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Molecular cloning and sequencing of a cDNA for olfactory marker protein

(neuronal protein/cDNA clone)

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ABSTRACT **cDNA clones corresponding to mRNA for rat olfactory marker protein (OMP) were isolated from a cDNA library. The library was constructed from olfactory mucosa poly(A)$^+$ RNA enriched for OMP mRNA and cloned into a pBR322-derived plasmid, pMG5. OMP cDNA clones were detected by using a 17-base oligonucleotide probe that contained all 16 possible sequences coding for a known partial amino acid sequence of rat OMP. The identity of these clones was confirmed by hybrid-selected translation and nucleotide sequencing. The sequence of one clone was determined and contained the complete OMP coding region of 486 nucleotides followed by 1630 nucleotides of the 3′ untranslated region. The 3′ untranslated region included the polyadenylylation signal 16 nucleotides upstream of the poly(A) tail. No other ATG-initiated open reading frame larger than 20 codons was present in register. RNA blot analysis of olfactory mucosa poly(A)$^+$ RNA using this clone as a probe indicated that the level of OMP mRNA, but not its size, declined significantly within a few days following olfactory bulbectomy. OMP mRNA was not detected in 14 nonolfactory rat tissues. Surprisingly, a small amount of OMP mRNA was observed in olfactory bulb. The presence of OMP mRNA in olfactory bulb was confirmed by *in vitro* translation and immunoprecipitation. These results suggest either that a previously undescribed population of neurons in the olfactory bulb synthesize OMP or that OMP mRNA is transported to the bulb by axonal transport.**

The mechanisms regulating cell-specific gene expression during neuronal development and differentiation are poorly understood. One approach to studying these mechanisms is to identify and characterize neuronal gene products that are developmentally regulated and whose expression is restricted to defined classes of neurons. One example is olfactory marker protein (OMP), a protein uniquely associated with the mature olfactory receptor neurons of many vertebrate species, and one that is developmentally regulated. This small (19 kDa) cytosolic protein has been purified to homogeneity and its physical properties and cellular localization have been thoroughly described (1–4). OMP has been shown to appear in differentiating olfactory neurons 7–8 days after the last [^{3}H]thymidine incorporation (5). It is also temporally correlated with the arrival of receptor neuron axons at their target in the olfactory bulb (6, 7) and with the appearance of the characteristic cellular complement of olfactory cilia (8). Recent studies have demonstrated that, although the presence of the target facilitates or stabilizes OMP expression, it is not absolutely essential (9–11). To investigate mechanisms that regulate the cell-specific developmentally regulated expression of OMP, we have begun to study its expression at the gene level. Previously, we have demonstrated that the synthesis of OMP is programmed by a very large polyadenylylated mRNA and that the primary translation product is indistinguishable from OMP isolated from olfactory tissues (12). In the study reported here, we describe the cloning, identification, and sequencing of the cDNA for OMP. In addition, the cDNA was used to determine the tissue distribution of OMP mRNA and to monitor the response of that mRNA to olfactory nerve lesion. Finally, the anomalous presence of OMP mRNA in the olfactory bulb is described.

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METHODS

Preparation and Construction of a cDNA Library. Poly(A)$^+$ RNA from rat olfactory mucosa was enriched in mRNA sequences coding for OMP and isolated by sucrose gradient fractionation as described (12). Double-stranded cDNA was synthesized by the method of Gubler and Hoffman (13) using poly(A)$^+$ RNA enriched in OMP mRNA sequences. The cDNA was tailed with dGTP and annealed to *Eco*RV-digested poly(dC)-tailed pMG5 plasmid. The construction of this pBR322 variant was prompted by the earlier observation that tailing of *Eco*RV-digested pBR with dCTP creates one new *Bam*HI site at position 184 of the plasmid (13). The salient feature of pMG-5 is its ability to create two new *Bam*HI sites upon tailing with dCTP. A G-tailed cDNA insert will thus be flanked by two *Bam*HI sites and can be excised from the vector by a single *Bam*HI digestion. Recombinant plasmids were used to transform *Escherichia coli* strain DH-1 as described by Hanahan (14). Bacterial colonies containing recombinant plasmids were screened for OMP cDNA by the methods of Hanahan and Meselson (15). A ^{32}P-labeled synthetic heptadecameric oligodeoxynucleotide with a degeneracy of 16 was used as a probe. This oligonucleotide was based on a partial amino acid sequence of OMP and has been described (12). Plasmid DNA was isolated by the method of Currier and Nester (16) except that LiCl precipitation was used to remove high molecular weight RNA, while low molecular weight RNA was removed by gel filtration on Sepharose Cl-2B (N. Itoh, personal communication).

