pheromones has been suggested (*16*), but these putative pheromones have never been isolated. Given the fact that sexually mature newts lead an aquatic life, a nonvolatile, but water-soluble, peptide is a reasonable form to expect as a pheromone in this vertebrate.

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5. A plastic container (diameter, 37 cm) was filled with 3000 ml of tap water. Eight test females received daily injections by us of 1 IU of prolactin and 25 IU of human chorionic gonadotropin for 7 to 11 days in order to secure their responsiveness to the sex attractant. On the day after the last injection, each test animal was put in a smaller cylinder of stainless steel mesh that was placed in the center of the plastic container. The container was divided into three sectors into which three sponge blocks with dimensions 5.6 cm by 7.3 cm by 3.4 cm (one per sector) were gently placed. One block contained the test substance dissolved in 100 ml of water, and the others contained control substances. Thirty seconds after the introduction of the sponge blocks, the inner cylinder was removed. The position of the snout of the test animal was observed, and the time spent by the snout in each sector was recorded by video for 10 min. In a series of tests, the test females were not used more than once. The percentage of the period spent by the snout of the test animal in each sector was analyzed statistically with Friedman's two-way analysis of variance, followed by the Wilcoxon matched-pairs signed-ranks test (*4*).
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8. Synthetic sodefrin was prepared by solid-phase chemistry (American Peptide, Sunnyvale, CA).
9. We generated an antiserum to sodefrin in a rabbit by injecting sodefrin that was extended on its COOH-terminus with Cys coupled to keyhole limpet hemocyanin (Pierce). Abdominal glands were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4°C. Frozen sections were cut at a thickness of 6 μm. The sections were incubated with 20% normal goat serum for 30 min before a 2-hour incubation with antiserum to sodefrin diluted at 1:1000 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) or with antiserum (1:1000 in 1 ml) absorbed with sodefrin (1 nmol). After the sections were washed with PBS, they were incubated with a mixture of rhodamine-labeled, affinity-purified goat antibody to rabbit immunoglobulin G (Jackson Immunoresearch) diluted with BSA (1%) in PBS and 4′,6-diamidino-2-phenylindole dihydrochloride (2 μg/ml) [K. Takata, T. Kasahara, M. Kasahara, O. Ezaki, H. Hirano, *J. Histochem. Cytochem.* **39**, 287 (1991)].
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17. Care of experimental animals was in accordance with institutional guidelines. We thank M. Ohashi and S. Kurono for their help with mass spectrometry. Supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (to S.K. and F.T.) and by a research grant from Waseda University (to S.K.).

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Epithelial Antibiotics Induced at Sites of Inflammation

Barry S. Schonwetter,* Ethan D. Stolzenberg, Michael A. Zasloff

The role of antimicrobial peptides in epithelial defense is not fully understood. An epithelial β-defensin, lingual antimicrobial peptide (LAP), was isolated from bovine tongue and the corresponding complementary DNA cloned. LAP showed a broad spectrum of antibacterial and antifungal activities. LAP messenger RNA abundance was markedly increased in the epithelium surrounding naturally occurring tongue lesions. This increase coincided with the cellular hallmarks of acute and chronic inflammation in the underlying lamina propria, supporting a role for epithelial antimicrobial peptides as integral components of the inflammatory response.

The epithelia of vertebrates provide the first line of defense between organism and environment (*1*). When this barrier is breached, microorganisms invade and an acute inflammatory response occurs (*2*). The physical barrier is fortified by the secretion of numerous antibacterial agents, including immunoglobulin antibodies, enzymes such as lysozyme, and proteins such as lactoferrin (*3*). Antimicrobial peptides have also been detected in barrier epithelial cells of several mammalian species, including mice, cows, and humans (*4*–*6*). Although the expression of antimicrobial molecules in epithelia suggests that they may participate in host defense, no direct evidence has been obtained to support such a role.

Mammalian tongue contains a dense epithelium that is constantly colonized by the microbial biota of the mouth, which includes bacteria, fungi, and viruses (*7*). Although abrasions to the surface of the tongue occur often, invasive infections in a normal host are rare, remain localized, and heal rapidly. Invasive infections of the tongue would interfere with the processes of chewing, swallowing, taste, and speech (*8*).

