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Isolation of an Olfactory cDNA: Similarity to Retinol-Binding Protein Suggests a Role in Olfaction

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Molecular cloning techniques were used to isolate and characterize a protein possibly involved in the signal transducing system in olfactory tissue of the frog *Rana pipiens*. A complementary DNA library was constructed with messenger RNA obtained from frog olfactory neuroepithelium. A 700–base pair complementary DNA clone encoding a protein with a molecular weight of 20,300 was identified by differential hybridization analysis with polyadenylated RNA from olfactory epithelium and nonsensory respiratory epithelium. The messenger RNA corresponding to this clone was abundant in the cells of Bowman's glands in olfactory tissue but not in respiratory epithelium nor in several other tissues. The predicted sequence of this protein is homologous to members of a family of proteins that bind and transport small molecules in serum, suggesting that this protein may also bind and transport odorants in the mucus secreted by Bowman's glands.

THE PRIMARY EVENTS OF THE OLFACTORY response—odorant recognition and signal transduction—occur in the receptor neurons of the olfactory neuroepithelium. The apical processes of these bipolar neurons lie at the luminal surface of the tissue and are covered with long cilia (*1*). The mucosal layer contains two other cell types—supporting cells and neuroblast-like progenitor basal cells from which mature neurons continually differentiate. In the submucosa just beneath the basal lamina are the Bowman's glands, which secrete the mucus that bathes the olfactory cilia. This mucous layer is thought to play a major role in the solubilization and concentration of airborne odorants (*2*).

Transduction appears to be mediated by odorant-specific protein receptors in the ciliary membranes (*1*), and several investigators have identified specific odorant-binding activities in olfactory tissue extracts (*3–5*). However, purified molecular components of the transduction system have yet to be fully characterized and their activities correlated with actual signal transduction in olfactory neurons.

We have used molecular cloning techniques (*6, 7*) to isolate and characterize individual products specific for olfactory tissue. We constructed a complementary DNA (cDNA) library of approximately 4500 clones using polyadenylated [poly(A)$^+$] messenger RNA (mRNA) obtained from olfactory mucosa of the frog *Rana pipiens*. Approximately 10% of 2400 clones screened were olfactory-specific; that is, they showed strong hybridization to a probe derived from olfactory mRNA, and weak to negligible hybridization to an mRNA probe derived from respiratory epithelium. The six clones that showed the most intense olfactory-specific hybridization were studied further by restriction mapping and nucleotide sequencing. Analysis with various combinations of restriction enzymes indicated that five of the six clones had similar restriction maps.

Analysis of the nucleotide sequence of both strands of clone 5B5, the longest of the five similar clones, revealed a single long open reading frame putatively encoding a 182–amino acid protein with a predicted molecular size of 20.3 kD (Fig. 1). The sequence contained no consensus *N*-linked glycosylation signal sites but had a single 13–amino acid hydrophobic region at the amino terminus, similar to the signal sequence of many secreted proteins. Hybridization analysis of total RNA from various tissues with an M13 strand-specific probe containing the central Pst I–Hind III fragment from the 5B5 coding region indicated that the approximately 850-base message represented by this cloned cDNA was produced in large quantity in the frog olfactory neuroepithelium but not detectable in frog brain, liver, or respiratory tissue (Fig. 2A).

The predicted properties of the 20.3-kD secreted protein that was specific for olfactory tissue coincided with those of a soluble protein of approximately 20 kD observed in extracts of olfactory tissue (Fig. 2B). This protein, not present in extracts of respiratory tissue, was also seen when total RNA from olfactory epithelium from frog was analyzed in a rabbit reticulocyte lysate in vitro translation system (Fig. 2C).