Hybrid-Selected Translation. Hybrid-selected translation was carried out as described by Parnes *et al.* (17). Plasmid DNA was linearized with *Eco*RI endonuclease, denatured with 0.1 M NaOH and bound to nitrocellulose filters. The filters were incubated with 10–20 μg of poly(A)$^+$ RNA for 4 hr at 50°C. Following extensive washing at room temperature in a buffer of 10 mM Tris·HCl/0.15 M NaCl/1 mM EDTA/0.5% $NaDodSO_4$, the bound RNA was eluted at 95°C with distilled water containing 100 μg of carrier tRNA per ml. The eluted mRNA was ethanol-precipitated and used to program a mRNA-dependent cell-free translation system. Finally, immunoprecipitated products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (12).

Nucleotide Sequencing. DNA fragments from restriction endonuclease digestions were 3′-end-labeled using [α-^{32}P]-

Abbreviation: OMP, olfactory marker protein.

dGTP and the large fragment of DNA polymerase I or 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP after removal of the 5′ phosphate groups with calf intestine alkaline phosphatase (Boehringer). Details of the enzymes used and the direction in which various fragments were sequenced are indicated in Fig. 1. Fragments with labeled ends were separated by polyacrylamide or agarose gel electrophoresis and sequenced by the methods of Maxam and Gilbert (18).

DNA Blot Hybridization Analysis. Plasmid DNA (0.5 μg) was digested with restriction endonucleases and electrophoresed on 0.8% neutral agarose gels. The resulting DNA fragments were electrophoretically transferred to nylon filters (ICN) and prehybridized at 37°C in 5× SSC/10× Denhardt's reagent/0.1% NaDodSO$_4$/500 μg of yeast tRNA per ml (1× SSC = 0.15 M NaCl/0.015 M sodium citrate; 1× Denhardt's reagent = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). Subsequently, the filters were hybridized in 10 ml of fresh prehybridization buffer containing 1× 10^6 cpm of ^{32}P-labeled oligodeoxynucleotide probe per ml labeled as described by Maniatis *et al.* (19).

RNA Blot Hybridization Analysis. Poly(A)$^+$ RNA from various tissues was electrophoresed on 1.0% agarose gels containing 2.2 M formaldehyde and electrophoretically transferred from the gel to a nylon filter. The filters were prehybridized in 50% formamide/5× Denhardt's solution/5× SSPE (1× SSPE = 180 mM NaCl/10 mM NaPO$_4$, pH 7.7/1 mM EDTA)/0.3% NaDodSO$_4$/250 μg of nonhomologous DNA per ml for 4 hr at 37°C. Random-primed labeling (20) was used to generate ^{32}P-labeled pOMP3 inserts, which were hybridized to the RNA on the filters.

Samples were prepared for RNA slot blots according to the method of White and Bancroft (21) and spotted onto nylon filters. Filters were prehybridized in a solution containing 50% formamide, 5× SSC, 20 mM Tris·HCl (pH 7.5), 0.05% NaDodSO$_4$, 5× Denhardt's solution, and 0.5 mg of tRNA per ml. Radioactive probe was prepared by random-primed labeling and added to the hybridization solution for a final concentration of 1 × 10^8 cpm/ml. Filters were hybridized at 37°C overnight.

RESULTS

Construction and Characterization of Clones Containing OMP mRNA. Two fractions from the sucrose gradient were obtained that represented a 2- to 3-fold enrichment of OMP mRNA. Prior analysis of the relative abundance of OMP mRNA by *in vitro* translation demonstrated that it represented 0.5% of the total poly(A)$^+$ mRNA (12). Double-stranded cDNA was synthesized from the OMP-enriched poly(A)$^+$ RNA and inserted into the *Eco*RV site of pMG5 as described. Approximately 1.0 × 10^3 ampicillin-resistant colonies of *E. coli* DH-1 were produced per 1.0 ng of double-stranded cDNA. These colonies were replica-plated and processed for *in situ* colony hybridization to the ^{32}P-labeled oligonucleotide probe. Four positive colonies were selected and screened at low density two additional times to ensure that each colony was derived from a single cell. Plasmids were isolated from these colonies, digested with various restriction endonucleases, and analyzed on neutral agarose gels. All four plasmids contained inserts of ≈2300 base pairs as well as internal *Bam*HI, *Ava* I, and *Pst* I cleavage sites. Cleavage with *Bam*HI produced two fragments of 900 and 1400 base pairs, while cleavage with *Ava* I and *Pst* I yielded fragments of 800 and 3000 base pairs, respectively. Cleavage of the plasmids with *Eco*RI produced a single band, demonstrating the absence of additional *Eco*RI cleavage sites. The ^{32}P-labeled oligodeoxynucleotide probe was shown to hybridize to the 900-base-pair *Bam*HI fragment, the 800-base-pair *Pst* I fragment, and the 3000-base-pair *Ava* I fragment.