Why is this exposed surface free of continuous infection? We approached this prob-

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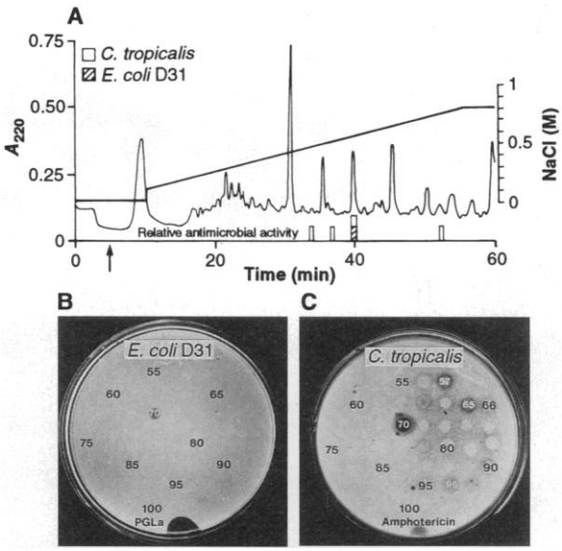


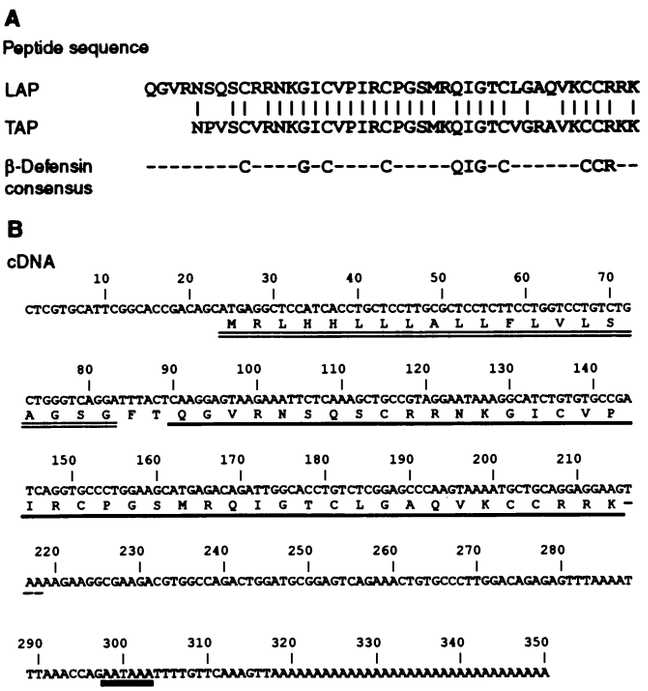
Fig. 1. Purification of lingual antimicrobial peptide (LAP). (**A**) Strong cation exchange chromatography of bovine tongue epithelial extract. (**B**) Antimicrobial assay of fractions against *Escherichia coli* D31. (**C**) Antimicrobial assay of fractions against *Candida tropicalis*. Antimicrobial activity against *E. coli* D31 was detected in fraction 70 (B), corresponding to a peak [in absorbance at 220 nm (A_{220})] eluting at 40 min (A). Antimicrobial activity against *C. tropicalis* was detected in fractions 57, 65, 70, and 96 (C), corresponding to peaks with retention times of 33.5, 37.5, 40, and 53 min (A), respectively. PGLa (5 μg) was used as a control for *E. coli* D31 activity (B), whereas amphotericin B (5 μg) is active against *C. tropicalis* (C).

lem by determining whether the epithelium of the tongue produces antibiotic agents capable of providing a broad spectrum chemical shield. Antibacterial and antifungal peptides were extracted from the dissected lingual epithelium of bovine tongue (Fig. 1) by a standard procedure for isolating antimicrobial peptides, which includes organic extraction as well as reversed-phase and strong cation exchange high-performance liquid chromatography (HPLC) (9). The most abundant antimicrobial peptide isolated (*10*), a member of the β-defensin class (Fig. 2A), exhibited both antifungal and antibacterial activity (Table 1) (*11*). This peptide, termed lingual antimicrobial peptide (LAP), is related in sequence to tracheal antimicrobial peptide (TAP) (*5*), which is expressed in the ciliated epithelium of the upper airway of the cow, as well as to bovine neutrophil β-defensins (BNBDs) (*12*).

