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Primary structure of cAMP-gated channel from bovine olfactory epithelium

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The complete amino-acid sequence of the bovine olfactory epithelium adenosine 3',5'-cyclic monophosphate (cAMP)-gated channel has been determined by cloning and sequencing its cDNA. It exhibits a high degree of sequence homology with the cGMP-gated channel of rod photoreceptors, suggesting that cyclic nucleotide-gated channels fall into a new family of genetically related proteins.

cAMP-gated channel; cDNA cloning; Olfactory epithelium; Signal transduction

1. INTRODUCTION

Vertebrate rod and cone photoreceptors [1,2] and olfactory cilia [3] contain cation-selective ion channels which are directly and cooperatively opened by cyclic nucleotides. In photoreceptors of the vertebrate retina cGMP controls channel opening and thereby serves as the intracellular excitatory messenger, whereas olfactory sensory neurons employ cAMP as messenger in odorant-stimulated signalling (for review see [4,5]). The rod photoreceptor channel is composed of a single polypeptide [6] which in its functional form exists as a homooligomer [7]. By cloning and sequencing of complementary DNA we have previously determined the primary structure of the cGMP-gated channel from bovine rod photoreceptors [7]. It shows no extensive amino-acid sequence similarity to other ionic channels but to cyclic nucleotide-binding proteins, in particular cGMP-dependent protein kinase.

We have now cloned DNA complementary to messenger RNA from the bovine olfactory epithelium encoding a polypeptide which shows a high degree of sequence similarity to the cGMP-gated channel of rod photoreceptor cells, suggesting that this protein represents the cAMP-gated channel of olfactory sensory neurons.

2. MATERIALS AND METHODS

Poly(A$^+$)RNA from bovine olfactory tissue was isolated by the guanidine thiocyanate method [10] on a CsTFA gradient followed by oligo(dT)-cellulose column chromatography [11]. Oligo(dT) and randomly primed libraries were constructed [8] from poly(A$^+$)RNA in a λ ZAP II vector [9] (Stratagene). Libraries were screened with DNA fragments which were either labeled radioactively by random priming [12] or with Digoxigenin according to the supplier's protocol (Boehringer).

First an oligo(dT) primed library was screened with a probe of the rod cGMP-gated channel which entails the putative cyclic nucleotide-binding site (*Acc*I(1491)/*Acc*I(1896) fragment from clone pRCG1 [7]; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage). Thus clone λ CHOLF1 (~ 2.2 kb) was isolated. It contains a region (~ 1 kb) with extensive similarity to the nucleotide sequence of the rod cGMP-gated channel but it is deficient in two respects: It lacks a poly(A$^+$) tract and it contains intron sequences within the coding region towards the 5' end of the clone. However, fragments from the homologous region of λ CHOLF1 were used to rescreen an oligo(dT) primed library to yield clone λ CHOLF2 and a randomly primed library to yield clone λ CHOLF3. Channel-specific cDNA of λ CHOLF2 and λ CHOLF3 was recovered in a pBluescript vector by in vivo excision from λ ZAP II [17] to yield pCHOLF2 and pCHOLF3. A recombinant carrying the complete protein-coding sequence of the olfactory cAMP-gated channel was constructed as follows. Clone pCHOLF2 was linearized by *Not*I and clone pCHOLF3 by *Xho*I. *Not*I and *Xho*I sites were filled-in by Klenow fragment followed by cleavage with *Bcl*I. The ~4850 bp fragment *Bcl*I(849)/*Not*I(vector) of pCHOLF2 and the ~1300 bp fragment *Xho*I(vector)/*Bcl*I(849) from pCHOLF3 were ligated to yield pCHOLF100.

Restriction fragments from pCHOLF2 and pCHOLF3 were subcloned into M13mp18 and/or M13mp19 and DNA sequencing was carried out on both strands by the dideoxy chain termination method [13] using [α-^{35}S]ATP and modified T7 DNA-polymerase [14] (sequenase version 2.0, USB). Some DNA sequencing was carried out on double-stranded DNA [15]. In both cases either M13-40 universal primer or appropriate oligonucleotide primers were used. The nucleotide sequence was confirmed by sequencing of independent clones. These clones differ in two positions at the 5' end of the coding region, falling into two classes. While five out of ten clones contain in position +128 a cytosine base and in position + 155 an adenine base, the other five clones contain an adenine and a guanine base, respectively (see Fig. 1). Both exchanges result in an amino-acid replacement (position 43 A → D, position 52 Q → R). Since the mRNA was isolated from the tissue of a few animals, we believe that

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these differences in sequence reflect certain individual genetic variations (alleles).