Hybrid-selected translation was carried out using the four recombinant plasmids. Clones 1–4 selected mRNA that directed the synthesis of a 19-kDa protein corresponding to OMP (Fig. 2, lanes 2–5). The quantity of OMP produced by clone 2 was consistently less than the quantity of OMP produced by the other three clones. Clone three was selected for all subsequent studies and identified as pOMP3.

DNA sequencing studies demonstrated that the cDNA insert consists of 2149 nucleotides, including a 3′ poly(A) tail of 29 nucleotides (Fig. 3). The consensus polyadenylylation signal, AATAAA (22), is present 16 nucleotides upstream of the poly(A) tail. The 5′ poly(C) tail that precedes the OMP coding region is derived from the construction of the recombinant plasmid. The absence of both an initiator ATG codon and a cap site indicates that the insert is not a full-length copy of the mRNA for OMP. Primer extension studies using a synthetic oligonucleotide indicate (data not shown) that an additional 100–150 nucleotides are present in the OMP mRNA 5′ to the beginning of the pOMP3 cDNA insert. However, the 5′ end of the insert contains an open reading frame of 486 nucleotides, predicting an amino acid sequence of 162 residues. This sequence is identical to that determined by protein sequencing for OMP isolated from rat (23). The open reading frame is followed by 1630 nucleotides, which do not contain any in register ATG-initiated open reading frames longer than 20 codons.

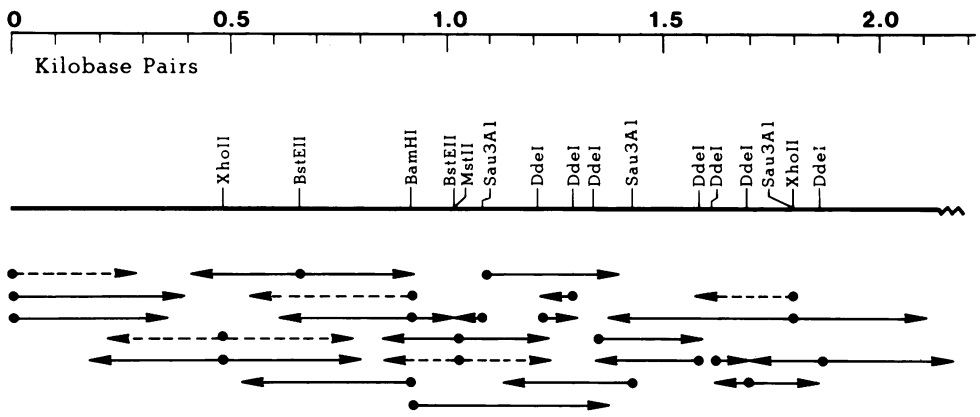

FIG. 1. Restriction map and strategy for sequencing analysis of the OMP insert in pOMP3. Sequencing was by the Maxam–Gilbert procedure (18). Solid lines indicate sequencing following 3′ end filling with [α-^{32}P]dGTP and the large fragment of DNA polymerase I. Broken lines indicate sequence analysis following 5′ end labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeling end of each fragment is indicated by the closed circle and arrows indicate the direction of sequencing. The position and length of the fragments are indicated by the number of bases across the ordinate. The jagged line represents the poly(A) tail.