A complementary DNA (cDNA) library was generated from bovine tongue epithelial polyadenylated [poly(A)$^+$] RNA and a cDNA for LAP was isolated (Fig. 2B) (*13*). The cDNA encodes a 64–amino acid precursor that is structurally similar to the prepro-peptide for the β-defensin TAP (*5*).

The site of expression of the LAP gene in normal tongue was examined by in situ hybridization with LAP sense and antisense RNA probes (Fig. 3) (*14*). The antisense probe revealed intense hybridization in the middle layers of the epithelium (Fig. 3B); the sense probe yielded no hybridization signal (Fig. 3A). LAP mRNA was detected on the dorsal surface of both the front and back of the tongue. In contrast, LAP transcripts were not detected in taste buds, the lamina propria underlying the epithelium, or neutrophils within the tissue specimen. Thus, the upper surface of the tongue is covered by an antibiotic-expressing epithelium.

We next assessed whether injury or infection of the tongue surface affects local expression of LAP. We studied lesions on the tongues of three otherwise healthy cows and two representative lesions are shown (Fig. 3, C to F). The lesions were several millimeters in diameter and probably caused by trauma during grazing and subsequent infection. Each lesion exhibited destruction of the normal epithelium as well as areas in the lamina propria that showed both acute and chronic



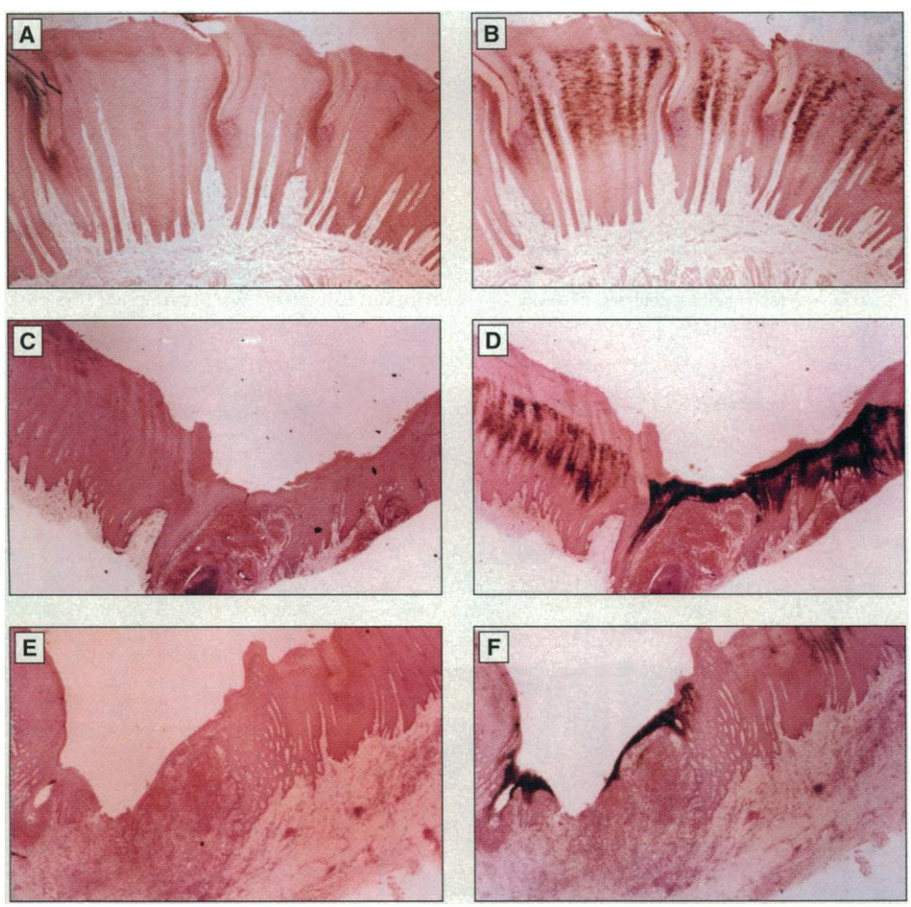


Fig. 2. Peptide and cDNA sequences of LAP. (**A**) Peptide sequences of LAP, tracheal antimicrobial peptide (TAP), and the consensus of bovine β-defensins. (**B**) Complementary DNA and amino acid (*27*) sequences of LAP. Double underline, putative signal sequence; solid underline, mature peptide; dashes, termination codon; and bold underline, polyadenylation signal.

