Identification of Non-functional Human VNO Receptor Genes Provides Evidence for Vestigiality of the Human VNO

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Abstract

In mammals, the vomeronasal organ (VNO) contains chemosensory receptor cells that bind to pheromones and induce a variety of social and reproductive behaviors. It has been traditionally assumed that the human VNO (Jacobson's organ) is a vestigial structure, although recent studies have shown minor evidence for a structurally intact and possibly functional VNO. The presence and function of the human VNO remains controversial, however, as pheromones and VNO receptors have not been well characterized. In this study we screened a human Bacterial Artificial Chromosome (BAC) library with multiple primer sets designed from human cDNA sequences homologous to mouse VNO receptor genes. Utilizing these BAC sequences in addition to mouse VNO receptor sequences, we screened the High Throughput Genome Sequence (HTGS) database to find additional human putative VNO receptor genes. We report the identification of 56 BACs carrying 34 distinct putative VNO receptor genes sequences, all of which appear to be pseudogenes. Sequence analysis indicates substantial homology to mouse V1R and V2R VNO receptor families. Furthermore, chromosomal localization via FISH analysis and RH mapping reveal that the majority of the BACs are localized to telomeric and centromeric chromosomal localizations and may have arisen through duplication events. These data yield insight into the present state of pheromonal olfaction in humans and into the evolutionary history of human VNO receptors. (Sequence data from this article have been deposited with the DDBJ/EMBL/Genbank Data Libraries under accession nos. AF305393–305416.)

Introduction

The vomeronasal organ (VNO) is an olfactory sensory structure that responds to soluble compounds such as pheromones and induces characteristic social and reproductive behaviors (Halpern, 1987; Buck, 2000). The VNO is located anterior to the primary olfactory system, residing within a tissue pouch in the septum of the nose. Neurons from the VNO project to the accessory olfactory bulb (AOB), which in turn sends neurons to the amygdala and hypothalamus. Since this neural pathway bypasses higher cognitive centers, it is believed that the pathway mediates innate behavioral and neuroendocrine responses. VNO sensory neurons have dendrites that terminate in specialized odorant-binding microvilli or cilia, within which VNO receptors are expressed (Dulac and Axel, 1998).

VNO receptors are seven-transmembrane-domain receptors distinct from the well-characterized multigene family of odorant receptors (OR) consisting of ~1000 genes (Strader *et al.*, 1995). It is believed that both ORs and VNO receptors

induce a G-protein-coupled signal transduction pathway after binding ligand, although ligands have not been identified for VNO receptors. Studies in mice have uncovered two evolutionarily independent families of VNO receptors that utilize different G-proteins and are expressed in two distinct subsets of VNO sensory neurons (Dudley and Moss, 1999; Kumar et al., 1999). The V1R VNO receptor family, which comprises an estimated 35 genes, lacks introns and has short N-terminal extracellular domains that act in combination with transmembrane domains to form a ligand-specific binding pocket (Dulac and Axel, 1995). The V2R VNO receptor family, which comprises an estimated 150 genes, contains introns and has very large N-terminal extracellular domains that may bind ligand (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Recent studies have uncovered a third family of highly conserved VNO receptors (V3R) that appear distantly related to V1Rs. Like V1Rs, V3Rs appear to

Tuble 1 Sequence and genomic characterization of non-ranetional naman who receptor gene sequences