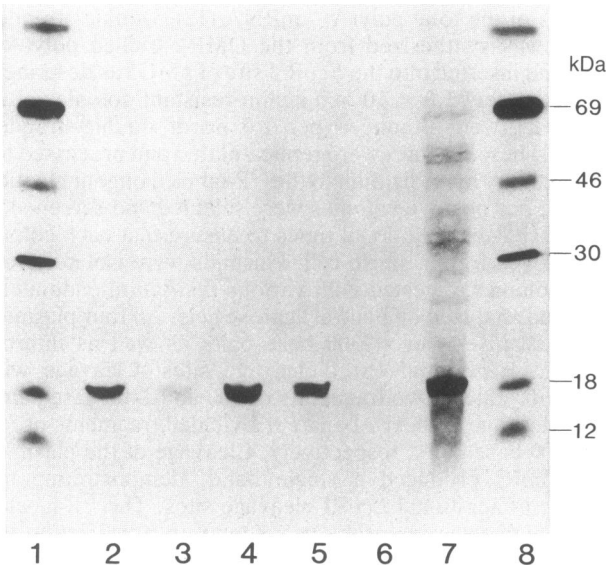

FIG. 2. Identification of rat OMP cDNA clones by hybrid-selected translation and immunoprecipitation. Fluorogram of a 12% $NaDodSO_4$/polyacrylamide gel used to fractionate the immunoprecipitated products from cell-free translation assays. Lanes: 1 and 8, molecular weight markers; 2–5, translated products of mRNA hybrid-selected by clones pOMP1, pOMP2, pOMP3, and pOMP4, respectively; 6, translated products of mRNA hybrid-selected by pMG5 containing no cDNA insert; 7, immunoprecipitation of cell-free translation products from olfactory epithelium RNA.

Size, Tissue Distribution, and Abundance of OMP mRNA. The tissue distribution of OMP RNA was evaluated by RNA slot blot analysis. Random-primed ^{32}P-labeled insert easily detected OMP mRNA in as little as 1.6 μg of total RNA from olfactory mucosa (Fig. 4, lane I, position E) as well as olfactory mucosa from bulbectomized animals (lane II, position E), where the level was significantly reduced. Low levels of OMP mRNA were also seen in 25 μg of total RNA from olfactory bulb (lane II, positions A and B). However, no OMP mRNA was detected in 25 μg of total RNA from 14 additional tissues (lane III, positions C–E), even on prolonged autoradiographic exposure.

To confirm the presence of OMP mRNA in the olfactory bulb, total RNA was isolated from three independent homogenates of olfactory bulb, translated *in vitro*, and the translation products were immunoprecipitated. A band (Fig. 5, lanes 1–3) can be seen at 19 kDa corresponding to the authentic OMP immunoprecipitate product (lane 5). Its authenticity was confirmed by immunoprecipitation in the presence of an excess of unlabeled OMP, which eliminated the radioactive product at 19 kDa (lanes 6–10).

Previous studies (4) have shown that the amount of OMP protein declines to ≈10% of the control value within 1 week after olfactory bulbectomy. Similarly, OMP mRNA levels declined significantly by 3 days, as shown by RNA blot hybridizations (Fig. 6, lane 3). In addition, the size of the OMP mRNA in olfactory bulb was the same as that in intact and bulbectomized olfactory epithelium (Fig. 6).

DISCUSSION

Adult central nervous system is composed of numerous cell types that express perhaps as many as 30,000 distinct proteins (24). To understand the mechanisms underlying differentiation and development of neuronal cell types, it is necessary to study the activation and expression of those genes whose products are developmentally regulated and neuronal specific. One example of a neuronal specific, developmentally regulated gene product is OMP. Therefore, we have begun investigating the expression of OMP by isolating four OMP cDNA clones from a rat olfactory mucosa cDNA library enriched for OMP sequences. These clones were detected on first-round screening with an oligodeoxynucleotide probe. Subsequent identification of the purified insert-containing plasmids was made from DNA blot hybridizations also using the synthetic probe. Since the probe was a mixed heptadecameric oligodeoxynucleotide, the clones were further characterized by hybrid-selected translation. From the hybrid-selection assay three clones were identified that hybridized to mRNA, which could be translated in a cell-free system to produce immunoprecipitable OMP.

Ultimate confirmation that the cloned cDNA corresponded to OMP mRNA was obtained by completely sequencing the cDNA insert of pOMP3. It contained an open reading frame of 486 base pairs, which predicted an amino acid sequence identical to that previously determined by amino acid sequencing of the isolated N-acetylated protein (23). The nucleotide sequence enabled us to evaluate our prior assignments of the only two potential ambiguities in the amino acid sequence. The sequence of the amino-terminal tripeptide, which had been assigned as Ala-Glu-Asp, was thus demonstrated to be correct. In addition, these data confirm our prediction that there is no discontinuity between Arg-53 and Leu-54. This was the only region of the amino acid sequence for which an overlap peptide had not been isolated. Thus, determination of the nucleotide sequence of pOMP3 complemented the previous protein sequencing efforts.