Fig. 3. Induction of LAP mRNA in areas surrounding sites of inflammation or infection. (**A** and **B**) Normal distribution of LAP mRNA as revealed by hybridization with sense (A) and antisense (B) RNA probes. Hybridization with the antisense transcript revealed that LAP mRNA is localized in the middle layers of the epithelium. (**C** to **F**) In situ hybridization of two naturally occurring bovine tongue lesions with sense (C and E) and antisense (D and F) RNA probes. Increased LAP mRNA abundance is apparent in areas surrounding sites of acute and chronic inflammation or infection. In (D), a region of normal LAP mRNA abundance (left) is adjacent to an area showing a markedly increased abundance surrounding a site of infection or inflammation (right). In (F), an increase in LAP mRNA abundance is apparent in areas surrounding an abscess (left). Magnification: (A and B), ×40; (C to F), ×20.

inflammation. Acute inflammation was characterized by the presence of hemorrhage, erythrocyte accumulation, and infiltration of polymorphonuclear leukocytes; areas of chronic inflammation were characterized by infiltration of mononuclear cells and the presence of granulation tissue.

Hybridization of these bovine tongue tissue specimens with the LAP antisense RNA probe revealed a marked increase in LAP mRNA in the epithelia surrounding areas of both acute and chronic inflammation (Fig. 3, D and F). The increase extended ~3 to 4 mm on either side of the central lesion. Normal tissue in these sections showed no apparent increase in LAP mRNA compared to uninvolved tissue from noncontiguous sites, nor was LAP mRNA present within the lamina propria of injured tissue or within white cells (Fig. 3, D and F). Bovine α-tubulin mRNA was distributed uniformly throughout the same tissue specimens, demonstrating the specificity of the LAP mRNA response.

Table 1. Minimal inhibitory concentrations (MICs) of lingual antimicrobial peptide (LAP) and magainin II against bacteria and fungi.

Microorganism	MIC (μg/ml)	
	LAP	Magainin II
Escherichia coli D31	16–32	13–25
Pseudomonas aeruginosa (27853)*	63–125	13–25
Staphylococcus aureus (29213)	63–125	50–100
Candida albicans (14053)	32–63	50–100
Candida tropicalis (13803)	16–32	13–25

*Numbers in parentheses refer to American Type Culture Collection strains.

LAP mRNA or closely related transcripts were detected in many of the exposed epithelial surfaces of the cow (Fig. 4), including sites in the conjuctivae, bronchi, colon, and urinary tract (*15*). The presence of LAP mRNA in so many epithelial surfaces suggests that antibiotic peptides may contribute to the defensive machinery of many mammalian epithelial surfaces that are exposed to microbes.

LAP mRNA was not detected in third-trimester fetal tongue but is abundant in tongues from cows and 4-month-old calves (Fig. 4A). Induction may occur as a result of exposure of the animal to microbial or viral agents, or, alternatively, it may be under developmental regulation. In either instance, LAP appears to be expressed at a constitutive level in bovine tongue after birth and is induced further in response to injury and infection.

Our observations in a mammal parallel the experimental data from insects demonstrating induction of cecropin mRNA in the epithelial cell layer of silkworm larvae after epicuticular and cuticular wounding (*16*). Induction occurs when abraded larvae are challenged with living bacteria or bacterial cell wall components. In primary cultured bovine tracheal epithelial cells, TAP mRNA abundance was increased at least fivefold by addition of lipopolysaccharide (LPS) to the culture medium (*17*). The genes for TAP and several insect antimicrobial peptides contain a binding site for nuclear factor κB (NF-κB) in the 5′ region; this site is implicated in LPS responsiveness of the antimicrobial peptide and other genes important in inflammatory reactions (*18–20*). TAP mRNA is also increased in cultured tracheal epithelial cells by cytokines such as tumor necrosis factor (TNF) (*21*). TNF expression by macrophages is stimulated by exposure to LPS (*22*). Thus, increased expression of LAP in the tongue epithelium at sites of injury or infection may result from direct stimulation by bacteria or from the production of cytokines at the injury site. Because defensins attract monocytes (*23*), the possible interplay between LAP expression and inflammation has the potential to generate a robust response to microbial and viral invasion.

