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Olfactory neuron-specific protein is translated from a large poly(A)$^+$ mRNA

(olfactory marker protein/cell-free translation/small cell-specific protein/large mRNA)

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ABSTRACT **Poly(A)$^+$ mRNA was isolated from rat olfactory mucosa and translated in a rabbit reticulocyte cell-free protein synthesizing system. Olfactory marker protein (OMP) of M_r 18,500 was faithfully produced by this system upon addition of mucosal mRNA. The protein was identified by radioimmunoprecipitation with specific anti-OMP serum and by competitive displacement of the radioactive product with authentic OMP. In addition, the immunoprecipitated product comigrated with OMP on NaDodSO$_4$/polyacrylamide gels and on HPLC. *In vitro* synthesized OMP represented 0.5% of the total translational products. Total olfactory mucosal poly(A)$^+$ mRNA is ≈1.5–21 kilobases in size, as determined by denaturing agarose gels. Translational assays of gel-fractionated poly(A)$^+$ mRNA demonstrated that OMP mRNA occurs in the 2.5- to 3.4-kilobase range. An mRNA of this size could code for a protein significantly larger than OMP. Since the *in vitro* synthesized OMP is indistinguishable in size from OMP isolated from tissue, our data indicate that OMP is synthesized directly without the intermediate formation of a larger polypeptide precursor. Thus, OMP mRNA contains untranslated regions that are four to five times larger than the coding region.**

Olfactory marker protein (OMP) is a small acidic protein of M_r 18,500 (1, 2). This protein is widely distributed phylogenetically (3) but is found solely in olfactory receptor neurons. It represents ≈0.1–1% of all cytosolic proteins of olfactory tissue (4) and is localized throughout the olfactory receptor cell, from perikaryon to synaptic terminal (5). OMP has been purified and well characterized in terms of its biochemistry, cellular ontogeny, and cellular localization (6–9). However, the physiological function of this protein is unknown. OMP initially appears in receptor neurons during the last trimester of gestation and reaches adult levels ≈2–4 weeks postnatally (10, 11). Although the protein has a relatively long half-life and is axonally transported at a slow rate, both the turnover and transport rates are faster in tissue of young animals or in regenerating tissue from adults than in normal adult olfactory epithelium (12). These observations demonstrate that OMP is a neuronal-specific gene product that is developmentally and physiologically regulated.

In the present study, we have investigated the *in vitro* biosynthesis of OMP using rat olfactory mucosal mRNA. Furthermore, we have characterized the mRNA encoding this protein and demonstrated its unusually large size.

METHODS AND MATERIALS

Tissue. Pregnant CD-1 Sprague–Dawley-derived rats (Charles River Breeding Laboratories) were farrowed in our animal facility. Pups were sacrificed with CO_2 and exsanguinated at 4–6 weeks of age. Olfactory turbinates were rapidly removed into liquid N_2 and stored over liquid N_2 until used.

mRNA Purification. Frozen tissue was homogenized directly in a minimum of 10 vol of 6 M guanidinium thiocyanate (13) with a Brinkmann Polytron tissue disintegrator. A 17-ml aliquot of the homogenate was layered onto a 13-ml cesium chloride (5.7 M) cushion and centrifuged in a type 60 Ti rotor (Beckman) at 40,000 rpm for 17 hr. Total RNA was recovered by alcohol precipitation in the presence of 0.3 M sodium acetate. Poly(A)$^+$ mRNA was isolated from total RNA by two passes through oligo(dT)-cellulose (P-L Biochemicals) as described by Aviv and Leder (14). The yield was 150–200 μg of poly(A)$^+$ mRNA per g of frozen tissue.

***In Vitro* Translation of mRNA.** A nuclease-treated rabbit reticulocyte lysate (Bethesda Research Laboratories) was used for *in vitro* protein synthesis. The translations were carried out in 30-μl volumes containing 10 μl of the lysate as supplied, 87 mM potassium acetate, 50 μM of each of 19 nonradioactive amino acids, and 50 μCi (1 Ci = 37 GBq) [^{35}S]methionine (Amersham) at >800 Ci/mmol. The reaction was initiated by the addition of mRNA and incubated for 60 min at 30°C. For determination of the radioactivity incorporated, aliquots of the incubation were added to 0.5 ml of 1 M sodium hydroxide containing 5% hydrogen peroxide and incubated for 10 min at 37°C. Proteins contained in the aliquots were precipitated by the addition of 2 ml of cold 25% trichloroacetic acid containing 2% casein. An aliquot of the precipitate was applied to a glass fiber filter (Gelman) and washed five times with 2 ml of cold 8% trichloroacetic acid. Incorporation of [^{35}S]methionine into total protein was measured by determining the radioactivity retained on the filter using scintillation spectrometry.