The nucleotide sequence of pOMP3 reported here includes almost the entire mRNA. The absence of the initiator codon and the cap site from the cDNA indicates that a portion of the 5′ noncoding region is missing from the cDNA, although the poly(A) tail suggests that the cDNA insert is complete at the 3′ end. The presence of a single guanine at the 5′ end of the cDNA could represent the third nucleotide of an ATG initiator codon. The difference between the size of the insert in pOMP3 (2149 bases) and the OMP mRNA (2300 bases) is ≈150 bases. This discrepancy suggests that a leader sequence of ≈150 bases, including the initiator codon, is missing from this clone. Although it is possible that this clone does not include the entire polyadenylylated region of the mRNA, primer extension studies (data not shown) indicate that 100–150 nucleotides are missing 5′ to the coding region. These data are consistent with the statistical studies of Kozak (25), indicating that 70% of 5′ leader sequences are fewer than 100 nucleotides long. Taken together, these data suggest that a full-length cDNA for rat OMP would be 2.3 kilobases.

The 1.6-kilobase untranslated 3′ region of the insert is 3 times longer than the OMP coding region but does not contain any ATG-initiated open reading frame longer than 20 codons. This untranslated region has a G+C content of 53.1%, compared to 58.9% for the OMP coding region.

The open reading frame for OMP is terminated by the pentanucleotide TGATG. This sequence contains a TGA termination signal frame shifted with an ATG initiator codon. Recent studies (D. Peabody and P. Berg, personal communication) indicate that such termination starts do occur in the *trp* operon of *E. coli*. The possible occurrence and significance of this mechanism in eukaryotes are unknown.

Using the insert from pOMP3 as a probe, we were unable to detect hybridization with RNA from 14 additional nonolfactory rat tissues under conditions that could have detected OMP mRNA at a level of 0.01%. This is consistent both with the lack of immunocytochemical staining in other tissues as well as with the lack of significant homology between the OMP amino acid sequence and any other known sequences contained in the NBRF data base (23). In addition, the failure of pOMP3 to hybridize with RNA of nonolfactory tissues suggests that the OMP mRNA is not derived from a gene that gives rise to differentially processed messages in various