In the context of mammalian mucosal defenses, which include immunoglobulin antibody production and secretion, T and B cell–mediated responses, and cytokine synthesis, the production of broad-spectrum antibiotics might contribute to the freedom from continuous infection that characterizes many epithelial surfaces of vertebrates. Inducible antibiotics such as LAP may play a role in the sterilization of injured tissue. In addition, they may participate in wound repair, given that defensins exhibit growth factor activity in vitro and in vivo (*23*). The association between epithelial injury or infection, inflammation, and defensin expression may have medical significance. For example, defensins inactivate many enveloped viruses that can infect or penetrate mucosal surfaces, including herpes simplex virus and human immunodeficiency virus (*24–26*). Elucidation of the regulatory mechanisms responsible for stimulation of the expression of epithelial defensins may have therapeutic applications in enhancing mucosal immunity.

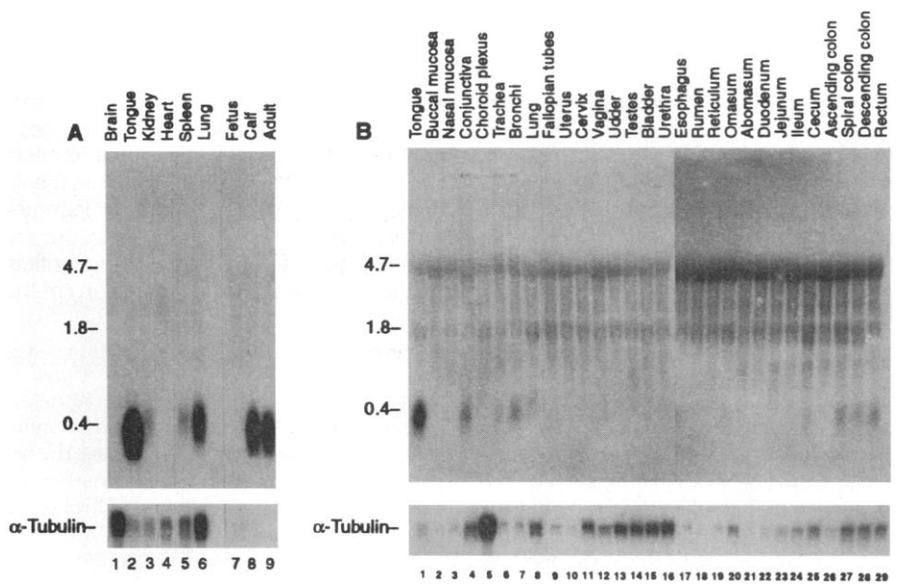


Fig. 4. Tissue and developmental distribution of LAP mRNA. (**A**) General tissue distribution of LAP mRNA among bovine tongue and other organs (lanes 1 to 6), and developmental stages of LAP mRNA in bovine tongue (lanes 7 to 9). (**B**) Tissue distribution of LAP mRNA organized by system: tongue and epithelia from the face (lanes 1 to 4), choroid plexus (lane 5), respiratory tract (lanes 6 to 8), male and female reproductive tracts (lanes 9 to 14), genitourinary tract (lanes 15 and 16), and gastrointestinal tract (lanes 17 to 29). Hybridization to a bovine α-tubulin probe is shown as a control for the amount of RNA present in each lane.

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nitrile and 1% trifluoroacetic acid (TFA), the extract was centrifuged, and the supernatant was lyophilized. The material was resuspended in 0.1% TFA and extracted with chloroform:methanol (2:1). The aqueous phase was lyophilized, resuspended in 0.1% TFA, and fractionated by P-30 gel filtration (Bio-Rad, Richmond, CA). Fractions were assayed for antimicrobial activity by a radial diffusion plate assay against *Escherichia coli* D31 and *Candida tropicalis* [M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5449 (1987)]. The active antimicrobial fractions were purified by reversed-phase (C_{18}) HPLC (Poly LC, Columbia, MD) followed by strong cation exchange HPLC (Poly LC). Salt was removed from each fraction with a C_{18} Sep-pak cartridge (Waters, Milford, MA), and the fractions were then dried overnight and assayed for antimicrobial activity against *E. coli* D31 and *C. tropicalis*.