RNA blot hybridization analysis was performed with 4 μg of poly(A$^+$)RNA separated on a denaturing 1.2% agarose/formaldehyde gel and transferred to a nylon filter by vacuum blotting. Immobilized samples were hybridized with a Digoxigenin-labeled RNA probe and identified by an enzyme-linked immunoassay using antibodies directed against Digoxigenin [16]. Filters were washed at 0.1 × SSC, 0.1% SDS at 68°C. Digoxigenin-labeled RNA probes were synthesized from channel-specific cDNA by in vitro transcription with T7 polymerase according to the supplier's instruction (Boehringer).

3. RESULTS AND DISCUSSION

The approach to isolate cDNA coding for the olfactory cAMP-gated channel was to screen a cDNA library of bovine olfactory epithelium by hybridization with a DNA probe derived from the previously cloned cGMP-gated channel of vertebrate rod photoreceptors [7]. Thus a clone λ CHOLF1 was isolated; fragments of this clone served as hybridization probes for the isolation of additional overlapping cDNA clones (λ CHOLF2 and λ CHOLF3, for details see Materials and Methods). Together they comprise the complete coding sequence and 5′ and 3′ non-coding sequences. Fig. 1A shows the complete 3,166-nucleotide sequence (excluding the poly(dA) tract) of the cDNA with an open reading frame that encodes a protein of 663 amino acids (Fig. 1A, B). The translational initiation site was assigned to the first ATG triplet which appears downstream of nonsense codons found in frame. At position −3, an adenine base is found, which is the most conserved residue of the eukaryotic ribosomal binding site [18]. The polyadenylation signal AATAAA (residues 2,729–2,734) is found 16 nucleotides upstream of the poly(dA) tract. The size of the specific message in the poly(A$^+$) RNA was estimated to be ~3,200 nucleotides by blot hybridization analysis of bovine olfactory poly(A$^+$) RNA with a RNA probe from cCHOLF100 (nucleotide positions −417 to 2,749 plus poly(A) tract; Fig. 2, lane 1, main band). There are two more distinct bands in this lane (~5,800 bp and ~7500 bp) which may represent unspliced precursor RNA species. The size of the main band is similar to that of the channel-specific in vitro transcript (Fig. 2, lane 2). A channel-specific signal was observed in poly(A$^+$) RNA from olfactory epithelium but not in control tissue (brain and liver, data not shown). The calculated M_r of 76,013 Da (including the initiating methionine) of the protein is similar to that of the rod cGMP-gated channel [7]. The amino-acid sequence of the encoded polypeptide is highly homologous to that of the photoreceptor cGMP-gated channel, especially in regions comprising 6 putative transmembrane segments H1–H6 and the cAMP/cGMP-binding domain, but the sequence similarity decreases at the C- and N-terminal ends (Fig. 1B). The strong similarity suggests that the protein represents the cAMP-gated channel of olfactory sensory cells.

The deduced amino-acid sequence has a hydropathicity profile similar to that of the photoreceptor channel (Fig. 3). There are 6 hydrophobic segments with a predicted secondary structure (α-helix/β-sheet; referred to as H1–H6) each comprising approximately 20 amino-acid residues. As in the photoreceptor channel, segments H4 and H5 show the highest hydrophobic index and, most likely represent transmembrane α-helices. Some or all of the 4 remaining segments may also span or interact with the membrane. A seventh hydrophobic region near the C-terminus contains the putative cyclic nucleotide-binding site and therefore is not considered a transmembrane segment. The olfactory channel has two potential N-linked glycosylation sites at positions 311 and 379. While the asparagine residue at position 311 is intracellular, position 379 would be extracellular according to a model which assumes 6 transmembrane segments and N- as well as C-terminus positioned inside [7]. A region similar to the extremely hydrophilic domain (~60-amino-acid residues) between the N-terminus and the first hydrophobic segment H1 observed in the photoreceptor channel also exists in the olfactory channel although its hydrophilic nature is much less pronounced.