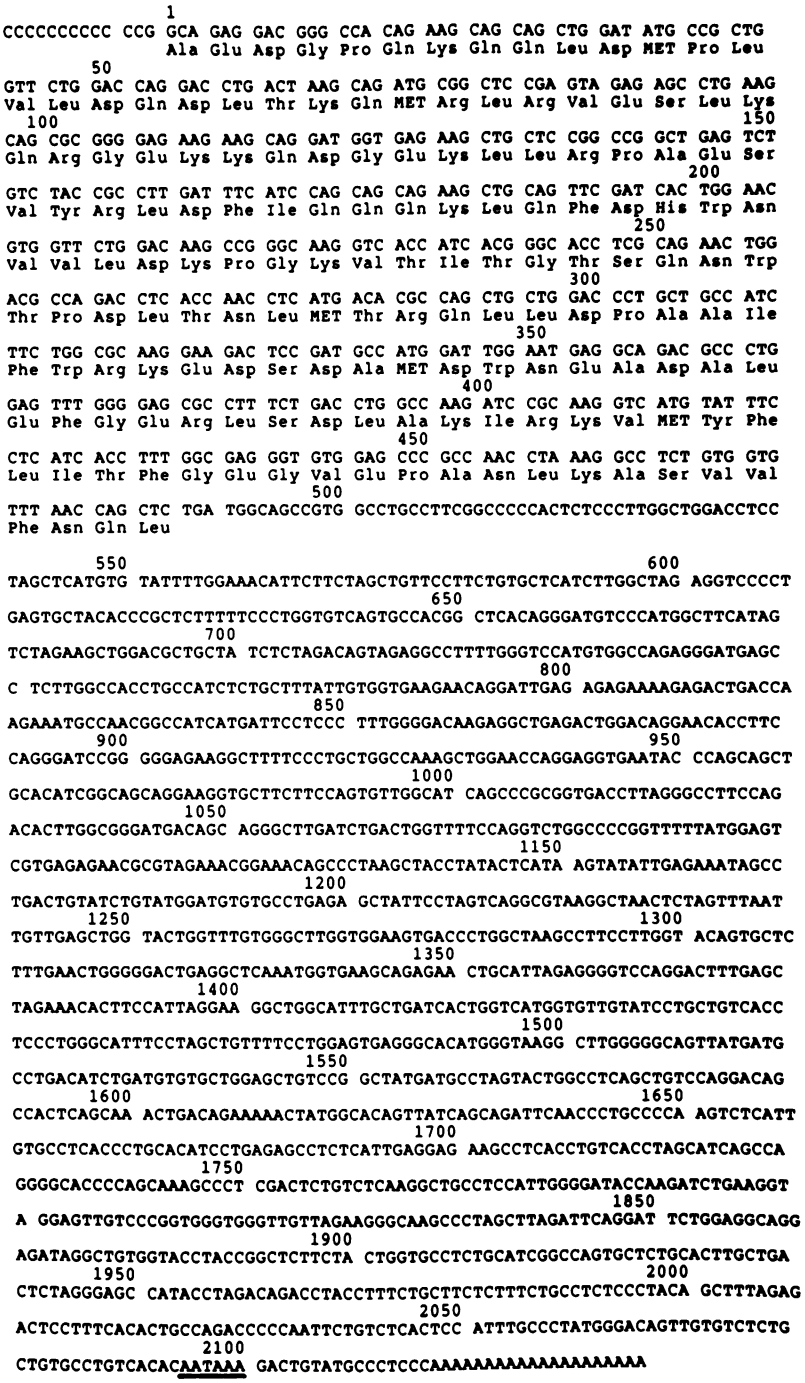

FIG. 3. Nucleotide sequence of the pOMP3 cDNA corresponding to rat OMP mRNA. The nucleotides are numbered in the 5′ to 3′ direction. The polyadenylylation signal sequence in the 3′ untranslated region is underlined.

tissues. A surprising observation was the detection of a small amount of OMP mRNA in olfactory bulb, since previous immunocytochemical studies had not demonstrated the protein in any cell type other than primary olfactory neurons. The presence of the OMP mRNA was a consistent observation with quantitatively similar amounts of OMP mRNA detected in three independently prepared rat bulb RNA preparations. One possible explanation is that the OMP mRNA may be located in the cytoplasm of the olfactory neuronal axons that innervate the olfactory bulb. Precedence for this postulate derives from the recent report of Giuditta *et al.* (26), which demonstrated that squid giant axons contain a complex class of mRNAs distinct from those in the neuronal cell body. An alternative explanation for the presence of OMP mRNA in the bulb is the existence of a small population of cells that contain the mRNA but have not been located by immunocytochemical staining. These two alternative suggestions may ultimately be resolved by *in situ* hybridization studies. Cloning of additional olfactory neuron-specific genes would also address the generality of this observation.

Finally, the changes in OMP mRNA levels following olfactory bulbectomy were examined by RNA blot hybridizations. OMP mRNA levels decreased dramatically within 3 days after bulbectomy and had returned to a significant percentage of their original level in ≈4 weeks. This rapid loss of OMP mRNA is consistent with our earlier studies on the decrease of OMP protein levels following bulbectomy (2, 4) and is in good agreement with previous studies, which determined that the numbers of olfactory cells in bulbectomized hamsters decreased to 25% by day 4 and returned to a

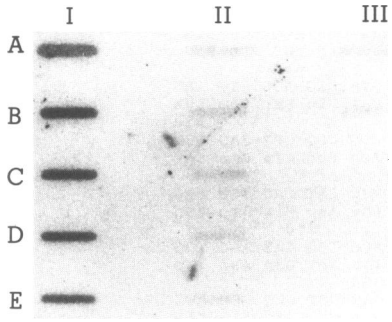

FIG. 4. Tissue specificity of OMP mRNA. Total RNA was isolated from various tissues of 4- to 6-week-old rats, spotted onto a nylon filter and hybridized with ^{32}P-labeled pOMP3. The blots were counter-probed with radiolabeled actin cDNA to demonstrate that, even in tissues where hybridization with pOMP was not observed, intact mRNA was present. Lane I, RNA from untreated rat olfactory epithelium; positions A–E correspond to 25, 12.5, 6.2, 3.1, and 1.6 μg of RNA, respectively. Lane II, RNA from rat olfactory epithelium 10 days after bulbectomy; positions A–E correspond to 25, 12.5, 6.2, 3.1, and 1.6 μg of RNA, respectively. Lane III, positions A and B, RNA from rat olfactory bulb; positions C and D, RNA from rat cerebral hemispheres; position E, RNA from rat lung. All positions correspond to 25 μg total RNA. In addition, no reaction was detected from 25 μg of RNA isolated from liver, muscle, kidney, cerebellum, salivary gland, thymus, eye, heart, spleen, skin, testes, or tongue.