10. The mass of the purified peptide was 4627.5 daltons by electrospray mass spectrometry, consistent with the size and amino acid composition of a β-defensin. Amino acid analysis was also consistent with a β-defensin. The sequence of ~25 amino acids from the COOH-terminus was determined after digestion of the purified peptide with trypsin, reduction and alkylation of the cysteine residues, and chromatographic separation of the peptide fragments. Microsequencing of individual fragments was performed by standard Edman degradation. The peptide sequence NKGICVPIRCPG-SMRQIGTCLGAQVK (*27*) was confirmed and completed by cloning of the full-length complementary DNA from a bovine tongue epithelial library.
11. Minimal inhibitory concentrations (MICs) were assessed in 96-well microtiter plates. Microorganisms were grown at log phase in 0.25× trypticase soy broth at a density of 1×10^5 cells per milliliter. For each organism, dilutions of peptide ranging from >500 to 1 μg/ml were tested. MICs were calculated on the basis of the lowest concentration of peptide that inhibited overnight growth.
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13. A polymerase chain reaction (PCR)–based strategy was designed to amplify a probe from tongue poly(A)$^+$ RNA. Assuming conservation of the signal sequence in defensin families (*6*), we used a PCR primer (5′-ATGAGGCTCCATCACCTG-3′) corresponding to the first six amino acids of the signal sequence of TAP (MRLMML) for the sense strand. Assuming nucleotide identity corresponding to amino acids in the COOH-termini of LAP and TAP (VKCC), we designed a degenerate (1:8) antisense primer [5′-ACAGCATTT-TAC(TC)TG(ACGT)GC-3′] with fully degenerate divergent amino acids (AQ). PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 58°C for 2 min, and 72°C for 3 min, followed by 72°C for 15 min. The products were purified on gels and subcloned into Bluescript vector that had been modified to accept PCR products [D. Marchuk, M. Drumm, A. Saulino, F. S. Collins, *Nucleic Acids Res.* **19**, 1154 (1991)]. The PCR product was sequenced and used as a probe to identify clones from a bovine tongue λZap II cDNA library (Stratagene, La Jolla, CA) constructed from 2 μg of bovine anterior tongue poly(A)$^+$ RNA. Inserts were selected by size from 0.1 to 3 kb. Phages (0.5×10^6) were plated over 10 plates; there were ~100 positive plaque-forming units per plate. Nylon membranes (Genescreen II, DuPont Biotechnology Systems, Boston, MA) were used for plaque lifts. Six phages were isolated after successive rounds of plaque purification. The inserts were ligated into pBluescript vectors by in vivo excision and sequenced by dideoxy sequencing from T3 and T7 primers. The final cDNA sequence was derived from sequencing of multiple clones and their complementary strands.
14. W. S. Young III, E. Mezey, R. E. Siegel, *Neurosci. Lett.* **70**, 198 (1986). Three tongues with visible, naturally occurring sores were selected from recently killed cows. The lesions and surrounding epithelia were fixed with 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, and mounted on sialanated slides, which were then subjected to standard in situ hybridization methods for RNA probes. Probes were prepared from the full-length LAP cDNA, which was subcloned into Bluescript and linearized with Sma I and Kpn I for sense and antisense transcripts, respectively. Hybridization was performed overnight at 42°C with 2×10^6 cpm per slide, after which slides were washed at 65°C with 1× standard saline citrate (SSC) containing 0.1% β-mercaptoethanol. Slides were exposed to Kodak NTB-2 emulsion for ~30 days, counterstained with hematoxylin and eosin, and photographed. Additional slides of the lesions were also hybridized with full-length RNA probes for α-tubulin (sense and antisense).
15. Adult bovine epithelial tissue specimens were obtained from five cows and frozen in liquid nitrogen. Fetal tongue RNA was pooled from five fully formed, third-trimester fetuses (Moyer Packing Co.). Calf tongue RNA was prepared from 4-month-old calves (Marcho Farms, Souderton, PA). Frozen epithelial tissue was extracted with guanidinium isothiocyanate [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutler, *Biochemistry* **13**, 5294 (1979)]. Cows differ from humans in having a four-part stomach that comprises the rumen, reticulum, omasum, and abomasum (proximally to distally). The transverse segment of bovine colon is identified as the spiral colon. For the poly(A)$^+$ RNA blot, oligo(dT) columns were used to isolate mRNA or bovine poly(A)$^+$ RNA was obtained from Clontech (Palo Alto, CA). Poly(A)$^+$ RNA (4 μg) from each tissue was subjected to electrophoresis on a 6.7% formaldehyde gel (Fig. 4A, lanes 1 to 6). Approximately 15 μg of total RNA was used per lane for tissue distribution (Fig. 4B) and developmental (Fig. 4A, lanes 7 to 9) studies. The RNA was transferred under neutral conditions to Zetabind membranes (Bio-Rad). The LAP 48-nucleotide oligomer was 5′-CCTCCTGCAGCA-TTTTACTTGGGCTCCGAGACAGGTGCCAATCT-GTCT-3′. Hybridization was performed overnight at 42°C in 6× SSC, 5× Denhardt's solution, 20% formamide, yeast RNA (200 μg/ml), and 0.5% SDS. The probe was end-labeled with ^{32}P by polynucleotide kinase to a specific activity of 1×10^8 cpm/μg. A full-length bovine α-tubulin cDNA was randomly primed to a specific activity of 0.5×10^9 cpm/μg. The blots were washed at 65°C with 1× SSC containing 0.1% SDS for LAP probe hybridizations, and at 65°C with 0.1× SSC containing 0.1% SDS for α-tubulin probe hybridizations.
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27. Abbreviations for the amino acid residues are as follows: 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.

