawa, 1983).

A different sort of issue is raised by comparisons of the other mouse 5' untranslated regions (UTRs). Initially, subcloning EcoRI and Xba I fragments from lambda clones suggested a relationship among genes 61, 10, and 25, because both the lengths of each subclone (about 4kb and 2.5 kb) and the positions of the coding regions within each are identical for the three (109 is also part of this group, but appears to differ due to mutations disrupting the pertinent restriction sites in the 3' UTR). Significant homology between untranslated regions of related genes is frequently observed (e.g., Scamps et al., 1996; Paine et al., 1994). In this instance, pairwise comparisons of genes 10 vs. 25 and 22 vs. 103 display only slightly more nucleotide homology between coding regions than between the 200 bp of 5' sequence upstream of the start codon (i.e., 91% vs. 86%, and 87% vs. 83%; comparisons between the 22/103 group and the 61/109/10/25 group reveal no readily quantifiable UTR homology). There are at least two somewhat competing interpretations for these data. First, perhaps the 5' UTRs are diverging relatively slowly, indicative of the conserved regulatory sequence elements predicted by a stochastic activation model for gene choice. One counterexample for this latter explanation is presented by the 5' UTRs of genes 25 and 22, which differ tremendously in sequence even though the receptors they encode are members of the same family of VNO receptors. An alternative explanation for the highly conserved 5' UTRs is that the coding regions diverge only slightly less rapidly than the noncoding

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[5] E.g., raising the overall nucleotide homology of 97% for the coding region to the 420th power yields an overestimate of the likelihood for the null hypothesis of equivalent variation between the sequences along their entire lengths. The calculation yields 2.8 x 10^{-4}%, and is extremely optimistic as it ignores the 240-bp identity upstream, as well as the rest of the coding region.

regions, i.e., that the coding regions of these genes evolve with unusual rapidity (definitive support for this model requires a comparison of silent mutations versus amino acid replacements, as in Tanaka and Nei, 1989). One would predict, for example, that receptors which bind pheromones would undergo positive selection to maintain binding affinities with those pheromones while they diversify--thereby providing one of many possible components of speciation.

Evidence for such interspecies diversification is suggested by the RT-PCR experiments performed upon gene 22, which it appears the spretus mice, but not the musculus, fail to express. This case, though perhaps extreme, corroborates the reasonable prediction made above: that speciation can be facilitated, or at least accompanied,[6] by a divergence of a pheromone receptor gene within two lineages, resulting in a critical difference in pheromone binding specificity; obviously, this model requires that the pheromones themselves coevolve, diverging concomitantly with the receptors. Such a model for positive selection must be distinguished carefully from that proposed, by Ngai et al. (1993a) for the catfish odorant receptors. Those authors emphasized "positive Darwinian selection for diversity," i.e., the adaptiveness of a multigene family which can expand its size, allowing for the development of a broader repertoire of environmental odorant recognition,[7] as well as modify already existing genes, allowing for adaptation to changes in odorants over time.[8] Within the mammalian main olfactory receptor gene family, an essential provision for tremendous expansion of diversity appears to have been the repeated duplication of an ancestral tandem array, such that the current family of as many as 1000 genes resides at

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[6] A qualification is made here because it is equally plausible that the critical divergence(s) involved in a speciation are not pheromone-related at all, the process proposed here merely being permissible because speciation has in essence already occurred. In fact, VNO receptor divergence need not be viewed as "the driving force" for it truly to facilitate successful speciation: members of subspecies which are becoming incompatible are undoubtedly more effective reproductively if they mate, care for young, etc. with an appropriate subspecies specificity.

[7] Of interest, therefore, is the evidence from Issel-Tarver and Rine (1996) that differential selection among breeds of dogs on the basis of olfactory acuity (as opposed to does not affect the number of genes per subfamily. The authors offer that increases in sensory acuity might result from an increase in the total number of olfactory neurons, the amount of diversity within subfamilies, or the levels of expression of the genes.

[8] Ongoing adaptation, of course, will not prove necessary if the odorant is constant: the MOE receptor gene I7, for example, is 99% homologous between mouse and rat (A. Chess, unpublished data), a level of divergence perhaps due to mutations which are either merely permissible or that refine the specificity of binding. Whether any similar cases will be found among the VNO receptors, as more are identified and exact homologs are rigorously sought, remains to be seen, but a role for the VNO in speciation mitigates against such a discovery.

numerous chromosomal loci dispersed throughout the genome (Ben-Arie et al., 1994; Sullivan et al., 1996).[9]

On the other hand, as the repertoire of pheromones to which the VNO must respond is circumscribed, unlike that of environmental odorants, one would predict that drastic expansion of the size of the family would not occur. Indeed, preliminary evidence suggests that most if not all identified VNO genes occupy the same genomic locus (L. Belluscio and R. Axel, personal communication). Here, a yeast artificial chromosome (YAC), containing approximately 800 kb of mouse genomic DNA, was found by PCR to contain genes 10 and 22 (A. Kreitzer, A.D., and A. Chess, unpublished data), and fluorescent in situ hybridization using lambda DNAs demonstrated chromosomal co-localization of clones 22 and 25 (B. Glidden, A.D., and A. Chess, unpublished data). Broader and hence more definitive data was sought through a Southern blot containing both genomic and YAC DNA, but hybridization stringency was not yet low enough to permit the detection of multiple bands with each probe. At any rate, a further possibility is that the confinement of the VNO receptor family to one locus might be required by whatever mechanism of receptor choice is operative in this system. It is unclear as of yet how the main olfactory system copes with its multiple loci: locus-independent models are appealing, but equally plausible is one involving sequential stochastic choices, first of a locus and then of a receptor gene at that locus which is acceptable in the particular epithelial zone (Chess et al., 1994; Sullivan et al., 1996).

A final sequence-related observation is that, dispersed throughout the coding region, there are several amino acid positions which exhibit differential conservation in mouse and in rat (e.g., position 178; see Figure 3). Why would such mutations spread throughout each family of genes? Positive selection for binding specificity is irrelevant, for such global substitutions represent not diversification, but homogenization. Instead, they may provide evidence of small-scale gene

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[9] The amphibian Xenopus laevis, in fact, may contain living evidence of the ancestral form of the mammalian MOE family (Freitag et al., 1995). The frog appears to possess a highly diversified set of fish-like receptor genes (for detecting water-soluble odorants) as well as a less refined class of mammal-like receptor genes (for airborne odorants).

conversion events occurring within diverged lineages, or they may represent modifications compensatory to mutations in the interacting G protein.

Expression of the Pheromone Receptor Genes: Confined to the VNO?