To demonstrate that this protein corresponded to the product of the olfactory-specific mRNA we had examined, we attempted to specifically arrest the translation of this species in vitro (Fig. 2C). Addition to the translation reaction of SP6-transcribed RNA complementary to the message specifically blocked the translation of the 20-

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Fig. 1. Nucleotide sequence and amino acid translation of clone 5B5. Clones were obtained with the ribonuclease H and DNA polymerase I method of Gubler and Hoffman (*14*). Tailed cDNA fragments were inserted into the Eco RV site of cloning vector pKP45, a pBR322 derivative with a deletion of base pairs 677 to 2364 (*15*). The 5′ Bam HI site was created upon insertion of the tailed cDNA insert into the Eco RV restriction site. Sequences were obtained from restriction fragments subcloned into M13 RF DNA. Sequence was confirmed with oligonucleotide-primed synthesis on denatured plasmid DNA template by the Sanger dideoxynucleotide method (*16*). The putative 20-kD protein sequence begins with the ATG codon at nucleotide position 18 and ends with the TGA termination codon at position 549. Amino terminal hydrophobic residues are shaded. Variations in nucleotide sequences and in the resultant amino acid sequences of other clones are indicated above and below the 5B5 sequence. Pst I and Hind III restriction sites are indicated.

kD protein from total mRNA from olfactory tissue. Addition of analogous SP6-transcribed sense-strand RNA had no effect on the translation of that species. A reciprocal experiment, in which the message for the 20-kD protein was isolated by hybridization to a complementary probe and translated in vitro, confirmed this result.

In situ hybridization of [^{3}H]uridine triphosphate–labeled RNA complementary to the 5B5 message to tissue sections indicated that this message is confined to the cells of the Bowman's glands (Fig. 3). This result is consistent with the hypothesis that the 20-kD protein, which we refer to as protein BG, is an abundant secretory product in the mucus of the olfactory epithelium.

The sequence of protein BG bears no similarity to that of olfactory marker protein, an olfactory neuron–specific protein recently sequenced by Margolis and coworkers (*8*). However, a computer search with the predicted amino acid sequence of protein BG indicated similarities to several soluble proteins, including serum protein α_1-microglobulin (also called protein HC) (21.4% identity over a 159-residue overlap), bovine β-lactoglobulin (BBLG) (17% identity over a 159-residue overlap), and human retinol-binding protein (RBP) (19.7% identity over a 173-residue overlap) (Fig. 4). These proteins are members of a recently described family of proteins. Pervaiz and Brew (*9*) analyzed the conservation of sequences among members of this family and proposed that members with homologous sequences may have similar structures and functions. All members of the family are thought to bind and transport small molecules in serum. Human RBP binds extracellular retinol (*10*). BBLG binds retinol and may bind and transport vitamin B–like molecules in milk (*9*). Protein HC appears to tightly bind a chromophoric molecule which has not yet been identified (*11*).

The sequence of protein BG is similar to those of the members of this family (Fig. 4). Cysteine residues that form disulfide linkages are conserved in the tertiary structure of RBP and BBLG (Cys^{75}–Cys^{180} and Cys^{125}–Cys^{134} in RBP; Cys^{75}–Cys^{180} and Cys^{125}–Cys^{139} in BBLG; Cys^{75}–Cys^{180} in protein BG) (*12*). Cys^{34} and cys^{136} in protein BG have conserved counterparts in protein HC and RBP, respectively. Also, alignment of protein BG with this family places the initial hydrophobic 10 to 15 amino acids on the amino terminal side of the initial residues of the other proteins. Signal sequences are normally removed by proteolytic cleavage during cotranslational processing; we therefore propose that the amino terminal sequence of protein BG is also cleaved during processing.

The question remains as to why an olfactory-specific protein resembles a family of serum transport proteins. A possible explanation lies in an examination of the role of the mucus in the olfactory system. The mucous layer represents the air-fluid interface at the surface of the transducing neuroepithelium. Therefore, its composition and its solubilizing capabilities probably have a role in modulating the olfactory response to particular odorants. Thus, protein BG may participate in the binding, transport, and concentration of odorants—particularly hydrophobic ones—in the mucus. Could this binding be specific for certain odorants? Sequencing of several clones revealed few variations (Fig. 1). This similarity suggests that sequence differences in protein BG do not provide for odorant discrimination. Therefore, although it is possible that other,