28. We thank W. Lane (Harvard University Microchemistry Facility, Cambridge, MA) for performing peptide characterization and microsequencing; C. Bevins (Children's Hospital of Philadelphia, Philadelphia, PA) for bovine α-tubulin cDNA; and C. A. Janeway Jr., T. Uzzell, A. Shinnar, T. Williams, P. Molinoff, and L. Jacob for critical reviews of the manuscript.

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Modulation of Serotonin-Controlled Behaviors by G_o in *Caenorhabditis elegans*

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Seven transmembrane receptors and their associated heterotrimeric guanine nucleotide–binding proteins (G proteins) have been proposed to play a key role in modulating the activities of neurons and muscles. The physiological function of the *Caenorhabditis elegans* G protein G_o has been genetically characterized. Mutations in the *goa-1* gene, which encodes an α subunit of G_o ($G\alpha_o$), cause behavioral defects similar to those observed in mutants that lack the neurotransmitter serotonin (5-HT), and *goa-1* mutants are partially resistant to exogenous 5-HT. Mutant animals that lack $G\alpha_o$ and transgenic animals that overexpress $G\alpha_o$ [*goa-1(xs)* animals] have reciprocal defects in locomotion, feeding, and egg laying behaviors. In normal animals, all of these behaviors are regulated by 5-HT. These results demonstrate that the level of G_o activity is a critical determinant of several *C. elegans* behaviors and suggest that G_o mediates many of the behavioral effects of 5-HT.

Changes in environmental conditions or physiological status often produce global changes in the behavior of animals. Two sets of experimental results suggest that seven transmembrane receptors (7-TMRs) and G proteins play a pivotal role in the modulation of behavior. First, synaptic signals produced by 7-TMRs are well suited to the task of promoting long-term changes in behavior because G proteins typically regulate the activities of neurons and muscles by means of a cascade of second messengers (*1*). Second, neurotransmitters that act on 7-TMRs (that is, metabotropic agonists) are potent modulators of many behaviors (*2*). However, the specific receptors and G proteins that mediate the response to a particular agonist are often not known.

We have genetically analyzed signaling by the metabotropic agonist 5-HT in *Caenorhabditis elegans*. It has been proposed that

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