In situ hybridization has confirmed that the mouse genes isolated here are homologs of the family of genes cloned by Dulac and Axel (1995). However, as for the rat family, and indeed still for the main olfactory receptor genes first cloned by Buck and Axel (1991), the receptors will remain putative until their function is explicitly demonstrated by the transduction of pheromone binding in to membrane potentials. It is dissatisfying that the riboprobes corresponding to genes 22 and 103 failed to hybridize; further experiments are required. Until hybridization is achieved, 22 and 103 remain possible pseudogenes--but this would contradict, of course, the interim RT-PCR data demonstrating expression of gene 22 in the VNO.

The more interesting question probed by the in situ hybridization experiments is the possibility of expression of a pheromone receptor by an occasional MOE neuron, which would complement the earlier finding of main olfactory receptor M12 expression in the VNO (Dulac and Axel, 1995). Further whole-mount hybridizations, though anatomically unwieldy for examining expression pattern in the VNO itself, will efficiently screen large numbers of MOE cells for rare pheromone receptor expression. Attempts were also made here to PCR the various VNO receptor genes from a cDNA library synthesized from mouse MOE, but again with no successful detection. From a statistical standpoint, however, one might in fact predict these difficulties: assuming frequency of such "leakage" is stochastic in a similar manner to "proper" expression, then the order of magnitude difference in receptor gene family size (as well as number of loci) should cause the frequency of VNO gene expression in the MOE to be only 1% as common as the converse. Thus, sufficient evidence may already exist to assert that expression leakage occurs in both directions. What is the significance of this? It may be an indication of some essential overlap between the gene regulatory mechanisms of the two systems, perhaps involving similar molecules. Yet even if so, implications towards a fundamentally similar mechanism for receptor choice, or towards a

common evolutionary descent of the two systems, are indeterminate; co-opting of distinct (see Introduction) but related components remains a formal possibility.

Are the Pheromone Receptor Genes Allelically Inactivated?

A crucial question towards relating the main olfactory and vomeronasal receptor systems remains unresolved, since the RT-PCR-based expression studies are inconclusive thus far in testing for allelic specificity of expression in the vomeronasal receptor genes. The complementary signature of monoallelic expression, replicative asynchrony of DNA, must also be assayed. Chess et al. (1994) found that, like X-linked genes (Taylor, 1960) and imprinted genes (Kitsberg et al., 1993; Knoll et al., 1994), the main olfactory receptor genes are found by fluorescent in situ hybridization (FISH) to be in a hemi-replicated state with unusually high frequency--in 25% to 45% of cells, as opposed to 10% for other genes. FISH is currently being attempted (B. Glidden, A.D., and A. Chess, unpublished data) to determine if the pheromone receptor genes are similarly asynchronously replicated.

Is it necessary, or even reasonable, to expect the VNO genes to be regulated in this manner? Available evidence suggests that the MO and VN systems utilize entirely distinct sets of signal transduction components, as well as different families of receptor genes; hence, any ancestral system common to the two may have diverged long enough ago that current similarities (e.g., duplication and divergence of genes in tandem arrays) are superficial consequences of common organizational motifs evolving within parallel lineages--i.e., of partially convergent evolution. In this scenario, it is impossible to predict whether the VNO receptors are allelically inactivated, imprinted, or even expressed biallelically. Is monoallelic expression required to construct a vomeronasal system of sufficient acuity? Allelic inactivation, by the current model, facilitates the integration of input from the periphery by ensuring the stochastic expression of only one receptor gene per sensory neuron, such that activity in any glomerulus of the olfactory bulb represents binding to a specific receptor. Nevertheless, if, as a consequence of stochastic selection of one gene from each allelic array, two pheromone receptors were expressed per cell in the VNO,

activity in any glomerulus in the accessory bulb would still faithfully signal binding of the receptor which all the cells projecting to it have in common. There would clearly be a decrease in resolution due to "background noise," but this would be nominal due to the relatively large (100) number of distinct VNO loci. Regardless of whether the vomeronasal receptor genes prove allelically inactivated, the result will be tremendously useful in subsequent elucidation of the mechanisms of receptor choice in both the main olfactory and the vomeronasal systems.

Sexual Dimorphism

Due to the role of the vomeronasal organ in many sex-specific behaviors, it has been tempting to speculate that certain of the pheromone receptor genes are expressed in a sex-specific manner. Preliminary in situ hybridization evidence (Dulac and Axel, 1995) uncovered no dimorphism in receptor expression. However, a second family of receptor genes was cloned recently and found expressed exclusively in the zone of G_o-positive cells (Herrada and Dulac, 1997; Matsunami and Buck, 1997); at least one subfamily of these genes displays a sexually dimorphic pattern of expression (Herrada and Dulac, 1997). Nonetheless, it remains reasonable to propose that at least some sex-specific responsiveness in the VN system is a consequence of developmental differences downstream, perhaps in the amygdala and hypothalamus.

Experimental Procedures

Screening of Genomic Library

A mouse genomic library prepared in Lambda Fix II (Stratagene) was screened according to standard protocols (Sambrook et al., 1989); low-stringency hybridization was carried out with a mixed probe composed of six subcloned PCR products amplified from rat genomic DNA using the

following primers: 1s-F, 5'-CGGAATTCTCTTGTAGACGATGGGATGACATC-3'; 1s-R, 5'-GCTCTAGAAGGCAATGCTGTCGAAGATAGAC-3'; 2-F, 5'-CGGAATTCGCATCTCAGA-ATGTGTGGAATGAC-3'; 2-R, 5'-GCTCTAGAGGATATGGTGCAGTCCAAGCAG-3'; 4-F, 5'-CGGAATTCGGATATGGGATTCTACCTCATGCC-3';4-R, 5'-GCTCTAGACCATCCTTG-AACTTCATCCTTGAG-3'; 5-F, 5'-CGGAATTCTTTATTTCTTGGAGGGGATGGG-3'; 5-R, 5'-GCTCTAGAGCTGTCGAAAATGGACATTAGGAC-3'; 6-F, 5'-CGGAATTCTTGAGGGG-TTTCAACCTTTGTG-3'; 6-R, 5'-GCTCTAGATTGCTGTGGGGATGCTTTTG-3'; 7-F, 5'-CG-GAATTCCCTTCCCATCACATCTCCTGTG-3'; 7-R, 5'-GCTCTAGATTCACACACATGGAC-CTCAAAGAG-3'. The underlined portions of the primer were appended to provide restriction enzyme recognition sites.