Cluster	BAC/HTGS	Start	Ch. localization	Degeneracy	V1R/V2R	Homology
1	AC025699.3	98421	1	2S/1F (?)/Alu	V1R2	45/130 (34%)
2	AC021022.2	104791	3	1S	V1R1	112/259 (43%)
3	AC011607.4	153581	3	2S/2F (-4, +1)	V1R2	114/305 (37%)
	AC011612.6	21981	ND	2S/2F (-4, +1)	V1R2	114/305 (37%)
	AC012018.6	158271	ND	2S/2F(-4, +1)/Alu	V1R2	114/305 (37%)
	AC024151.13	32400	3	1S/2F(-4, +1)	V1R2	62/74 (83%)
4	AC024199 1	74100	4	Short	V1R2	29/77 (37%)
5	ΔC0222024	169038	7	1F (_1)/Δlu	V1R5	71/163 (43%)
6	AC022202.4	105050	7 7n11 2 (D752/129)	1F (_2)	V1R5	28/76 (36%)
0	071010					
	709D1 07FF11			ND		
7	970FTT					
/	354A1		/pii.i, /qii.i	IF(-Z)	VIRS	33/89 (37%)
8	3/86		/q11.1 (D/S2/2/)	TS/Alu	VIR2	43/119 (36%)
_	575A8		/q11.1 (D/S2/2/)	1S/Alu	V1R2	43/119 (36%)
9	AL022344.1	16754	10	95	V1R2	61/207 (29%)
	AL031601.2	45983	10	ND	ND	ND
	AL023808.1	345891	10	9S	V1R2	61/207 (29%)
	AL354975.6	190600	10	95	V1R2	61/207 (29%)
10	AL157387.1	106681	10p11.21–10p12.1	5S/1F (?)/Alu	V1R2	35/103 (33%)
11	AC022860.3	140704	11	4S/1F (4)	V1R1	80/240 (33%)
12	AC022860.3	15916	11	1S/1F (–7)/Alu	V1R2	69/217 (31%)
	AC011823.3	77194	11	1S/1F (7)/Alu	V1R2	69/217 (31%)
13	AL161418.3	120744	13	2F (?, ?)/Alu	V1R2	48/137 (35%)
	AL159971.2	36171	13	Short/Alu	ND	ND
14	AC060814.1	31291	15	2F (7, 7)	V1R1	38/97 (39%)
15	AC011821 4	118613	15	1S/2F (7 7)	V1R2	67/206 (32%)
16	AC011821.4	131597	15	$15/2F(+1 -5)/\Delta lu$	V1R2	60/136 (44%)
10	AC011817 3	160263		Short	ND	
17	AC011017.5	62205		$25/1E(\pm 7)$	V/1 <i>R</i> 1	80/259 (30%)
17	AC020079.2	702235		$23/11(\pm 7)$	V 1 N 1 V 1 D 1	20/259 (50 /0) 20/250 (20%)
	AC021303.3	20407	16	$23/11^{(\pm 7)}$		00/239 (30 %)
10	ACU25191.1	50467	10			
10	679A7		16p11.2	IF (+3)	V I K5	45/134 (33%)
19	92FTT		16011.1 (D165408)		VIKI	51/125 (40%)
	28688		16q11.1 (D165408)	Short	ND	
	190C5		16q11.1	1F (-11)	VIRI	51/125 (40%)
20	AC022323.1	491	1/	1S/2F (?, +1)	V1R2	29/68 (42%)
21	428E8		19q13.11 (D19S766)	1S/1F (+1)	V1R1	28/66 (42%)
22	AC025953.2	121025	ND	1F (–1)/Alu	V1R1	49/135 (36%)
	AC015480.3	11661	ND	1F (–1)/Alu	V1R1	49/135 (36%)
23	AC023338.1	70301	2	2S/1F (–1)	V2R4	52/98 (53%)
24	AC023338.1	891	2	4S/2F (+11, -1)	V2R3	88/249 (35%)
25	182G10		7q31.3 (D7S530)	1S	V2R14	25/56 (44%)
26	AL353729.1	13676	9	4S/4F (?, ?, ?, -1)	V2R3	54/75 (72%)
	AL353791.1	46882	9	4S/2F (?, -1)	V2R3	127/155 (81%)
	AL353794.1	127077	9	Short	ND	ND
27	111A2		16q23 (D16S516)	2S/1F (-1)	V2R4	51/98 (52%)
	139G7		16a23 (D16S516)	25	V2R4	80/96 (83%)
	608D8		ND	25	V2R4	80/96 (83%)
	AC0091394	34770	16	2S/1F (_1)/Alu	V2R4	80/96 (83%)
28	834F7	51770	ND	1S/2F (_1 _1)	V2R7	42/66 (63%)
20	895F/		ND	15/21 (1, 1)	V2R1/	69/78 (88%)
	291C6		19q13.4 (D19S1124)	15	V2R14	69/78 (88%)
29	34G7		19q13.4 (D19S418)	1S/1F (–1)/Alu	V2R2	25/55 (45%)
30	AC011457.1	41301	19	2S/3F (?, ?, –1)/Alu	V2R5	79/132 (59%)
31	AC011457_1	62568	19	2S/1F (-1)	V2R14	89/186 (47%)
32	AC010422 4	80288	19	4S/3F (-1 ? ?)/ΔΙυ	V2R3	59/167 (35%)
33	AC016588.4	37975	19	4F (?, ?, -1, ?)/Alu	V2R4	75/255 (29%)
34	139H10	2.2.3	21a22 3 (D2151979)	15	V2R3	27/54 (50%)
	1331110		- 1922.3 (DZ 1313/3)		v 2113	

contain short N-terminal extracellular domains and are expressed in the apical half of the VNO neuroepithelium (Pantages and Dulac, 2000).

To date, pheromonal olfaction has not been well characterized in humans. It has been traditionally assumed that the human VNO is a vestigial structure that had degenerated during the development of higher cortical centers responsible for sexual behavior and function. For instance, studies attempting to characterize sensory cells or nerve fibers in the adult VNO epithelium either failed or were inconclusive (Doving and Trotier, 1998). Moreover, an anatomical study of 562 adults failed to locate the vomeronasal vestibule in 70% of subjects, and other studies have failed to locate the AOB in humans (Keverne, 1999). Despite these results, recent studies have raised the possibility that the human VNO may be structurally intact and possibly functional. Volumetric measurements of the human fetal VNO suggest that it contains sensory receptor-like cells and does not atrophy during development (Boehm and Gasser, 1993; Smith et al., 1997). Also, whole-cell recordings of isolated microvillar human VNO cells suggest that voltagedependent inward currents take place in response to androstadienone, estratetraenol and pregnadienendione. Functional magnetic resonance imaging studies indicate that VNO exposure to these compounds induces activation of the hypothalamus, amygdala and other centers responsible for emotion and innate behavior (Monti-Bloch et al., 1998). The contradictory results of these studies imply that further work is necessary to characterize the human VNO.