A region has been identified [7] in the photoreceptor channel (residues 498–577) as a putative cGMP-binding domain on the basis of sequence comparison with other cyclic nucleotide-binding sites, in particular from cGMP-dependent protein kinase (cGK). The respective region (residues 475–554) in the olfactory channel is highly conserved: 68 out of 80 amino-acid residues are

Fig. 1. (A) Nucleotide sequence of cloned cDNA encoding the cAMP-gated channel from bovine olfactory epithelial cells. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet encoding the initiating methionine. Preceding residues in the 5′ non-coding region are indicated by negative numbers. Numbers indicate the position of the residue on the left side of each line. Nucleotide 2749 is followed by a poly(dA) tract connected with the vector DNA sequence. The initiating ATG triplet and the stop triplet are underlined. (B) Alignment [26] of the deduced amino-acid sequences (in one-letter code) of the bovine olfactory (olf) and the bovine rod photoreceptor channel (rod). For sequence data of the rod photoreceptor cGMP-gated channel and numbering of nucleotide and amino-acid residues, see [7]. Numbers of the amino-acid residues at the right-hand end of each line are given. Identical residues are indicated by colons and conservative substitutions by single points. Conservative substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, G; N, D, E, Q; H, R, K; M, I, L, V; F, Y, W [25]. Potential N-glycosylation sites are indicated by arrow heads and threonine residues 537 (olf) and 560 (rod) by an asterisk. The hydrophobic segments (H1–H6) are overlined; the termini of each segment are tentatively assigned as in the amino-acid sequence of the rod photoreceptor channel [7]. The putative cAMP/cGMP-binding region is indicated by a grey bar. The positions in which sequences of individual clones differ from each other are indicated by a dot above the respective nucleotide and amino-acid residue, see Materials and Methods.