Immunoprecipitation of Translation Products. *In vitro* translation reactions were terminated by the addition of NaDodSO$_4$ to a final concentration of 2%. The reaction mixture was subsequently diluted 1:5 with 50 mM Tris·HCl, pH 7.4/0.15 M NaCl/6 mM EDTA/2.5% Triton X-100/1.0 mM methionine followed by the addition of 1 μl of nonimmune goat serum and 200 μl of a 10% IgGsorb suspension (The Enzyme Center, Boston, MA) (15). Following 30 min at room temperature, the insoluble material was collected by centrifugation and discarded. The supernatants were treated with the same quantities of immune goat serum (3, 7, 10) and IgGsorb and incubated at 4°C for 17 hr followed by centrifugation. Immunoprecipitates were washed five times with 1 ml of 20 mM sodium phosphate (pH 7.4) containing 150 mM NaCl, 1 mM methionine, 0.1% NaDodSO$_4$, and 0.5% Nonidet P-40. Immunoprecipitates were resuspended in 50 μl of 50 mM Tris·HCl, pH 6.8/1% NaDodSO$_4$/0.2% 2-mercaptoethanol/1% glycerol, heated at 95°C for 5 min, and centrifuged

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Abbreviation: OMP, olfactory marker protein.

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to remove the IgGsorb. Molecular weight markers were also diluted into this buffer. Immunoprecipitates and molecular weight markers were electrophoresed in 10% polyacrylamide/NaDodSO$_4$ slab gels (16). Gels were infused with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 film at −70°C for varying periods of time. For analysis by HPLC, immunoprecipitates were dissociated in 50 μl of 6 M guanidinium thiocyanate in the presence of 10 μg of carrier OMP, heated to 95°C for 3 min, and centrifuged to remove IgGsorb.

HPLC. The reversed-phase chromatographic analysis system consisted of a model II Chromat-a-trol (Eldex Laboratories, Menlo Park, CA) with LP-II rationing valves equipped with a 10-ml mixing chamber to form the low-pressure gradient generation system at a flow rate of 1.5 ml/min. A Vydac C_{18} column (The Separations Group, Vydac, Hesperia, CA) of 4.6 × 250 mm, 300-Å pore, and 5-μm particle size together with an M441 absorbance detector (Waters Associates) were used for separation and monitoring elution profiles. The mobile phase buffer A was 0.1% trifluoroacetic acid (Pierce), whereas 90% acetonitrile in 0.1% trifluoroacetic acid was used as the organic modifier in buffer B. All eluents were kept under helium pressure of 10 psi (1 psi = 6.89 kPa). Chromatograms were developed in a 60-min linear gradient from 43% to 55% buffer B. Fractions were collected (0.5 ml) and radioactivity was determined.

mRNA Size Fractionation. Poly(A)$^+$ mRNA was fractionated on methylmercury agarose gels (17). The mRNA was denatured in 10 mM methylmercury hydroxide at 65°C prior to electrophoresis through 1.2% low-melting temperature agarose (FMC) containing 5 mM methylmercury hydroxide. Approximately 20 μg of mRNA was used in each lane of a 0.3 × 5 × 8 cm minigel (18). Following electrophoresis, the gel was washed according to the procedure of Lonberg and Gilbert (19) and sliced into 2-mm segments. Each segment was again split into two pieces and stored in liquid nitrogen until used. Each gel piece containing mRNA was used for translation assays in the rabbit reticulocyte lysate system without removal of the agarose. Translational assays were performed as described with the exception of the incubation temperature, which was raised to 37°C to prevent gelling of the agarose.

Alternatively, nondenaturing sucrose gradient density centrifugation was used to size fractionate the mRNA. Sucrose gradients of 5–40% (wt/vol) made in 20 mM sodium acetate/2 mM EDTA, pH 6–7, were prepared by using the freeze-thaw technique (20). Poly(A)$^+$ mRNA to be fractionated was denatured by heating to 95°C for 60 s prior to application to the gradient. A companion gradient was loaded with *Escherichia coli* rRNA markers. Gradients were centrifuged in an SW40 rotor (Beckman) at 28,000 rpm for 20 hr. Fractions of 0.5 ml were collected, pooled in groups of three fractions, precipitated with ethanol, and used for translation assays.