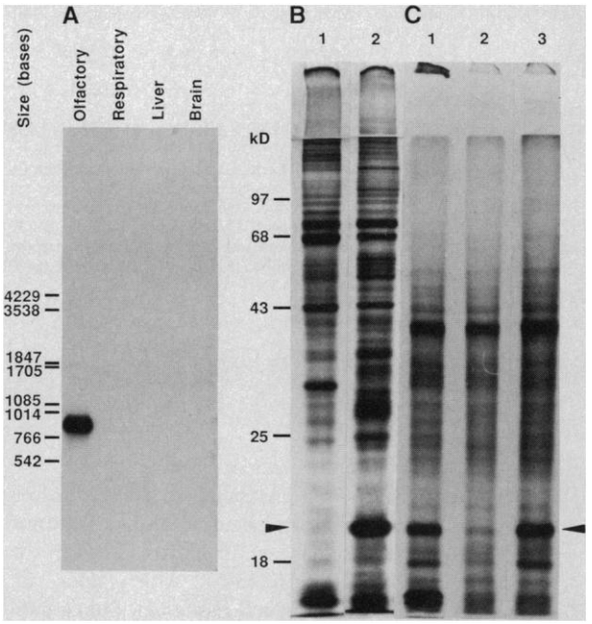

Fig. 2. (**A**) Northern blot experiment. Total RNA was extracted from frog olfactory, respiratory (palate), liver, or brain tissue in the presence of guanidium thiocyanate (*17*). RNA samples (10 μg) were treated with glyoxal and subjected to electrophoresis on a 1% agarose gel in 10 m*M* phosphate buffer, *p*H 7.0. The RNA was transferred to Nytran (Schleicher & Schuell) and hybridized to a single-stranded DNA probe labeled by primer extension. The probe contains the central 450-bp Pst I–Hind III restriction fragment from clone 4A1. After hybridization overnight (12 hours) at 42°C, the filter was washed twice for 30 minutes at 67°C in 2× standard saline citrate buffer containing 0.1% SDS (*18*), air dried, and autoradiographed. Standard saline citrate buffer consists of 150 m*M* NaCl and 15 m*M* sodium citrate. Molecular sizes were determined from ^{32}P-labeled SV40 DNA restriction fragments run in adjacent lanes. Hybridization with a *Xenopus laevis* ribosomal DNA clone confirmed the presence of undegraded RNA in each lane. (**B**) Silver-stained SDS-polyacrylamide gel of frog respiratory (lane 1) and olfactory (lane 2) tissue extracts. Extracts were prepared by disrupting tissue samples with a Polytron homogenizer in TME buffer (*19*), centrifuging at 4000*g* for 5 minutes, and 85,000*g* for 90 minutes to obtain soluble fractions. TME buffer contains 30 m*M* tris-HCl, *p*H 8.3, 10 m*M* $MgCl_2$, and 0.1 m*M* EDTA. Samples (80 μg) of the supernatants from the second centrifugation were analyzed on a 10% SDS-polyacrylamide gel (*20*). Protein bands were visualized by silver stain (*21*). Arrows indicate the 20-kD protein band. (**C**) Hybrid arrest experiment. Single-stranded sense or antisense transcripts of the 450-bp Pst I–Hind III fragment of clone 4A1 were obtained with the in vitro transcription plasmids SP64 and SP65 (*22*). One microgram of total RNA from frog olfactory tissue was incubated with approximately 2.5 μg of SP64-450 (sense strand) or SP65-450 (antisense strand) RNA. The sample was cooled from 75°C to 58°C over the course of 1 hour and then translated in vitro in a rabbit reticulocyte lysate (*23*). Low molecular weight species that were also apparently arrested may represent prematurely terminated translation products. Lane 1, olfactory RNA plus SP64-450; lane 2, olfactory RNA plus SP65-450; lane 3, olfactory RNA, no additions.

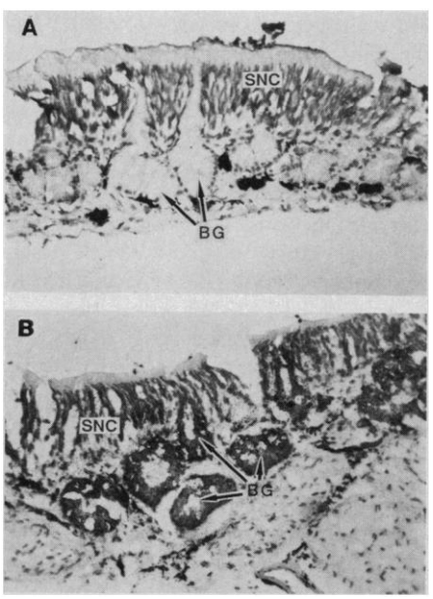

Fig. 3. In situ hybridization. Tissue samples were fixed in 1% glutaraldehyde, sectioned to 10-μm thickness, exposed to photographic emulsion for 20 days, and stained with hematoxylin according to the procedure of Angerer and Angerer (*24*). (**A**) [^{3}H]Uridine triphosphate–labeled SP64-450 sense RNA (control). (**B**) [^{3}H]Uridine triphosphate–labeled SP65-450 antisense RNA. SNC, supporting and neural cell layer; BG, Bowman's glands. Apparent darkness of the Bowman's gland structures is due to intense localization of exposed emulsion grains in and out of the plane of focus.