The identification of human VNO receptors may aid in resolving the controversial existence of the human VNO. Recently, a single human putative VNO receptor gene known as *V1RL1* was identified and reported to be expressed in the olfactory mucosa (Rodriguez *et al.*, 2000). Given the gene's low homology to mouse VNO receptors (28% identical and 47% similar to the mouse V1ra2 product at the amino acid level) as well as its tissue specificity, it remains to be shown whether V1RL1 is a VNO receptor or simply an odorant receptor with pheromone-like ligand properties. To identify additional human VNO receptor

genes, we scanned a human Bacterial Artificial Chromosome (BAC) library and the GenBank High-Throughput Genome Sequencing (HTGS) database with human pheromone-receptor-like cDNA sequences and with mouse V1R and V2R genes. We report the identification of 56 BACs carrying 34 distinct putative VNO receptor gene sequences, all of which appear to be pseudogenes.

Materials and methods

Identification of putative human VNO receptor sequences

All GenBank entries were searched for the keywords 'human VNO receptor' and 'human pheromone receptor'; the search yielded five different human cDNA sequences homologous to mouse VNO receptor gene sequences. The five cDNAs include two that had been isolated from Schwannoma tumor cells (AA553508, AA551068), one from a testis cDNA library (AA442630) and two (AA969989, AA806860) from a combination of three cDNA libraries (testis, fetal lung and B-cell).

Screening of human BAC library

The five cDNA sequences were aligned using the program Pileup (University of Wisconsin, GCG 8.1 software package). Using Primer3, primers were designed to amplify short fragments corresponding to transmembrane domains. The primers include

- (i) 442630, spanning transmembrane region 3
 (Forward 5'-TCACAGCCAACACCTTCCTCC-3' Reverse 5'-GTGGACAAGGGCCAGGTGAC-3');
- (ii) 551068, spanning transmembrane region 5
 (Forward 5'-ACCAGCCTCTCCCCAAGACC-3' Reverse 5'-TGCCCATAGCGAGGTTGAGG-3');
- (iii) 989AD, spanning transmembrane region 7
 (Forward 5'-GCCTGACACCTTCAATAAGTCAA-GTTC-3', Reverse 5'-GGGCAAAGATGCAGCCAAG-3'); and
 (iv) 060080 graphing transmembrane ration 7
- (iv) 969989, spanning transmembrane region 7
 (Forward 5'-CCTT CCCCTTGATGCTGTGG-3', Reverse 5'-GGATACCGGGGGCTCCTTGAC-3').

Notes to Table 1

Listed are the CIT Library-A and HTGS BACs carrying human putative VNO receptor genes. The *BAC/HTGS* column lists the accession numbers of HTGS BACs and the location of CIT-A BACs. BACs with identical sequences and/or restriction digest fingerprinting patterns are clustered together (e.g. 378E6 and 575A8). The *Start* Column lists the 5' starting location of the VNO receptor sequences within HTGS BACs. The *Ch. localization* column denotes cytogenetic localizations of the pseudogenes based on FISH, followed by the closest RH marker in parentheses. The *Degeneracy* column lists sequence characteristics for each pseudogene: 'S' indicates stop codons, where values preceding 'S' indicate the total number of stop codons in the corresponding reading frame; 'F' denotes frameshift mutations, where values within parentheses indicate the number of base pair insertions '(+)' and/or deletions '(-)' causing the frameshifts. '(?)' indicates that the number of base pairs involved in the frameshift mutation could not be determined. The 'Short' symbol denotes psuedogene. The *V1R/V2R* column lists the putative mouse VNO receptor genes that share the closest homology to the BAC sequences. The *Homology* column lists the number of matched amino acids/total number of amino acids spanning the region of homology, followed by percentage identity. 'ND' indicates that the chromosomal localization and/or sequence properties of a psuedogene could not be determined. See GenBank entries for more detailed analyses of these clones.

The primers were used to screen a $4 \times$ human genomic BAC library (CIT-A) by polymerase chain reaction (PCR) (Kim *et al.*, 1996). After identifying BACs, we confirmed that the BACs carried putative VNO receptor genes by cloning the PCR fragment (Promega-T Easy Vector System) and sequencing it via T7/SP6 sequencing primers. BACs were presumed to contain putative VNO receptor sequences if BlastX and BlastN suggested homology to mouse VNO receptor sequences. These confirmed BACs were further sequenced by primer walking. (These sequences have been deposited with GenBank under accession numbers AF305393-AF305416.) Additionally, all BACs were fingerprinted via restriction mapping (*Hin*dIII and *Eco*RI, separately) to determine overlap.