Routine molecular cloning techniques were performed using standard procedures (Sambrook et al., 1989), with two exceptions: for subcloning entire lambda clone inserts, the DNA Ligation Kit (Takara) was used, and for PCR-product subcloning, the PCRscript Amp SK(+) cloning kit. Except for the PCRscript vector, pBluescript KS II(+) was used in all subcloning. Complete forward and reverse sequencing of coding regions of VNO receptors was accomplished using the rat primers above to generate PCR products (annealing temperature of 48°C), T3 and T7 primers to begin sequencing lambda subclones from the plasmid polylinkers, and the following mouse-specific primers: 10.6, 5'-AAAGCACTGCATGGGAGAACCAGC-3'; 10.7, 5'-GGACACTCAACATGCTGGTGGTAC-3'; 10.8, 5'-ACATGGTGGTCCTCTTGTG-3'; 10.9, 5'-TTTGCTCTGCGGATGCTTTGGG-3'; 10.10, 5'-AGCATGAGTGTGGTCTGTGG-3'; 10.11, 5'-GGCAAAAACTACCTCATAGGG-3'; 22.1 AGGAGTTTCTTGGACTTAGG-3'; 22.2, 5'-GCTCTCCTATGGAGTCACAAG-3'; 22.3b GATCTGCCAGCTTCTACCTC-3'; 22.4b, 5'-TTTTGCCAGCTCGGTGTTGGTTGG-3'; 22.9, 5'-CGTTCCCAAAATGTCATGAT-CC-3'; 22.20, 5'-TGTTGCTGTGAAGGTGCTGG-3'; 25.1, AAACAGGAGCTTCTGGGACT-GAGG-3'; 25.2, 5'-CCCTTGAGTTACGTCATGC-3'; 25.4, 5'-ACTGATGCAGCCACCATA-GG-3'; 25.7, 5'-GGGAAAGGCTGATACCTTG-3'; 25.9, 5'-CATGACTGATAGTTCTGATG-CC-3'; 61.1, 5'-TGCTGGTGGTACAAAGGGAG-3'; 61.2, 5'-CCTAAAAACAGCCCCAGAG-

3'; 61.4, 5'-CCAAGGAGGATGGGTGAGATATTG-3'; 61.7, 5'-TGAAGTTGAGAGGACCA-TGAG-3'; 61.9, 5'-TCTGATGCCAGCACATTCCC-3'; 103.1, 5'-CACCTGAGATATGATGA-GGAG-3'; 103.2, 5'-CCCTTTGTGCTACCTGTCTG-3'; 103.3, 5'-CATGAGCTTCTTTGTGG-TTC-3'; 103.4, 5'-TGGTGCTTGGTGTTGGTTG-3'; 103.5, 5'-CCTGAGTGGGTATGCTATT-TAG-3'; 109.1, 5'-CATATAGAGGACACTCAGGAA-3'; 109.2, 5'-CATGGTCCTCTCAACTT-CATAC-3'; 109.3, 5'-GGATGCTTTGAGGGAAAG-3'; 109.4, 5'-GCTCAACTTAGGAAGGG-TAAG-3'.

RT-PCR Assays

Total RNA was isolated from vomeronasal organs dissected from adult C57/BL6 mice by guanidium isothiocyanate and phenol-chloroform extraction using RNAzolB (Tel-Test). An intron in the 5' UTR of clone 22 was identified: avian myoblastosis virus reverse transcriptase (Promega) and both primer 22.9 and oligo-dT, in separate reactions, were used to synthesize cDNA; PCR was performed with Pfu polymerase (Promega), using an initial denaturation of 3 min at 95°C and subsequent cycles of 30 s at 95°C, 1 min at 54°C, and 1 min at 72°C. First-round PCR contained 45 cycles and used primers 22.1 and 22.6, 5'-AGGCAGGAAAAGGGCTGATC-3'; a second round of 30 cycles was performed with 22.1 and 22.7, 5'-AAGGACATTCAGCAGGCA-GG-3'. Spretus sequence for the corresponding genomic region was obtained using the same PCR protocol, but with an extension step of 2 min at 72°C.

RT-PCR experiments in which clone 22 was found untranscribed in spretus mice were performed as above, with the following modifications: cDNA was synthesized using Superscript II RT (Gibco BRL) and either the primer 22.15, 5'-GGGTGATGGTCCAAAGGACATTC-3' or oligo-dT; PCR used Biolase DNA polymerase (ISC BioExpress) and primers 22.10, 5'-TTCTGTTTCAGGCAGGAAAAGG-3' and 22.13, 5'-TGTGCATGTGGTAGAATCCCATC-3' in the primary round, primers 22.11, 5'-GCATTAGGTGGGTTAGGGAGAAG-3' and 22.12, 5'-GCAGGAAAAGGGCTGATCTGTC-3' in the secondary round.

In Situ Hybridization

Standard in situ hybridization, upon sections cut from frozen vomeronasal organs from adult C57/BL6 mice or heads of E16.5 animals, was performed according to standard procedure (Scheren-Wiemers and Gerlin-Moser, 1993), with subcloned fragments of lambda genomic clones containing VNO receptor genes serving as templates for synthesis of digoxigenin-labelled cRNA probes, both antisense and sense. Sections were hybridized for ~14 hr at 65°C, washed in 0.2X SSC at 72°C, incubated with alkaline phosphatase-conjugated anti-digoxigenin F(ab) (1:5000 dilution) overnight at 4°C, and reacted with 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) at ambient temperature for two days. Whole-mount in situ hybridization was also performed: adult mice were perfused with 4% paraformaldehyde and intact skulls cut medially were postfixed overnight, in preparation for the procedure (Harland, 1991; Vassar et al., 1993). An antisense probe made from the coding region of the main olfactory receptor M12 served as a positive control for effectiveness of both standard and whole-mount procedures.

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Figure Legends

Figure 1. Anatomical Distinction of the Vomeronasal and Main Olfactory Systems

(A) A sketch of a parasagittal section through the skull of a rat (the mouse skull differs only slightly in gross morphology). The turbinates upon which lies the main olfactory epithelium (MOE) are found deep inside the nasal cavity (NC); the vomeronasal organ (VNO) is anterior to the MOE, just above the palate (P) and embedded in a pouch against the septum. Axons from MOE sensory neurons send axons to the main olfactory bulb (OB), whereas the VNO projects to the accessory olfactory bulb (AOB), which is anatomically distinct from the OB.

(B) A coronal section of the above.

(Dulac and Axel, 1995)

Figure 2. Homology-Derived Relationships of Mouse and Rat Pheromone Receptors

(A) Dendrogram of the six cloned mouse VNO receptors, calculated from comparisons of deduced amino acid sequences. Numbers at branchpoints indicate percent amino acid identities: for branches with multiple termini, the number given is the arithmetic mean of all pertinent pairwise percentages.

(B) Analogous dendrogram of the seven rat VNO receptor genes cloned in Dulac and Axel, 1995.

(C) A compilation of the rat and mouse dendrograms, treating all thirteen sequences indiscriminately.