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                10        20        30        40        50        60
BG     * ---QCQADLPPVMKGLEENKVTGVWYGIAAASNCKQFLQMKSDNMPAPVNIYS---LNNGHMKSS
RBP       -ERDCRVSSFRVKENFDKARFSGTWYAMAKKDPEGLFLQDN-IVAEFSVDETGQMSATAKGR-VR
BBLG      ------LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELK---PTPEGDLEI
HC        GPVPTPPDNIQVQENFNISRIYGKWYNLAIGSTCPLKIMDRMTVSTLVLGEGA-----TEAEISM

                70        80        90        100       110       120       130
BG        TSFQTEKG-CQQM--DVEMTTVE-KGHY-KWKM----QQGDSETIIVATDYDAFLM-EFTKIQMG
RBP       LLNNW--DVCADMVGTFTDTEDPAKFKMKYWGVASFLQKGNDDHWIVDTDYDTYAVQYSCRLLNL
BBLG      LLQKWENGECAQKKIIAEKTKIPAVFKIDA--------LNENKVLVLDTDYKKYLL--FCME-NS
HC        TSTRWRKGVCEETSGAYEKTDTDGKFLYHKSKW-----NITMESYVVHTNYDEYAI--FLTKKFS

                          140       150       160       170       180       190
BG        AEVCV--TVKLFGRKDTLPEDKIKHFEDHIEKVGLKKEQYIRFHTKAT-CVPK
RBP       DGTCADSYSFVFSRDPNGLPPQAQKIVRQRQEELCLARQYRLIVHNGY-CDGRSERNL
BBLG      AEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI
HC        RHHGPTITAKLYGRAPQLRETLLQDFRVVAQGVGIPEDSIFTMADRGE-CVPGEQEPEPILIPR
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* Sequence of N terminus = MIRIIAIVVLFFL

Optimal alignment score (with respect to protein BG): HC, 130.0 ± 20.7 (SD); BBLG, 125.0 ± 9.2; RBP, 63.0 ± 3.6.

Fig. 4. Sequence alignment of protein BG with proteins of the retinol-binding protein family. Alignments were made initially with the FASTP program described by Lipman and Pearson (*25*) on a VAX/VMS operating system, and final alignment adjusted for all four proteins, based on the alignment between protein HC and protein BG. Protein HC and BBLG were the proteins with greatest homology to protein BG identified in a search of the National Biomedical Research Foundation library; RBP is included because of its similarity to HC and BBLG (*9*). The pairwise optimal alignment scores (*25*) as well as their significance in standard deviation units were calculated with the FASTP and RDF programs of Lipman and Pearson. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M. Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

more variant clones exist but were not detected, if protein BG plays a role in the binding and solubilization of odorants, it most likely is a nonspecific one.

Other investigators have reported the purification of odorant-binding proteins from bovine sources. Pelosi and his co-workers described the binding of pyrazine compounds to protein extracts from bovine olfactory mucosa (*5*). Pevsner *et al.* further characterized this pyrazine-binding activity and have implicated a specific protein, OBP (for odorant-binding protein), for this role (*13*). OBP is also a mucous protein synthesized in the Bowman's gland cells and appears to bind several other odorants, including amyl acetate and methyldihydrojasmonate. Its site of synthesis and its reported molecular size of 19 kD is similar to the predicted properties of frog protein BG. Extensive sequence homology between frog protein BG and bovine OBP or demonstrations of odorant-binding activity by purified protein BG would imply that these proteins have similar roles in the binding of odorants and presentation of odorants to receptors.