RNA Transfer Blot Hybridization. A mixed heptadecameric probe with a degeneracy of 16 was predicted from the amino acid sequence of a tryptic peptide (21) and was synthesized by using the solid-support phosphite methodology (22, 23). The peptide and the predicted oligodeoxynucleotide are

$$-\text{Gln}-\text{Phe}-\text{Asp}-\text{His}-\text{Trp}-\text{Asn}-$$

$$3'\ -\text{GT}\begin{matrix}\text{T}\\\text{C}\end{matrix}\ \text{AA}\begin{matrix}\text{A}\\\text{G}\end{matrix}\ \text{CT}\begin{matrix}\text{A}\\\text{G}\end{matrix}\ \text{GT}\begin{matrix}\text{A}\\\text{G}\end{matrix}\ \text{ACC}\ \text{TT}-5'.$$

T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham) were used to label its 5′ ends to a specific activity of 6.8 × 10^8 cpm/μg. Approximately 20 μg of sucrose gradient-fractionated RNA was electrophoresed in 1.2% agarose gels in the presence of methylmercury hydroxide as detailed above. The mRNA was transferred to nitrocellulose sheets as described (24). Filters were prehybridized for a minimum of 3 hr at 34°C in 0.75 M sodium chloride/0.075 M sodium citrate, pH 7.0/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.2% bovine serum albumin/0.1% NaDodSO$_4$/denatured salmon sperm DNA at 100 μg/ml. Hybridizations were carried out at 37°C for 24 hr in the same buffer containing 25–50 ng of the radioactively labeled probe per ml. The filters were then washed at room temperature three times for 10 min in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0, dried, and autoradiographed at −70°C. *Hin*dIII λ DNA digest-size markers (P-L Biochemicals) were run in parallel lanes and visualized by staining with ethidium bromide subsequent to transfer onto a nitrocellulose membrane.

RESULTS

The recovery of total RNA from rat olfactory mucosa by the cesium chloride cushion method (13) was ≈20 mg/g of wet weight of tissue. Protein contamination was low, as evidenced by A_{260}/A_{280} ratios that were routinely >1.9. DNA was undetectable on agarose gel electrophoresis with ethidium bromide staining. Poly(A)$^+$ mRNA purified by oligo(dT)-cellulose chromatography represented 1.5–2.0% of the total RNA. This poly(A)$^+$ mRNA contained no detectable tRNA and only low levels of rRNA.

Olfactory mucosal poly(A)$^+$ mRNA directed the incorporation of 1.3–3.0 × 10^6 cpm of [^{35}S]methionine per μg of poly(A)$^+$ mRNA into total protein. The M_rs of these total translational products ranged from 5000 to 100,000 (Fig. 1, lane 7). Immunoprecipitation of the translational products with OMP antibody produced a single radioactive band that migrated to the 18,500 M_r position of native OMP in NaDodSO$_4$/PAGE (Fig. 1, lane 2). Addition of nonimmune

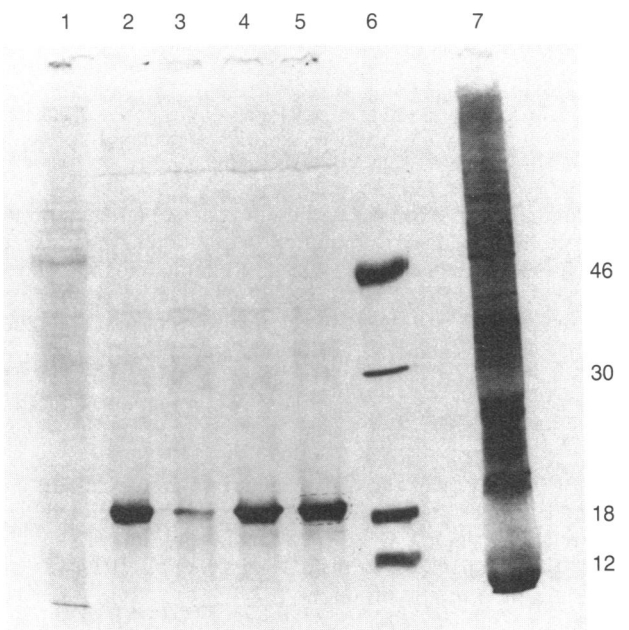

FIG. 1. Fluorography of NaDodSO$_4$/PAGE of *in vitro* translational products. Translations and immunoprecipitations of rat olfactory mucosa poly(A)$^+$ mRNA were scaled up 5-fold. Lane 1, proteins precipitated with nonimmune serum. Lane 2, protein precipitated with anti-OMP serum. Lane 3, same as lane 2 except that 10 μg of nonradioactive OMP was added prior to immunoprecipitation. Lane 4, same as lane 2 except that 40 μl of a 10% cerebellum extract was added prior to immunoprecipitation. Lane 5, same as lane 2 except that 10 μg of nonradioactive OMP was added subsequent to immunoprecipitation. The gel was stained with Coomassie blue to locate the OMP prior to fluorography; ---- indicates the position of nonradioactive OMP. Lane 6, ^{14}C-labeled size markers shown as M_r × 10^{-3}. Lane 7, 2.5 μl of total translational products.