(D) Tables containing all pairwise amino acid identities.

Figure 3. Deduced Amino Acid Sequences of the Vomeronasal Receptors

Peptide sequence alignments of pheromone receptors, with consensuses independently derived for mouse and rat. In mouse sequences, residues common to at least five of the six proteins are denoted in white lettering on a black background; in rat, at least five of the seven are required. Transmembrane domains, as predicted by Dulac and Axel (1995), are indicated as I-VII.

Figure 4. Restriction of Mouse Pheromone Receptor Expression to Stochastically Distributed Subpopulation of Cells in VNO Epithelium

(A)-(F) In situ hybridization to 20-μM coronal sections of a dissected adult VNO. Probes were digoxigenin-labelled transcription products from plasmids containing the entire coding region and 1.5 kb of adjacent genomic sequence: (A), antisense of 61; (B), sense of 61; (C), antisense of 25; (D), sense of 25; (E), antisense of 10; (F), sense of 10.

(G) Identical procedure performed upon coronal section of head of E16.5 mouse, using antisense RNA probe from receptor 10. Compare anatomy to Figure 2b.

(H) Close-up image of (G).

Figure 1

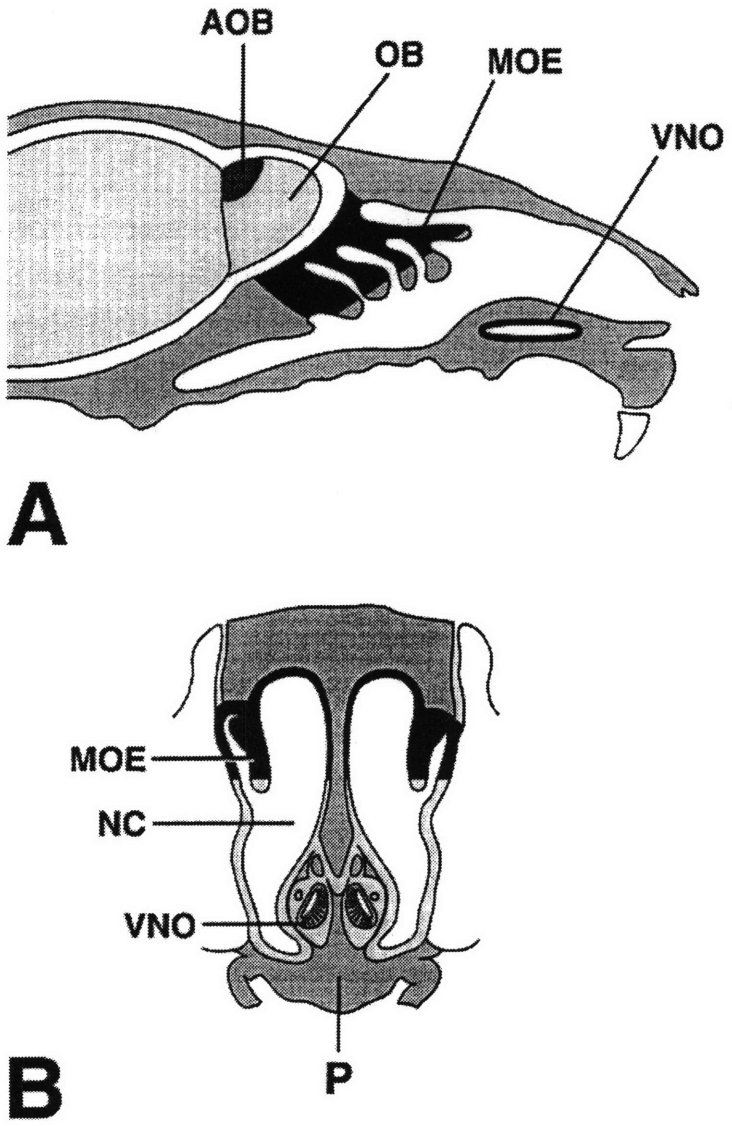


Figure 2A

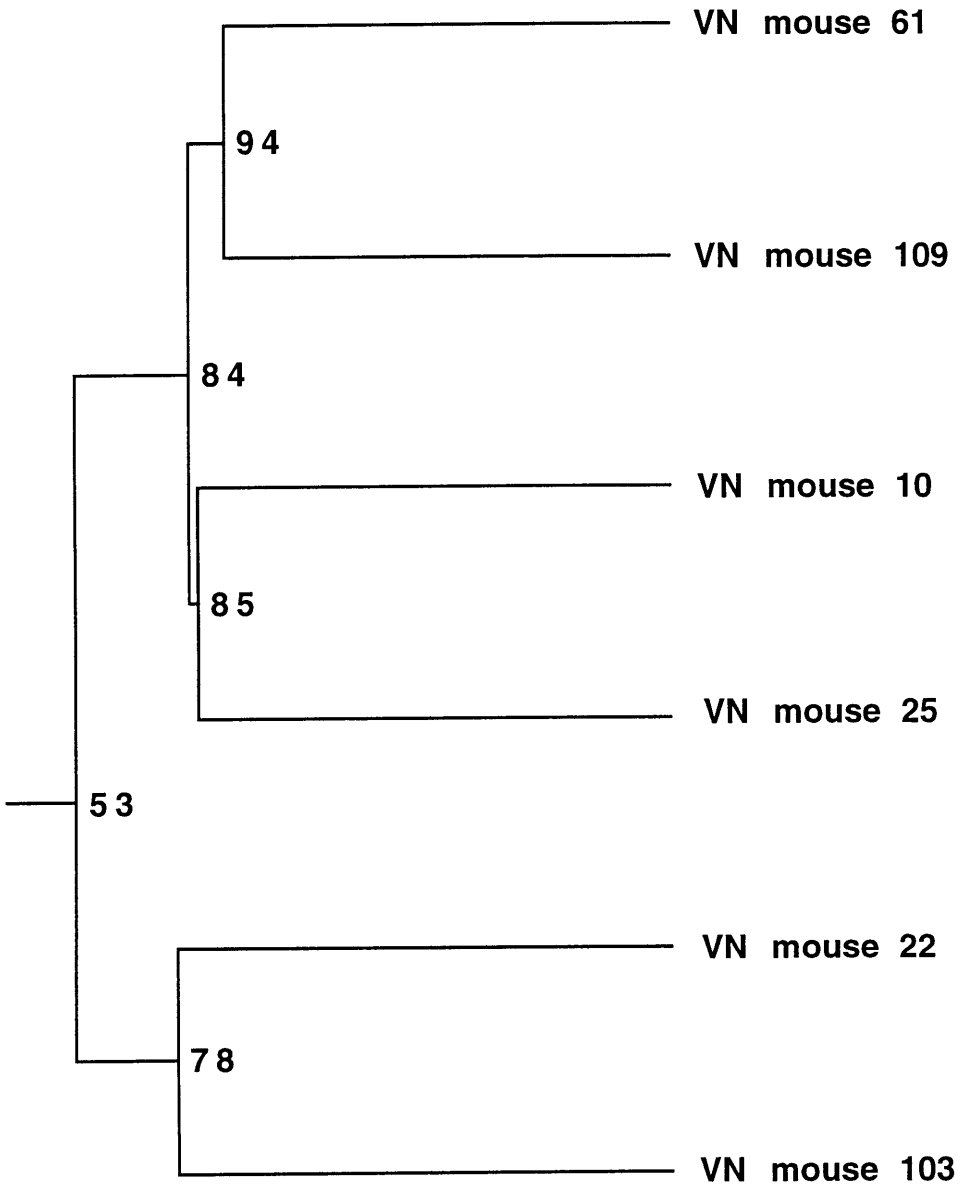
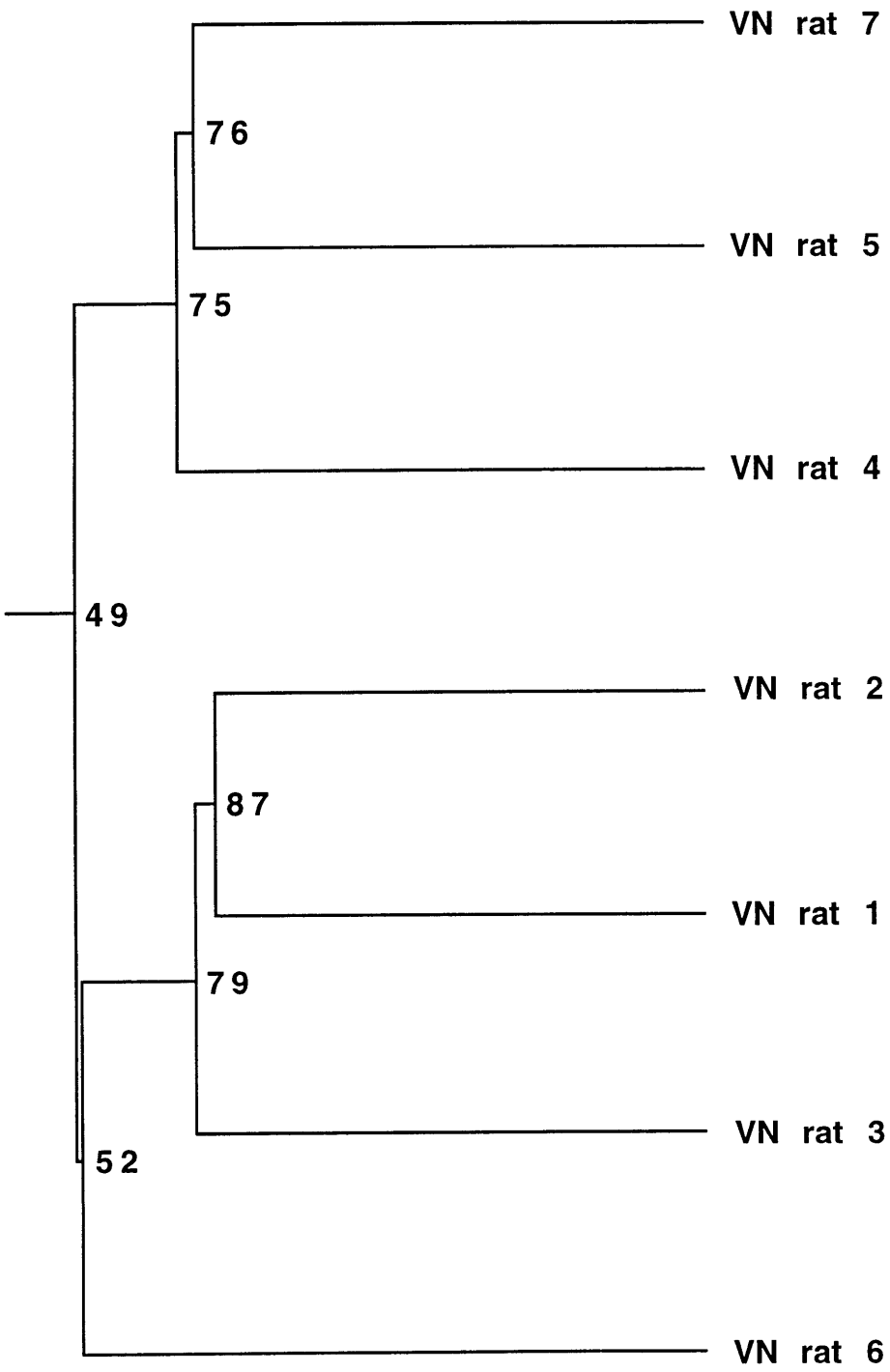

VN mouse 61
9 4
VN mouse 109
8 4
VN mouse 10
8 5
VN mouse 25
5 3
VN mouse 22
7 8
VN mouse 103