Sequence analysis

The BlastX program was used to determine sequence characteristics of the putative VNO receptor genes, including the presence of nonsense and frameshift mutations. Any resulting frameshift mutations were verified through DNA sequence alignments with mouse and rat VNO receptor genes. All putative human VNO receptor gene sequences (both DNA and amino acid) were aligned with mouse VNO receptor sequences using ClustalX, the Windows interface for the ClustalW multiple sequence alignment program. This program was also used to generate phylogenetic trees from the alignments. In the case of V2R-like human pseudogenes, intron/exon structure prediction was not performed because the various tools used did not yield reproducible data. The mouse VNO receptor genes (and their corresponding GenBank entries) used in the alignment were V1R1 (Y12725), V1R2 (Y12724), V1R5 (M21, Y17566), V1R6 (M24, Y17567), V2R1 (AF011411), V2R2 (NM_009492), V2R3 (AF011413), V2R4 (NM_009493), V2R5 (AF011415), V2R6 (AF011416), V2R7 (AF011417), V2R8 (NM_009494), V2R9 (NM_009495), V2R10 (NM_ 009486), V2R11 (NM_009487), V2R12 (NM_009488), V2R13 (AF011423), V2R14 (NM_009489), V2R15 (NM_ 009490) and V2R16 (NM_009491).

Chromosomal mapping

Chromosomal localization of CIT-A BACs was determined by radiation hybrid (RH) mapping and by fluorescence *in situ* hybridization (Cox *et al.*, 1990; Trask, 1999). For RH mapping, BAC ends were sequenced with either the primers 5'-pBAC108L and 3'-pBAC108L, or 5'-pBeloBAC and 3'-pBeloBAC, depending on the BAC vector used. These BAC-end sequences were then used to design primers for PCR amplification of DNA from the Stanford G3 and Genebridge 4 panels. With the exception of 679A7, at least two of these approaches were used to establish a BAC's chromosomal location.

HTGS screening

Once all BACs carrying putative VNO receptors had been

mapped and characterized, the sequences were used to search the HTGS database to identify additional sequences. Mouse VNO receptor genes (see above) were also used to scan the database. Sequence analysis of the positive HTGS clones was performed as described above. Additionally, BlastX was performed using 1–2 kb sequence upstream and downstream of the putative genes in order to identify additional exons and other entities such as Alus. The chromosomal locations of these BACs was based on their GenBank database entries.

Results

A PCR screen of human BAC library CIT-A was carried out using four primer sets designed from human cDNA sequences homologous to mouse VNO receptors. The screen yielded a total of 22 BACs, and restriction mapping indicated that 11 of these represented distinct putative VNO receptor gene sequences. Utilizing these 11 BAC sequences, as well as mouse VNO receptor gene sequences, we scanned the human HTGS database and identified 38 additional putative VNO receptor sequences carried on 34 BACs. Sequence alignment analysis indicated that 23 of these represented distinct sequences, yielding a total of 34 distinct gene sequences carried on CIT-A and HTGS BACs (Table 1).

Analysis of the 34 distinct HTGS and CIT-A sequences revealed that 27 carried frameshift mutations, 24 carried nonsense mutations, and 19 carried both nonsense and frameshift mutations. Thus, 32 of the 34 distinct putative VNO receptor sequences were pseudogenes. The two sequences that were not pseudogenes included AC024199.1 and AC023191.1, which contained only 260 and 144 bp of coding sequence respectively. Further sequence analysis suggested that of the 34 distinct sequences, 22 were homologous to mouse V1R genes whereas 12 were homologous to mouse V2R genes (Figure 1).

Radiation hybrid and fluorescence in situ hybridization mapping revealed that the CIT-A BACs mapped predominantly to pericentromeric and subterminal locations on chromosomes 7, 16, 19 and 21. Although the cytogenetic locations of the HTGS BACs were not known at the time of the search, the BACs are annotated to indicate that they are widely distributed over chromosomes 1, 2, 3, 4, 7, 9, 10, 11, 13, 15, 16, 17 and 19 (Table I). In some cases, BACs carrying distinct putative VNO receptor sequences mapped to the same cytogenetic positions. These include three sets of BACs that mapped near the centromere of chromosome 7 (7p11.1 and 7q11.1), three sets that mapped around the centromere of chromosome 16 (16p11.2 and 16g11.1), and two sets of BACs that mapped near the telomere of chromosome 19 (19q13.4—the V1RL1 gene also mapped to this location). Interestingly, four BACs each carried two putative VNO receptor gene sequences separated by 21-124 kb.