level of 60–70% of the original number by day 28 (27). Therefore, our data demonstrate that OMP mRNA is produced by newly formed olfactory neurons, even in the absence of their normal target. However, it is probable that the presence of the target tissue is necessary for the maintenance of expression of normal levels of OMP mRNA, as suggested by the studies of Chuah and Farbman (9).

The isolation and characterization of pOMP3 has enabled us to confirm the amino acid sequence of this unique neuronal protein and to investigate the properties and expression of OMP mRNA. In addition, it will prove to be an invaluable tool for studying the developmental regulation of OMP mRNA. Finally, pOMP3 can now be used to identify OMP sequences in genomic libraries. This will permit the study of the regulation of expression of a unique neuronal protein at the level of the gene.

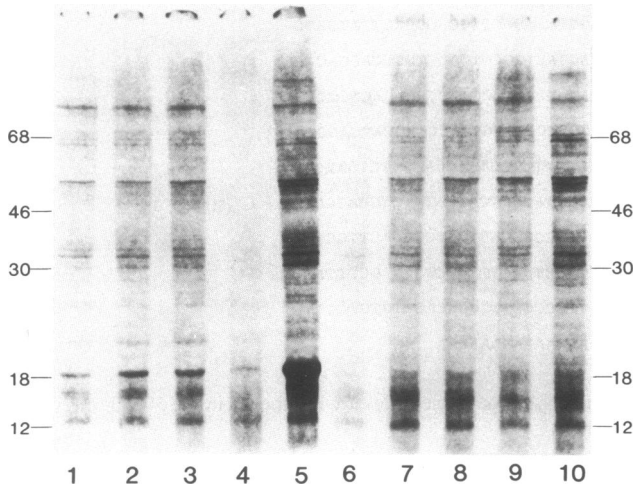

FIG. 5. Cell-free translation and immunoprecipitation of OMP from rat olfactory bulb RNA. RNA from various rat tissues was translated in a rabbit reticulocyte lysate and immunoprecipitated with goat anti-OMP antibodies. The translation products were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis after immunoprecipitation followed by fluorography. Immunoprecipitated OMP from three independent bulb RNA preparations (lanes 1–3), immunoprecipitated products from rat cerebral hemispheres RNA (lane 4), immunoprecipitated products from rat olfactory epithelium RNA (lane 5). Immunoprecipitation of OMP in the presence of 7 μg of nonradioactive authentic OMP, from three independent bulb RNA preparations (lanes 6–8), from cerebral hemispheres RNA (lane 9), from olfactory epithelium RNA (lane 10).

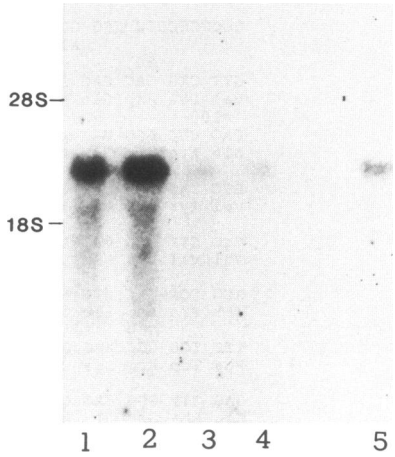

FIG. 6. Influence of bulbectomy on OMP mRNA levels. Surgery was performed on 4-week-old rats. Total RNA (5 μg) isolated from olfactory epithelium of control and lesioned rats was electrophoresed and blotted as described. The filter was hybridized with ^{32}P-labeled OMP cDNA. Lanes: 1, RNA from untreated 4-week-old rats; 2, RNA from untreated 8-week-old rats; 3, RNA from animals 3 days after bulbectomy; 4, RNA from animals 7 days after bulbectomy; 5, RNA from animals 28 days after bulbectomy.

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