Figure 2B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VN rat 4
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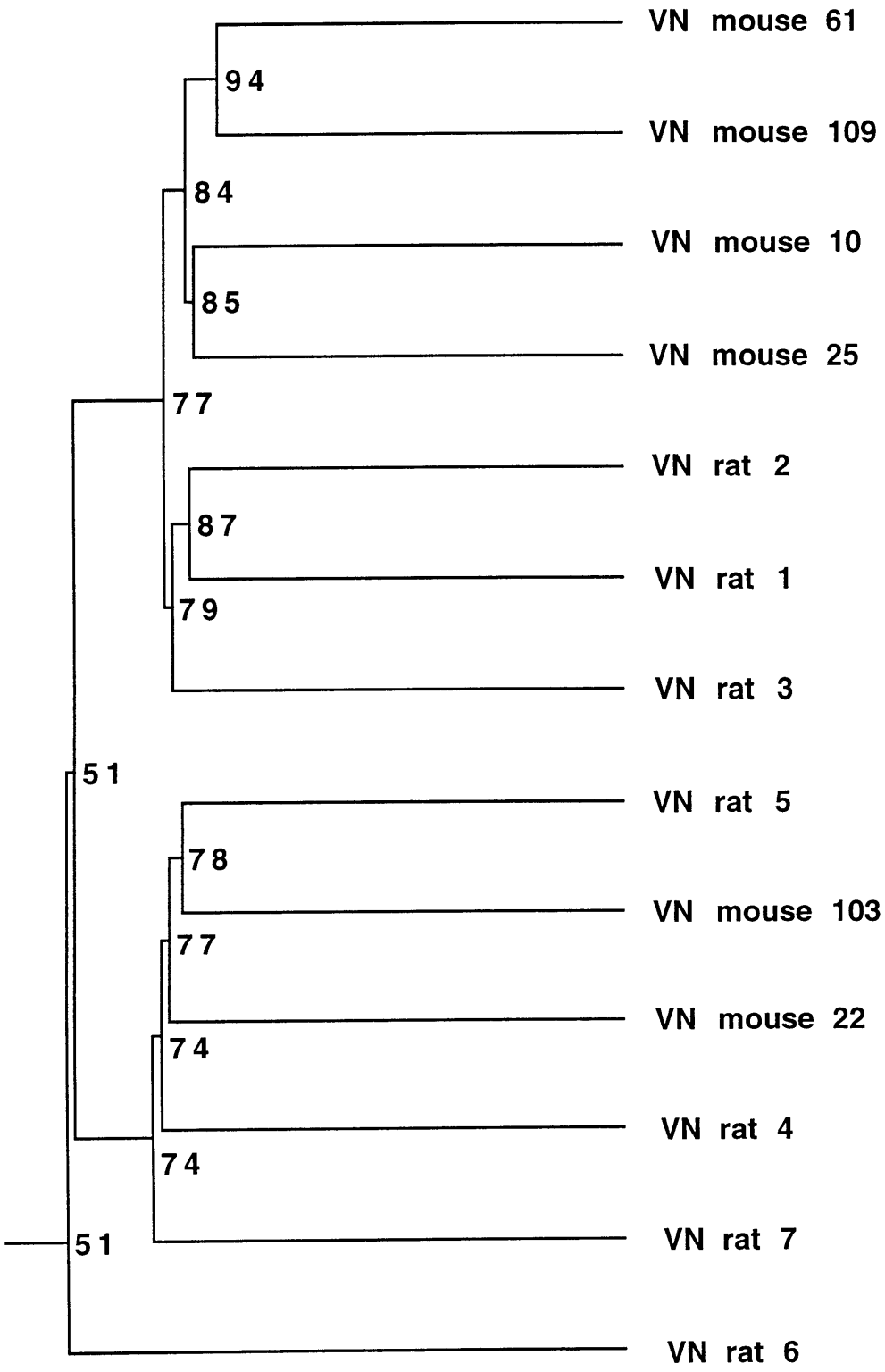
VN rat 2
87
VN rat 1
79
VN rat 3
52
VN rat 6

Figure 2C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VN mouse 109
84
VN mouse 10
85
VN mouse 25
77
VN rat 2
87
VN rat 1
79
VN rat 3
51
VN rat 5
78
VN mouse 103
77
VN mouse 22
74
VN rat 4
74
51
VN rat 7
VN rat 6

Figure 2D

Mouse

	10	22	25	61	103	109
10	---					
22	55	---				
25	85	53	---			
61	84	54	80	---		
103	54	78	52	52	---	
109	88	54	82	94	53	---

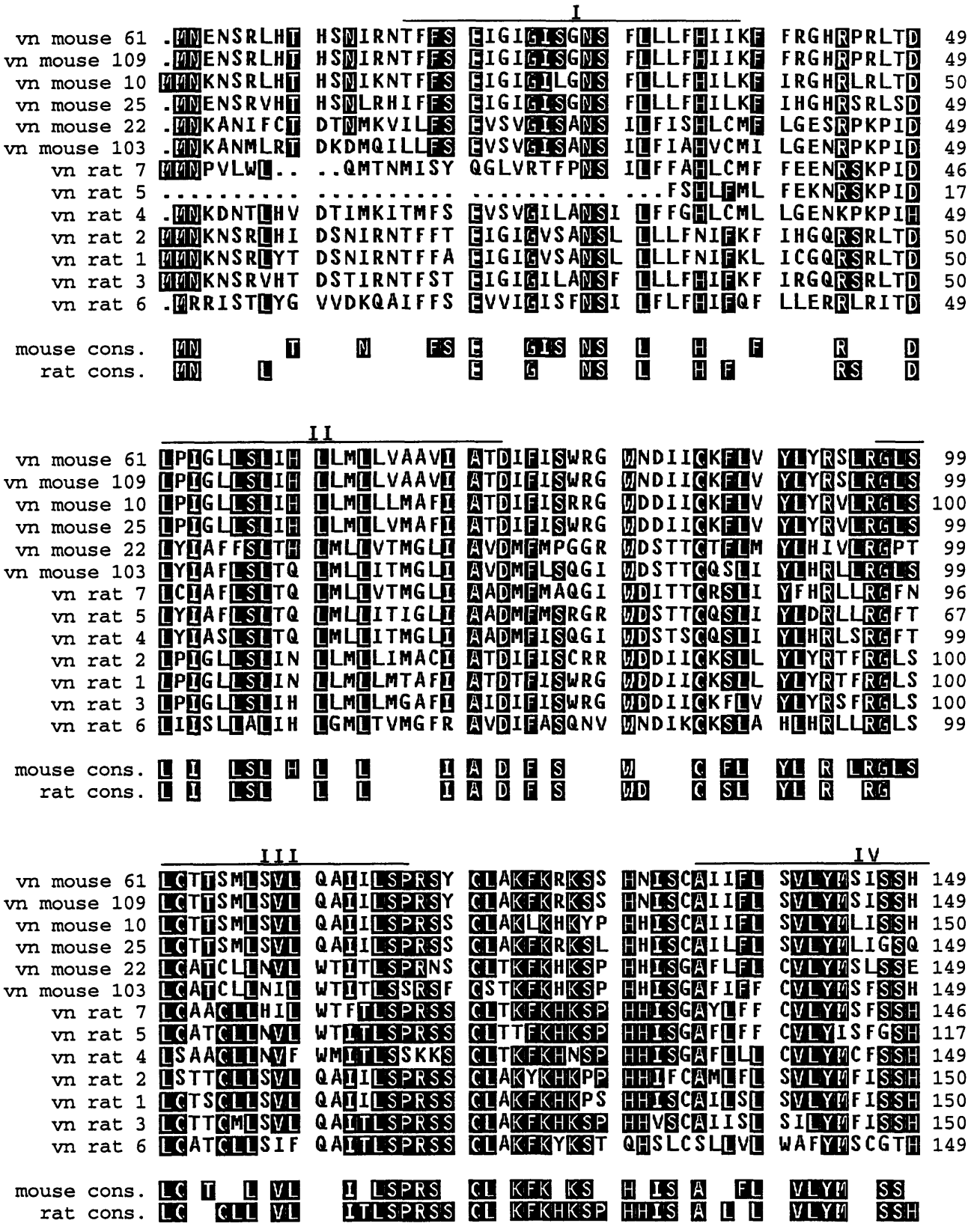
Rat

	1	2	3	4	5	6	7
1	---						
2	87	---					
3	80	78	---				
4	49	48	48	---			
5	52	51	50	77	---		
6	52	52	52	46	46	---	
7	48	47	48	73	76	50	---