BlastX analysis indicated that a number of putative VNO

Figure 1a

	*	20	*	40	*	60	*	80	*	100	*	120	
AC020679.2				MKNASHF	GIGISANTFLL	FCVFLLF	REERTYDPV	CHVALIHMVVL	DTMVELS	-POLFESLN	FONDFREE	SFYLRRVI-R	LSI
AC060814.1				MKNASHF	GIGISANTFLL	LFCVFSFFLDI	LRPERTYLPV	CHVALIHMVVL	LIMVELS	- POLFESLN	FQNDFKYEA	SFYLRRVI-R	VLSI
679A7					TANTFLL	FHIFTLLEN	RPKPRDLLT	CHLALVHIOML	LTAVDF	PLDIFESLH	FGNDFKCKA	LFYTNRAM-K	GLSI
AC011821[118613]	EVTLMMEVLLVVFFS		MNSFHLTLSF	KSAFFAQA	DVGVSTNILLF	FSCIMTLIND-	-LKLTDLT	SHLDLVHIMML	ETIVEL	SODLFKSLH	FQDDFKCK	RGD-E	GLSI
354A1			LMTL	KIALFSOA	GIRLTANTFLL	FFCICTLPUD	RPKPTDLIT	CHLALVHLVML	UTVSFL	SLDLFESOY	FONDFKCKV	FEYMHRGM-R	LSI
656F5					TANTFLL	FFCICTLPUD	RPKPTDLIT	CHLALVHLVML	ITVSED	SLDLFESQY	FONDFKCKV	FFYMHRGM-R-	LSI
AC022860[15916]			DDHVPVKVL	TIALLFOR	GIGLRANNFLL	FFQIFSLLQD	IRPKPTDPI T	CHLALVHLGML	LIVVFL	SPDLFESLY	FQNDFKCKA	FFCMHRVM-R	SLSI
428E8					TANTFLL	FH-IFTTLID	IKPKSTDHII	CHLALVHL-ML	LIAVEL	SPDLFK-LH	LONNLKCKA	FYMYRVM-R	SLSS
AC011821[131597]	I	SGLQNVSSMS	LGTHISHVIF	QKAFCFOA	GIGISANIFLL	LWHIFTFFKD	KLKNHDLII	CHLVSVYIVML	VMAAEL	SLOVFESON	FQNNFRCKA	VFYIYKVM-R	FILI
AC22202.4		EHTFHMLS	L	KKAFYF <u>Q</u> A	GIGISANIFLL	LWHIFIFFKDE	IKPKNHDLI I	CHLTFVHILML	VIAAKU	SPDGFESQN	FQNNFRCKA	VFYTYKAM-RO	GL LI
AL161418.3		S	F	KNTFNCOA	SIRISANIFHL	LFHIFTEFQDE	IRPKTHDLVI	CHLAFVHLVML	FTAMEF	SPDMFESLN	FQNNFRCKA	FFYLHKVM-RC	GLSI
AL157387.1					ITLAIT			LAFIHLVML	UIVMDLI	SPDVFESLH	FWNDFRCK-	VFYLSRVT-RO	GLSI
VIR1		MMMNE	NSRVHTHSN	RHIFFSEI	GIGIS <mark>G</mark> NSFLL	FILKING	RSRLSDLPI	GLUSUIHLIML	LVMAF	ATDIFISWR	GWDDIICKF	LVYLYRVL-RO	GLSL
V1R2		MMNK	NSRLHTHSN	KNTFFSEI	GIGILGNSFLL	LEHILKEIRG	RLRLTDLPI	GLUSIATHIAML	Intro AF	ATDIFISRR	GWDDIICKF	LVYLYRVW-RO	GLSL
AC011607.4		-AYFPHKMNK	NNKPSSFIA	RNAAFSEV	GIGISANAMLL	FHILTCLICK	IRTKPADLIV	CHVALIHIILL	PTEF	ATDIFGSQD	SEDDIKHKS	VIYRYRLM-RO	GLSI
AC021022.2		FPIFMNK	NNKLSSFIA	RNAAFSEV	GIGISANAILL	FHVLTCLICKY	RTKPTDLI	GHVALIHIVLL	RDM PKGF	ATDISASOD	SGDDIKHKS	7IYRYRLM-RO	∂1'F1
92F11													
AC022860[140704]				SLKNTS	FFQAGVGIST	NELIEFTICLO	RPKPTDLTI	NHLAFIHIVMM	VSIP	SPGVFESLY	FQNDFKCKV	FSYLNNIII-RC	CFSS
378E6													
AC024199.1													
AC025699.3										VFESLN	FWKDFKCQA	LFSMNRVM-RO	-TAI
AC025953.2			NK	KKNLFSQA	TIGLLANTFFL	FENIEICODO	OKSKPHDLIS	CNSAFIHVVMF	LTVVDAV	PPDMPESLH	LGNEFKFKS	SYINRVR-M	61 CI
AC022323.1										SLH	LGNEFKCKS	LSYINRVT-T	6I
AL022344.1				KMPLFFPK	LPLDFDP	FFNIFTELDHF	R-SKHHGUIS	YHIMFINIPHC	GGP	VSRYA	ITALREVQT	LVIHROSED	эвсп