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-417 5'-GCTCACCTACTGGCAGGCTGGGGTGTGCAGGCCCCACAGTGAGAGGGTCAGACATCC
-360 AGCCAGTGGGCAGAACTGTTTCTCCAGTGGGCAAGTGCTGCCCTGGGCCGAGGCCTCGAC
-300 CTCGCCTCAGCTGTAGGCATGGGCTCCTCTGAGTCACTGCCGGCCTCCCCTGCTGGCCTC
-240 AGGCAGGGCGGGCAGCTCTTTGACTGAGAGGCTGAGAAGCTGCGTGGGGATTCGGGGATC
-180 CCCCTGGTCTGGGGACAGGAGCTTTGGGAGGTCTCTACTGTCCCTTGGTGCTGATGAGCT
-120 CCCGAGGGTTCGTCTCTGACTGGAAGCTTCTTGGACAGACCTATAGCCTGTGGCCAAGGG
 - 60 ACACCCTGCCTCGGAATCCGTCTGGTGAGGAAAGGTGAGGGTCCTGGTTGTACATGGAGG
    1 ATGACAGAAAAAGCCAATGGCGTGAAGAGCTCCCCAGCCAATAACCACAACCACCATGCC
   61 CCTCCTGCCATCAAGGCCAGTGGCAAAGATGACCACAGGGCCAGCAGCCGGCCACAGTCT
  121 GCTGCTGCTGATGACACCTCCTCAGAGCTACAGCAACTGGCAGAGATGGATGCCCCCCAG
  181 CAGAGGAGGGGTGGCTTCCGCAGGATTGCCCGCCTGGTGGGGGTCCTCAGAGAGTGGGCT
  241 TACAGGAACTTCCGTGAGGAGGAGCCTAGACCTGACTCATTCCTTGAGCGTTTCCGGGGG
  301 CCTGAGCTCCACACCGTGACAACACAACAAGGAGACGGCAAAGGCGACAAGGACGGCGAG
  361 GGCAAGGGCACCAAGAAGAAGTTTGAACTCTTTGTCTTGGACCCAGCCGGGGACTGGTAC
  421 TACCGCTGGCTTTTTCTCATTGCCTTGCCCGTCCTCTACAACTGGTGCCTATTGGTGGCC
  481 AGAGCCTGCTTCAGTGACCTGCAGAAAGGCTACTACATAGTGTGGCTGGTGCTGGATTAC
  541 GTCTCAGATGTGGTCTACATCGCAGACCTCTTCATCCGACTGCGCACAGGTTTCTTGGAG
  601 CAGGGGCTACTGGTGAAAGACACCAAGAAGTTGCGGGACAACTACATCCACACCATGCAG
  661 TTTAAGCTGGATGTGGCCTCCATCATCCCTACAGACCTGATCTATTTTGCTGTGGGGATC
  721 CATAACCCTGAGGTGCGCTTCAACCGCCTGCTACACTTTGCCCGCATGTTTGAGTTCTTT
  781 GACCGCACTGAGACACGCACCAGCTACCCCAACATCTTCCGAATAAGCAACCTGATCCTC
  841 TACATCTTGATCATCATTCACTGGAATGCCTGCATCTACTATGCCATCTCCAAGTCCATC
  901 GGCTTTGGGGTAGACACCTGGGTTTACCCCAACATCACTGACCCTGAGTATGGCTACCTG
  961 TCTAGGGAGTACATCTATTGCCTTTACTGGTCTACACTGACCCTCACCACCATTGGGGAG
 1021 ACACCACCCCCTGTAAAGGATGAGGAGTACCTGTTTGTCATCTTTGACTTCCTGATTGGT
 1081 GTCCTCATCTTTGCCACCATCGTGGGAAATGTGGGCTCCATGATCTCCAACATGAATGCC
 1141 ACCCGGGCTGAGTTCCAGGCCAAGATTGATGCTGTCAAACATTATATGCAGTTCCGAAAG
 1201 GTCAGCAAGGAGATGGAAGCCAAGGTCATTAGGTGGTTTGACTACTTGTGGACCAATAAG
 1261 AAGAGTGTAGATGAGCGAGAAGTCCTCAAAAACCTGCCAGCAAAGCTCAGGGCTGAGATA
 1321 GCCATCAACGTCCACCTGTCCACACTCAAGAAAGTGCGCATCTTTCAGGACTGTGAGGCT
 1381 GGCCTGCTGGTGGAACTGGTATTAAAGCTCCGGCCTCAGGTCTTTAGCCCTGGGGACTAC
 1441 ATTTGCCGCAAGGGGGATATTGGGAAGGAGATGTACATAATCAAGGAGGGAAAATTGGCA
 1501 GTGGTGGCTGATGACGGTGTCACTCAGTATGCCCTGCTCTCGGCTGGGAGTTGCTTTGGA
 1561 GAGATCAGTATCCTTAATATTAAGGGCAGCAAAATGGGCAATCGGCGCACAGCCAACATC
 1621 CGCAGTCTTGGCTACTCTGATCTGTTCTGCTTGTCCAAGGATGATCTTATGGAAGCTGTG
 1681 ACTGAGTACCCTGATGCCAAGAGGGTCTTGGAGGAGAGAGGCCGGGAGATTCTGATGAAG
 1741 GAGGGCTTGTTGGATGAGAATGAGGTGGCAGCCAGCATGGAGGTAGATGTGCAGGAAAAG
 1801 CTAGAACAGCTGGAGACCAACATGGACACCTTGTACACTCGTTTTGCCCGCCTGCTGGCC
 1861 GAGTACACGGGAGCCCAGCAGAAGCTCAAGCAGCGCATCACAGTTTTGGAAACGAAGATG
 1921 AAGCAGAATAATGAGGATGACTCCCTGTCAGATGGGATGAACAGCCCAGAGCCACCTGCC
 1981 GAGAAGCCATAATGGCTTGGCCCAATTGCCCCTCCAGCCTTGGCTTTGACCCCAGGGCTG
 2041 GAAGAGCTGTGTAGGTCCCCACATATATATGCATTACCACATCCCCTTGAATTCTCCCAG
 2101 AAGCCTCTCTGCTGGAAGGTTTAGGGCTCGATCATCCAGAAGCCCTCCTCCAAGTCCGAC
 2161 TAACAGCTAATCTTGTGCAGGGCATAGACTGTGCTTAGCTCGGCTTCCAGAAGCTTCAGC
 2221 CTGTCTAAGTTTGAGGAAGAAAGAAAAGAGGAGCATCTCTCCAGGCTCTTTTGCATCTAG
 2281 TTACCTCCTACTTGATTCTTTTCTAATATGTGTTCTGAATATTTCCATTTCCCTGCAGCA
 2341 GTATGTAGTTAGAACACTGGCTGCAGACACCCAGCACTGGTCCCAGTGTCCTTTTCCCAA
 2401 GGCAGGCAAAAGGTGTGGAGGGGGGCAAGGAGGAGATGCTCACTCCAAGTCCTGCCGTGC
 2461 TGATTCCTCCGCCTGTCTGCCAACCCAGAGTGGGAGCCCTGTGGTCTTTTCTGAACCAGG
 2521 GGGAGGAGGATGCTCCTGGTCTCCAATCCATCCCAGGACATGGAGTGAGGAACTAGCAGT
 2581 TGGCCAGCAGGCAAGGCACCTGGAGAAGGTGGTGGGCAGGAGCCTGGCCATCACCCCTCT
 2641 ATGCAGAGTGTTTCTCAGGAGGCCCTGAGGCTGATGGTGGGTGGATGACTCTTCAAGTTA
 2701 ACATCTGCAGTAGAGACACTTACAAGTTAATAAATTCCTCTGAACTTTT-----3'
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Fig. 1, part 1 – see legend on p. 25.