Mouse vs. Rat

	10	22	25	61	103	109
1	78	51	79	73	51	75
2	78	51	77	71	50	74
3	82	50	79	75	50	78
4	51	71	48	48	75	48
5	52	75	52	50	78	50
6	51	51	54	51	50	52
7	50	74	52	52	74	51

Figure 3


I
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vn mouse 10 MMNKNSRLHT HSNIKNTFFS EIGIGILGNS FLLLFHILKF IRGHRLRLTD 50
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vn mouse 22 .MNKANIFCT DTNMKVILFS EVSVGISANS ILFISHLCMF LGESRPKPID 49
vn mouse 103 .MNKANMLRT DKDMQILLFS EVSVGISANS ILFIAHVCMI LGENRPKPID 49
vn rat 7 MMNPVLWL.. ..QMTNMISY QGLVRTFPNS ILFFAHLCMF FEENRSKPID 46
vn rat 5 ....FSHLFML FEKNRSKPID 17
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vn rat 3 MMNKNSRVHT DSTIRNTFST EIGIGILANSF LLLFHIFKF IRGQRSRLTD 50
vn rat 6 .MRRISTLYG VVDKQAIFFS EVVIGISFNSI LFLFHIFQF LLERRLRITD 49
mouse cons. MN T N FS E GIS NS L H F R D
rat cons. MN L E G NS L H F RS D
II
vn mouse 61 LPIGLLSLIH LLMLLVAAVI ATDIFISWRG WNDIICKFLV YLYRSLRGLS 99
vn mouse 109 LPIGLLSLIH LLMLLVAAVI ATDIFISWRG WNDIICKFLV YLYRSLRGLS 99
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vn mouse 25 LPIGLLSLIH LLMLLVMAFI ATDIFISWRG WDDIICKFLV YLYRVLRGLS 99
vn mouse 22 LYIAFFSLTH LMLLVTMGLI AVDMFMPGGR WDSTTCTFLM YLHIVLRGPT 99
vn mouse 103 LYIAFLSLTQ LMLLITMGLI AVDMFLSQGI WDSTTCQSLI YLHRLLRGLS 99
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vn rat 5 LYIAFLSLTQ LMLLITIGLI AADMFMSRGR WDSTTCQSLI YLDRLLRGFT 67
vn rat 4 LYIASLSLTQ LMLLITMGLI AADMFISQGI WDSTSCQSLI YLHRLSRGFT 99
vn rat 2 LPIGLLSLIN LLMLLIMACI ATDIFISCRR WDDIICKSLL YLYRTFRGLS 100
vn rat 1 LPIGLLSLIN LLMLLMTAFI ATDTFISWRG WDDIICKSLL YLYRTFRGLS 100
vn rat 3 LPIGLLSLIH LLMLLMGAFI AIDIFISWRG WDDIICKFLV YLYRSFRGLS 100
vn rat 6 LIISLLALIH LGMLTVMGFR AVDIFASQNV WNDIKCKSLA HLHRLLRGLS 99
mouse cons. L I LSL H L L I A D F S W C FL YL R LRGLS
rat cons. L I LSL L L I A D F S WD C SL YL R RG
III
IV
vn mouse 61 LCTTSMLSVL QAIILSPRSY CLAKFKRKSS HNISCAIIFL SVLYMSISSH 149
vn mouse 109 LCTTSMLSVL QAIILSPRSY CLAKFKRKSS HNISCAIIFL SVLYMSISSH 149
vn mouse 10 LCTTSMLSVL QAIILSPRSS CLAKLKHKYP HHISCAIIFL SVLYMLISSH 150
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vn mouse 103 LCATCLLNIL WTITLSSRSF CSTKFKHKSP HHISGAFIFF CVLYMSFSSH 149
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vn rat 1 LCTSCLLSVL QAIILSPRSS CLAKFKHKPS HHISCAILSL SVLYMFISSH 150
vn rat 3 LCTTCMLSVL QAITLSPRSS CLAKFKHKSP HHVSCAIISL SILYMFISSH 150
vn rat 6 LCATCLLSIF QAITLSPRSS CLAKFKYKST QHSLCSLLVL WAFYMSCGTH 149
mouse cons. LC T L VL I LSPRS CL KFK KS H IS A FL VLYM SS
rat cons. LC CLL VL ITLSPRSS CL KFKHKSP HHIS A L L VLYM SSH

Figure 3 (cont.)