	* 7	140	* 160	*	180	*	200	*	220		240	*
AC020679.2	CTTCLLDMLQ	VNISPSISWI	VREKWKSTIFTFH	LESWSLSF	PVSSSLTFYT	VASSNVTQIN-	LHVSKYC	SLFPINSIIF	GLEFTLSLF	RDVFLKQ-I	MLFSSVYMMTI	DOELOEILV
AC060814.1	CTTCLLGMLQ	VNISPSISWL	VRFKWKSTIFTFH	LFSWSLSF	LVSSNLIFYT	VASSNVTXIN-	LHVSKYC	SLFPINSII	RLVFTL			
679A7	CTTCLLNMLQ.	AISISPSTSWL	ARFKHKSTNYILHV-		.s							
AC011821[118613]	CITCLLSMLQ!	AIIISPRTSWL	VREKYKETNHISHV-	LVFIWSFST	SCSSYTTFYT	VVSSNTTHTSF	LTLNKYC	LISPRKSSSF	LIFLTLSLS	RDVSFVG-T	MLLSSAYIVEL	ICR-HQWSO
354A1	CTTCLRSVLQ.	AVTITEGTSWL	ARIKOKFTHCIFH	BLGFSVSF	/sv							
656F5	CTTCLRSVLQ	AVTITPGTSWL	ARIKOKETHCIF									
AC022860[15916]	CTTCLLSMLQ	AVAISPGTSWS	ARIKQKFKGYIFHS-	FEFLWVILSI	SLSSNLLSST	VASSNGTKTVV	LSISKYS	LSSISYI	LSELPLE	-NVEFVA-T	MOPSSAYMVII	LFRHQRQSQ
428E8	SITFILITVLO	/ITISPSTSWL	VKTK									
AC011821[131597]	CTTSLLSMLQ	ITISPSTSWL	VREKEKIHILGLL	FFWSLNL	SFNSDMIIYI	VGFSSV-TQI	LNVSEYC	SLSPMNVTI	RLFVTLSLS	RDVFLVG-I	MPLSSAFMVIL	LSRHQRCSQ
AC22202.4	CTTSLLSMLQ)	AITISPSTSWL	VRFKHKITKYNILA-	L-LLWSLNF	SFNSDMIIYI	VGFSSV-TQI	LNVSKYC	SLSPMNVTIF	RLFVTLSLS	RDVFLVG-I	MPLSSAYMVII	LSRHQRRSQ
AL161418.3	CTTCLLSMLQ	AITISLSTSWL	VREKHKETKYDILG-	LF VFWFSNL	SFSSDMIIYT	WGYSNTQII	LNISKYC	TFFPMNVIII	TLFLMLSLS	RDAFFIG-I	TLLSSVYMVIL	LSRHQRHSQ
AL157387.1	CTSCLLSTLQ/	AITINPSTSWL	ARVKHKSTNYIIHV-	FFSFWFLNL	FSRSNMIMYT	VAYFKHRPGNQ	IPTSKYC	SFSVISIS-	GUIFILEF	PNVFFVE-I	MLLSIVCMVVI	LFNHQR-SH
V1R1	CTTSMLSVLQ/	AIILSPRSSCL	AKEKRKSLHHISCA-	ILFLSVLYM	LIGSQULVSI	IATPNLTTNDF	IYVIQSC	SILPLSYVMC	SMESTLLVI	RDVFLIS-L	MVLSTWYMVAL	LCRHRKKTQ
V1R2	CTTSMLSVLQ/	AIILSPRSSCL	AKLKHKMPHHISCA-	IIFLSVLY	LISSHILLSI	IATPNLTRNDF	LYVTQSC	SILPLSYVMC	SMASTILAL	REVFLIS-L	MVLSTLYMVVL	LCRHRKOAQ
AC011607.4	STTCLLSILP	AITCSPRSSCL	AVFRFSHHQPRC-	FLFIWVFHT	SISDSFLVST	LPIK MASNSI	TFVTQSC	SAGILSCFLF	QTIFUMTE	QDVSLAG-L	TAPSSGYMVII	LSRRNRQSQ
AC021022.2	STTCLLSVLQA	AINLTPRSSRL	AMERDPIITNRV-	A SCOGSST	YPLVEASSPL	FPQK-CCLNSG	TFVDQSC	SAGPLSCFILC	QTIFULMTE	QDVSLXG-L	MAPFSGYMVIL	LCRHNRQSQ
92F11					·ILYT	IASSSVTLSN-	-LLHIS	KYCS-LSIIF	RLFLTLSL	RDVLLIR-I	MLLSSTCMVVI	LFRHOMKFW
AC022860[140704]	CTTCLLSVLQ	AIIISPSSFWL	VRENHKSTVFTCHFF	FFSCF R	FFF VV	ISSCTLLLPVP	AIFCIS	VNTVRFSIIF	RLFLTLSLF	RDVLLIR-I	MLLSSTCMVVI	LFRHOMKFW
378E6						-TTSGACLKS-	7	LVQRSC	ALFLILSLS	RDVFFCRTR	CLLSSAYMVVI	LFRHQROPO
AC024199.1												ККК
AC025699.3	TTTCLLSMLQ.	AITISPSILWV	R		KN	INLPSVGDFCS-	LS	SPMSSITC	GUIFINNEF	QNIEFSG-I	MLLSSMYTVIL	FRHERYOS
AC025953.2	CNICLLSIHQ/	ANTISPNNFCL	ARLKOKFTNNIIMSS	FFSFFFWSINL	SFSYNIVFFT	VASSNVTQNSL	PKGQYC	SLSPMKSFMF	KVFFTLTLS	RDVFIIG-I	TLHSIAHMVIL	VSRHETOSO
AC022323.1	CNTCLLSVHQ.	ASTISPSNCCL	ARLKOKSQISLS-	-VSFFFWSVNF	SFSHNIIFFT	VASSNVTQTSL	LKVSKYC	SLSPVKSFMF	KVFLFLTLS	RDVFIIG-I	TLHSIAHMVII	LSRHQRQSQ
AL022344.1	CNTCLLSVHP	ASTISPSNCQL	AREKOKETNNIITV-	-ISSYFF	FWS	VCLSVVIYSSI	WLLPNCIPVVI	RSANTVPFUF	SPFFTLTLS	GDVFIIG-I	TLHSITHMVIF	LSOHOROSO