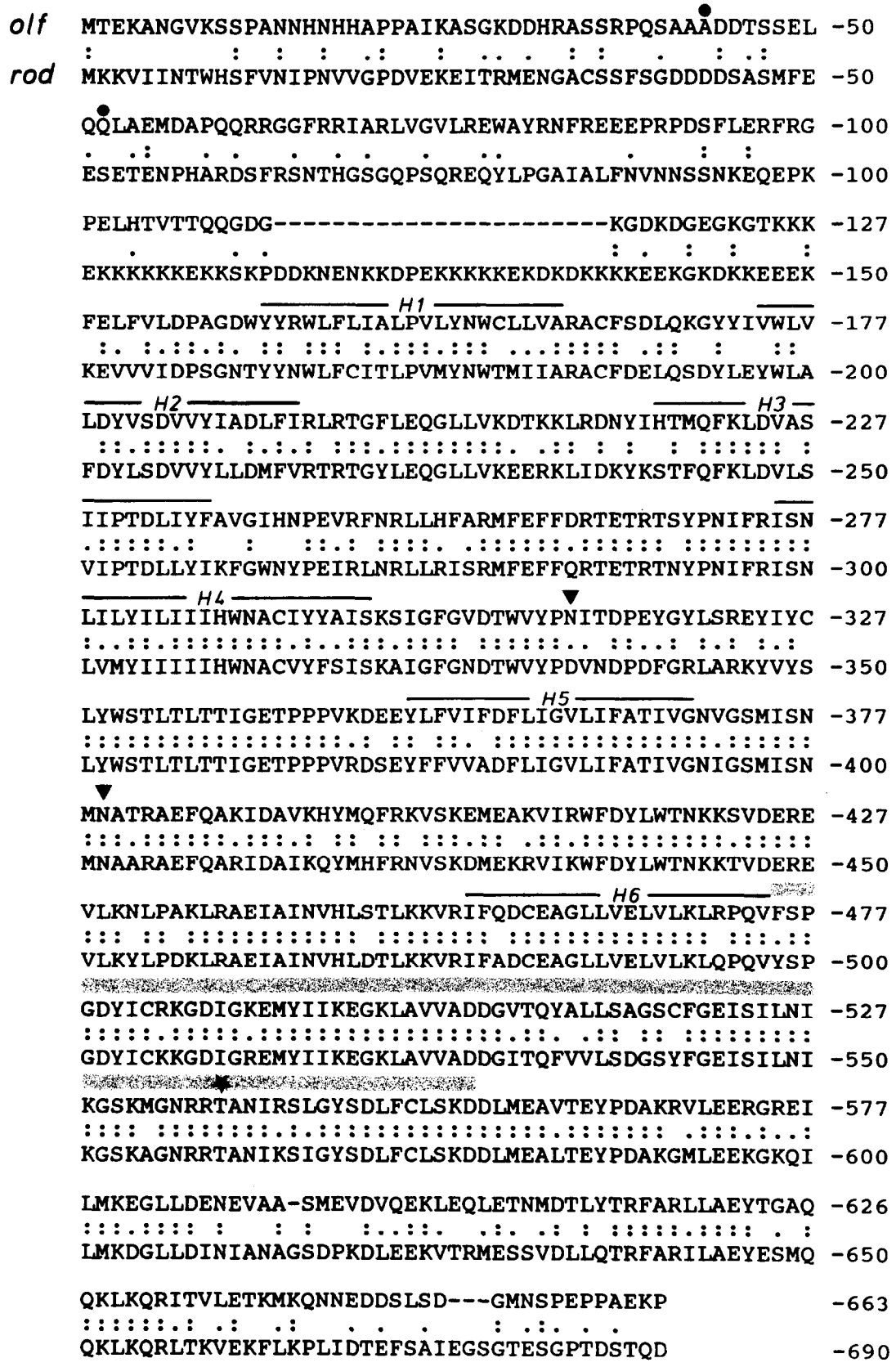

Fig. 1, part 2 – see legend on p. 25.