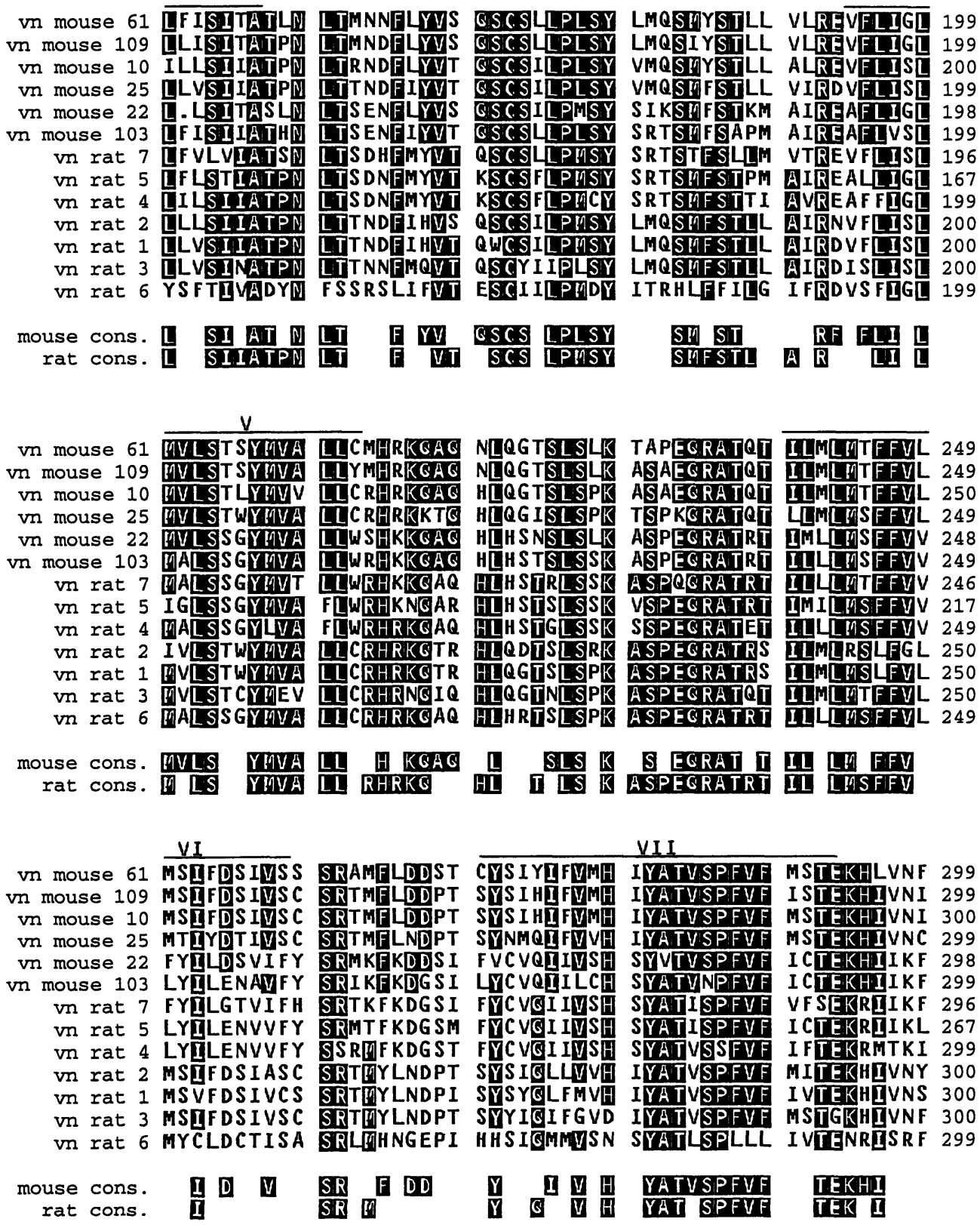


Figure 3 (cont.)

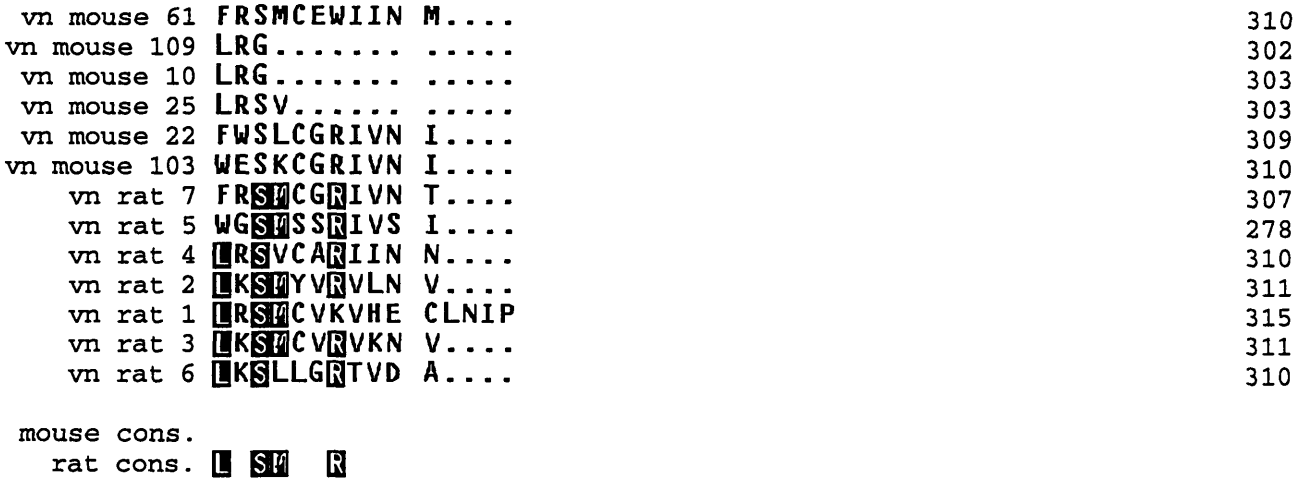
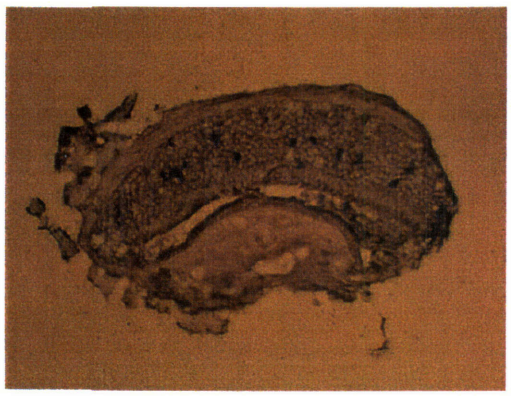
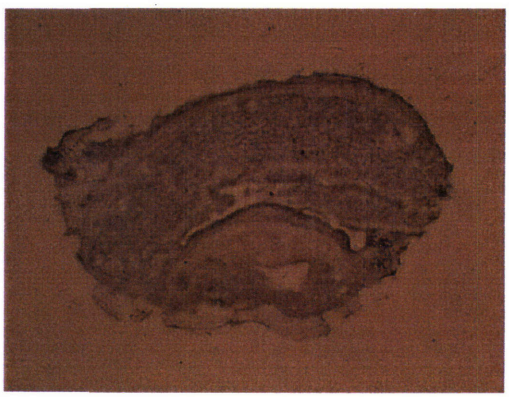


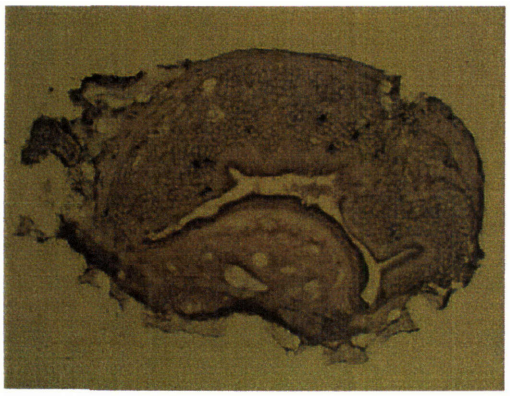
Figure 4



A



B



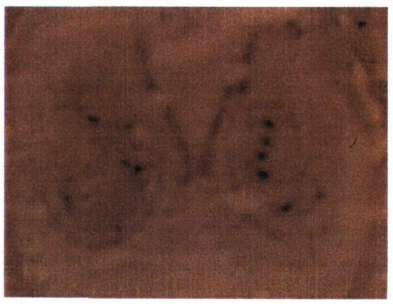
C

D

E

F

G



H

Biographical Note

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Teaching Assistant, Introductory Biology, Massachusetts Institute of Technology, fall 1996
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offered National Science Foundation Graduate Fellowship, 1995
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