Figure 1a (cont)

	260		280	*	300	*	320		340	
AC020679.2	PSQPQPEPKDI	LCRGKS-HQH	ILLPVSFSVG	YKMDFILST	SSTLPWAYDR -	GVREV	SVYTINRF	LLRSDKRV	NVM	
AC060814.1										
679A7										
AC011821[118613]	HLHSTRLSPR	SPEK								
354A1										
656F5										
AC022860[15916]	YLHRTNLSPR	SPEKR-TIL	LLVSCFLVMY -							
428E8			- 							
AC011821[131597]	HIHSTSFLLR	SPEKR-ATK	TILLLVSFFVV	MYSLDLIVS	SSKMLLWVFSPV	DSVHKFV	/NAYATVSPM	VLIRSEKRI.	STUPKVHWKCH	PFL-
AC22202.4	HLHSTSFLLR	SPEKR-ATK	TILLLVSFFVV	MYSLDLIVS	SSTMLLWVFSP	VIYSVHKFM	/NAYATVSPM	VLIRSDKRI:	SILPKVH	
AL161418.3	HFHSSSLILR	ISLVKM-ARK	TILMLVNSFVL	MYSVDFILS	SSTMLLWVIGP	VTYGVH	NAYATVSPL	VLIRSDERI	INILQKFQWKCH	l
AL157387.1	HLHSPSLCSSI	PSPAKR-AAH	AILLLVICEVV	M						
VIR1	HLQGISLSPK	SPROR-ATC	TLLMLMSFFVL	MTIYDTIVS	- CSRTMELNDP	TSYNMOIFV	HIYATVSPF	FMSTEKHI	NCLRSV	
V1R2	HLOGTSLSPK	SAEQR-ATC	TILMLMTFFVL	MSIFDSIVS	-CSRTMFLDDP3	TSYSIHIFY	HIYATVSPF	VFMSTEKHI	NILRG	
AC011607.4	HFHSTNLSPK)	PPEKM-ATC	TILLLVSCFVI	VYVLDCVVA	SCSGLVWNSDP	VRHRVOMLVI	ONGYATISPS	VIVSTEKMI	KVTSMW	
AC021022.2	HLHSINLSPK	PPDKR-AIC	SILLLVSEFVF	MCLF						
92F11	YFHSTSLSPR	PSPEKS-ATC	TILLLVSIFVV	IYWVDFIIL	FISISLWAYDP	VVLCVQRLV	NVYATVSPF	VLLRSDKKI :	SWAKTVRQKVN	KL
AC022860[140704]	YFHSTSLFPR	PSPEKS-ATC	TILLLVSIFVV	IYWVDFIIL	FISTLLWAYDP	VVL <mark>G</mark> VQRLV	NVYATVSPF	VLLRSDKKI	SWAKTVRQAVN	KL
378E6	YLHSTSLSPR	PSPEQS-ATC	TILLLVSFFVV	VYSVDFIIS	FSSTSLWAYDP	VVL <mark>G</mark> VQRLV	TVYATVSPL	FLRSDKRI	INVAGCGGSSL-	
AC024199.1	SPQ1	esl <u>okk</u> rtak	TILLLVSFFVV	TYWMDLSLS	SYSIPISTYDP	VIL <mark>GVQR</mark> RV	SAYATVNPL	VLIRS <mark>E</mark> KRI	DILQMMRQKYH	
AC025699.3	-LRNTSRSPR	SPEKRAMOT	SLCLLVSCSVV	TYSVDVITS	SSLTMWWVHGP	VTRDVQMPV	TLCATVCPL	VQIIPDRRI:	NIPKKYSNKVP	PIFN
AC025953.2	HLHSISISPQ	FPEKR-AAC	TIPLLVSYCLV	MCWVDLIIS	SSSTLLWTCNP	VFLSMONLV	DVYATVVLL	EQISSOKNI	TDILQNMQSAIK	
AC022323.1	QLHSTSFSP-	FPEKR-ATC	TIQULS							
AL022344.1	HLHSTRISPR	EPEKR-ATC	TILLIVSYEG-	- CWVDUTTS	CSSTLLWTYNTY	SVON W	NUSATVVPI	RIS		



Figure 1 Sequence alignment analysis of non-functional human VNO receptor sequences with mouse V1R and V2R genes. Shown are ClustalW alignments of non-identical putative human VNO receptor sequences are darkened according to the following consensus percentages: black = 50%; dark gray = 25%; light gray = 5%. (a) Alignment of 22 distinct putative VNO receptor sequences homologous to the V1R family along with mouse V1R1 and V1R2 sequences. (b) Alignment of nine distinct putative VNO receptor sequences homologous to the V2R family along with mouse V2R1 and V2R2 sequences. Not included among the latter alignment are 182G10, 139H10 and AC011457.1 [41301] due to short sequence length relative to the other nine V2R-like psuedogenes. Values within brackets correspond to the 5' starting location of the genes within HTGS BACs, as shown on Table 1.