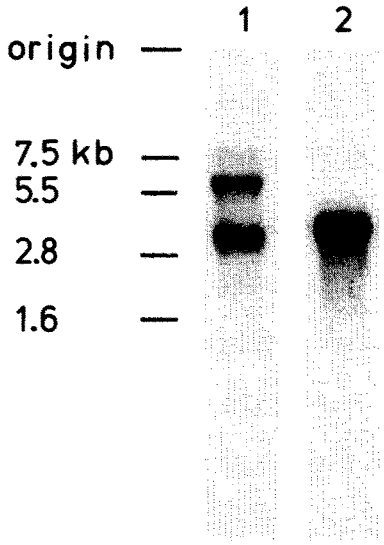

Fig. 2. Northern blot analysis of RNA from olfactory epithelium tissue. RNA samples were electrophoresed on a 1.2% agarose/formaldehyde gel. 4 μg of poly(A^+) RNA from olfactory tissue (lane 1) and 100 pg in vitro of transcript of clone pCHOLF100 (lane 2) were hybridized with a RNA probe that contains the entire protein coding and non-coding sequence, including the poly(dA) tract. An RNA ladder (Boehringer) was used as size marker.

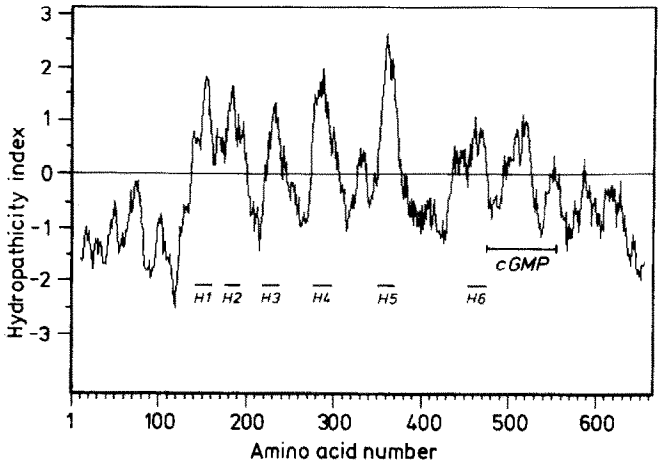

Fig. 3. Hydropathicity profile of the cAMP-gated channel from olfactory epithelium. The averaged hydropathicity index [24] of the nonadecapeptide composed of amino-acid residues $i-9$ to $i+9$ is plotted against i, the amino-acid number. Putative transmembrane segments H1–H6 are indicated by lines, and the putative cAMP/cGMP-binding region by a horizontal bar.

identical to those of the photoreceptor channel and the remaining 8 residues are conserved. It has been suggested [19] that a threonine residue (positions 537 and 560 in the olfactory and photoreceptor channel, respectively) in the cyclic nucleotide-binding site is characteristic for binding of cGMP, while an alanine residue is found at the homologous position in cAMP-dependent protein kinase (cAK). In this respect the region in the olfactory channel should be classified as a cGMP-binding domain. However, the olfactory channel is activated by both cAMP and cGMP at similar concentrations ($EC_{50,cAMP} \sim 1.6$ μM and $EC_{50,cGMP} \sim 2.3$ μM, [3]), whereas the cGMP-gated channel of photoreceptors is much less sensitive to cAMP ($EC_{50,cAMP} \geq 1$ mM and $EC_{50,cGMP}$ from about 10 μM to over 50 μM, [20,21] and references therein). These results question the importance of a threonine or alanine residue at the respective position as a critical determinant of the binding affinity for cAMP or cGMP.

Both the photoreceptor and olfactory cyclic nucleotide-gated channels contain a sequence motif (-R-L-N-R-L-L-R-I-S-R- and R-F-N-R-L-L-H-F-A-R, respectively) between H3 and H4 which is reminiscent of the voltage-sensor motif in voltage-gated channels [22]. Since the photoreceptor and olfactory channels are gated by cGMP or cAMP [1,3] and not by voltage, the functional significance of this voltage-sensor sequence motif is uncertain. The existence of a messenger RNA in the olfactory tissue which is highly homologous to the cGMP-gated channel of rod photoreceptors is consistent with the observation of cAMP-gated channel activity [3,27] and odorant-stimulated adenylyl cyclase activity [5,23]. Based on this analogy it is likely that cAMP stimulates changes in membrane potential by directly opening ionic channels in olfactory membranes and that cyclic nucleotide-gated channels represent a new family of functionally and structurally related proteins which may arise from a common ancestor.

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