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RNA Complementary to a Herpesvirus α Gene mRNA Is Prominent in Latently Infected Neurons

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In initial attempts to define the molecular events responsible for the latent state of herpes simplex virus, in situ hybridization was utilized to search for virally encoded RNA transcripts in latently infected sensory neurons. The use of cloned probes representing the entire viral genome indicated that transcripts encoded within terminal repeats were present. When the α genes encoding ICP-0, ICP-4, and ICP-27 and the γ_1 gene encoding VP-5 were employed, only RNA transcripts hybridizing to the ICP-0 probe were detected. In latently infected cells, the ICP-0–related transcripts were localized principally in the nucleus; this was not the case in acutely (productively) infected neurons or in neurons probed for RNA transcripts coding for actin. In Northern blotting experiments, an RNA of 2.6 kilobases was detected with the ICP-0 probe. When single-stranded DNAs from the ICP-0 region were used as probes, RNA from the strand complementary to that encoding ICP-0 messenger RNA (mRNA) was the major species detected. This RNA species may play a significant role in maintaining the latent infection.

HERPES SIMPLEX VIRUS (HSV) establishes latent infections in the neurons of sensory ganglia, and the natural history of recurrent disease is characterized by reactivation of active infection from these cellular reservoirs (*1*). The molecular mechanisms operating during establishment and maintenance of latency and during reactivation from the latent state are not understood. On the basis of information available about other DNA viruses that establish latent infections, including the related Epstein-Barr virus, one would predict that the replication cycle of the latent virus is restricted or blocked at some early point. Although the precise mechanisms are not defined, in these other systems it is generally thought that at least some viral encoded proteins that are detectable in latently infected cells function to maintain the latent state (*2*).

Many laboratories, including our own, have searched for HSV transcripts and proteins in latently infected sensory neurons. At least three groups have detected transcripts (*3*), and there is one report that a specific protein, the α polypeptide ICP-4, occurs in trigeminal ganglia of latently infected rabbits (*4*). In spite of this latter finding, proteins are generally difficult to reproducibly demonstrate in latently infected cells harboring the HSV genome.

As a first step in defining the molecular nature of the latent state, we have determined which viral genes are transcribed during latent infection in mice. Using the complementary technologies of in situ and RNA blot hybridization, we have shown that transcripts from the genomic region encoding the α protein ICP-0 were present in relative abundance in latently infected sensory neurons. These were the only RNAs readily detected by in situ methods, and they were localized mainly in the neuronal nucleus. ICP-0–related RNA transcripts in acutely (productively) infected neurons and actin gene transcripts in uninfected neurons were not localized in the nucleus. Further investigation showed that the RNA transcripts in the latently infected neurons derived from the DNA strand opposite to that encoding the ICP-0 messenger RNA (mRNA).

To establish which regions of the viral genome were transcribed, we utilized in situ hybridization on frozen sections of murine spinal ganglia latently infected with the HSV-1 strain KOS-M, which does not readily invade neural tissue (*5*). Spinal ganglia from latently infected mice were cut on a cryostat and subjected to in situ hybridization with methods specific for viral RNA. The four groups of probes represented essentially the entire genome; they included Hind III fragments I, O, and J; Hind III fragments A, K, and L; Hind III fragments D+M and Eco RI fragment H; and Eco RI fragment J+K (*6*). All were nick-translated with [^{3}H]nucleotides. A positive signal was obtained with the pools of labeled probes that contained Hind III D+M and Eco RI H, and Eco RI J+K (all probes were positive on acutely infected ganglia) (Fig. 1). These results suggested that genetic information in the terminal repeat regions might be expressed during the latent state. Included in these regions and in adjacent areas in the long and short unique regions are the genes encoding the five α polypeptides (that is, those proteins that are the first to be synthesized during a productive infection). Three of these genes are the ICP-4 gene [0.83 to 0.86 and 0.96 to 0.99 mu (map units)], which codes for a multifunctional regulatory protein that controls the synthesis of both itself and later genes (*7*), and the ICP-0 (0.79 to 0.81 and 0.01 to 0.03 mu) and ICP-27 genes (0.74 to 0.75 mu), which encode proteins that regulate later genes (*8*) by an unknown mechanism.

We then tested whether specific HSV transcripts were detectable in latently infected cells by employing probes for the three α genes and the γ_1 gene (0.24 to 0.27 mu) VP-5. Transcripts from the VP-5 gene (en-

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