receptor sequences had been inactivated via Alu insertion. Two BACs (AL161418.3 and AC010422.4) contained Alu sequences within coding regions, while four others (AC025953.2, 378E6, 34G7, and AC011457.1) contained Alu sequences immediately adjacent to coding regions. An additional eight BACs (AC012018.6, AC022202.4, AC025699.3, AL157387.1, AC022860.3, AC009139.4, AC011457.1 and AC016588.4) contained Alu sequences further upstream or downstream of the putative VNO receptor gene (Table 1).

Discussion

Our data suggest that human VNO receptors throughout the genome have undergone significant degeneration as a result of missense mutations, frameshift mutations and Alu insertions. These results are consistent with an independent, small-scale search for human VNO receptor genes in which seven psuedogenes were identified (Giorgi *et al.*, 2000). The work here extends the implications of the study by providing greater evidence that human VNO receptor genes throughout the genome have undergone significant degeneration. It should be noted that a more recent HTGS screening for putative VNO receptors was performed, yielding an additional set of 16 sequences that were all identified as pseudogenes (data not included).

Although nearly all putative VNO receptors in this study appear to be pseudogenes with deleterious mutations, it is nonetheless possible that some of these pseudogenes are expressed in the VNO or olfactory epithelium as truncated proteins. This possibility seems unlikely given the high degree of sequence degeneration and the extremely short coding regions of the pseudogenes. One may make the argument that the pseudogenes are expressed since we screened a BAC library with primers designed from human cDNA sequences. Because the cDNA sequences were from testis as well as from several tumor cell lines, it is indeed possible that the original cDNA sequences were in fact misexpressed pseudogenes. It remains to be shown, however, that these pseudogenes are expressed in the VNO and that they play functional roles in human pheromonal olfaction. In addition to being misexpressed pseudogenes, it is also possible (but less likely) that the cDNAs were simply not VNO receptor genes or that they arose from genomic contamination of the cDNA library.

Chromosomal mapping of the sequences in this study revealed clusters of putative VNO receptors in subterminal and pericentromeric localizations. This pattern, plus the observation that four BACs carried two VNO receptor genes separated by 21–124 kb, suggests that VNO receptors may be genomically clustered like odorant receptors (Sullivan *et al.*, 1996; Trask *et al.*, 1998). Thus, like odorant receptors, VNO receptors may have arisen through duplication events. Such a claim may be challenging to verify due to the sequence divergence that has resulted from the degeneration of these genes over time. On the other hand, analysis of the level of degeneration between duplicated pairs of genes could yield insight into the evolutionarily decline of human VNO receptors.

Our data support the theory that most human VNO receptors have degenerated over time, which further suggests that the human VNO is a vestigial structure. Since our data do not prove that all human VNO receptors are pseudogenes, it is possible that humans have retained a few VNO receptors that have irreplaceable functions. Thus, the recently identified VIRL1 gene may have played a significant role in pheromone olfaction and was thus under selective pressure not to degenerate. The role may not be that of the conventional mammalian VNO receptor, however, since V1RL1 exhibits only 28% identity to the closest mouse VIR gene and is expressed in the olfactory mucosa. It is possible that V1RL1 was once a VNO receptor which evolved into an odorant receptor with pheromone-like ligand properties, but further work will be necessary to prove this point. The pseudogenes in our study, which exhibit greater homology to mouse VNO receptors, may not have been under such selective pressure in humans. They may have served as conventional pheromone receptors in the VNO, an organ that is believed to have degenerated in humans once higher cortical centers began to replace its functions (Keverne, 1999). It should be noted that there exists a lack of direct evidence that V1R and V2R receptors act as chemosensory pheromone receptors, though the complete absence of human counterparts to rodent VnRs seems to add support to such a role.

This experiment offers data that can be used in future experiments to characterize human VNO receptor genes and the human VNO. Conserved regions within these human putative VNO receptor gene sequences can be used to generate additional primers for DNA library screening. Degenerate primers designed from conserved regions in mouse VNO receptors can also be used for such screening. As additional mouse VNO receptor genes are identified and characterized (e.g. the recently identified V3R family), more accurate alignment analyses and synteny comparisons can be performed, which may aid in locating additional human VNO receptor genes. Also, evolutionary studies may be performed by searching for the homologs of these putative human VNO receptor genes in other primates. By comparing the structural properties of these pseudogenes and the presence of Alu sequences across species, it may be possible to elucidate the evolutionary degeneration of human VNO receptors. The latter may yield insight into the evolutionary history of the human VNO, the functional existence of which remains unclear. Additional work to characterize the human VNO may include *in situ* hybridization experiments with primer sequences from this study as well as neural tracing experiments to characterize VNO sensory neurons.

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