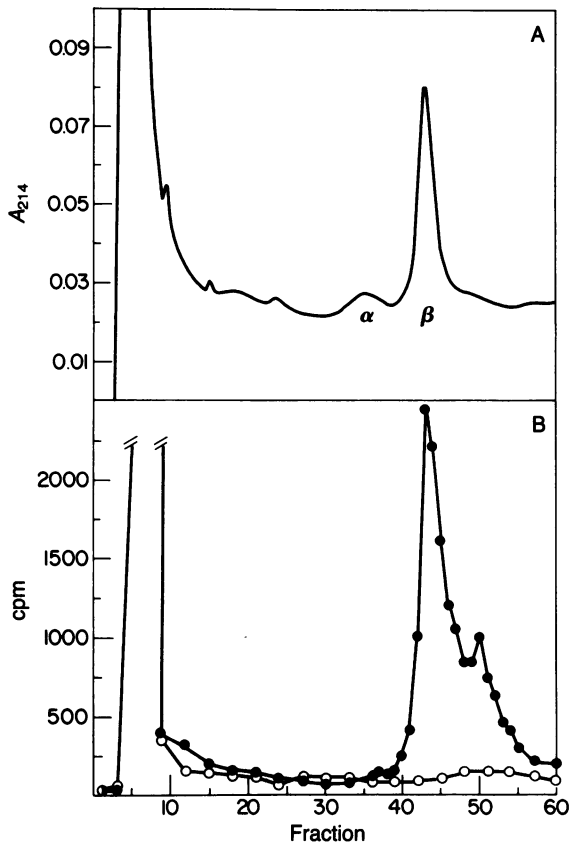

FIG. 2. Identification of immunoprecipitated product on reversed-phase HPLC. Translational products were immunoprecipitated with anti-OMP serum. Precipitated proteins were released from IgGsorb by treatment with 6 M guanidinium thiocyanate at 95°C for 3 min. (*A*) Profile of absorbance at 214 nm; β indicates carrier OMP in fraction 43. The minor peak (α) at fraction 35 is also OMP that is present in isolated preparations. (*B*) Radioactivity profile of collected fractions. ○, Products precipitated with nonimmune serum. ●, Products precipitated with anti-OMP serum.

serum to a translation assay did not produce an immunoprecipitate (Fig. 1, lane 1). Immunoprecipitation in the presence of excess unlabeled OMP virtually eliminated the band of radioactivity (Fig. 1, lane 3), indicating effective competition by authentic OMP. However, addition of a cerebellum extract under identical conditions had no effect on the immunoprecipitation (Fig. 1, lane 4). Finally, carrier OMP added to the assays subsequent to immunoprecipitation migrated to the identical position of the radioactive product (Fig. 1, lane 5).

HPLC analysis of the dissociated immunoprecipitate produced a single major radioactive peak that coeluted with authentic carrier rat OMP (Fig. 2). In addition, a shoulder peak was seen in the translational products that was not present in the native product.

The translation assay was optimized for OMP production with regard to both time and mRNA concentration. Incorporation of [^{35}S]methionine into total protein and OMP was linear for nearly 30 min. About 90% of the OMP product was generated during the initial 25 min of the reaction (data not shown). Varying amounts of olfactory poly(A)$^+$ mRNA (0.25–2.5 μg) were added to the translation assay, which was found to saturate at ≈1.0 μg. A concentration of 0.5–1.0 μg of mRNA per 30-μl assay was chosen for subsequent studies. Under these conditions, OMP represented ≈0.5% of the total translation products.

Poly(A)$^+$ mRNA fractions separated on methylmercury agarose gels were analyzed for their ability to direct the synthesis of OMP. When total translational products were electrophoresed, the molecular weight range of the protein products increased in proportion to the size of the mRNA fraction (data not shown). In addition, proteins having M_rs of 15,000–20,000, excluding OMP, were translated primarily by mRNA of <1700 nucleotides. Subsequent immunoprecipitation of translational products indicated that the OMP mRNA was contained in four consecutive gel slices (Fig. 3). The peak of OMP activity was found in the gel slice corresponding to a size of 3400 bases. Results from sucrose density gradient centrifugation (Fig. 4) indicate that, under the conditions used, the mRNA for OMP is ≈18–21 S, which is consistent with the data obtained from the agarose gel fractionation. Evaluation of [^{35}S]methionine incorporation per μg of poly(A)$^+$ mRNA indicated that gradient-fractionated mucosal mRNA had been enriched 2- to 3-fold with regard to OMP mRNA.

Finally, fractions of olfactory mRNA from sucrose gradient centrifugation were electrophoresed in an agarose gel and transferred to nitrocellulose. Hybridization with the radioactive synthetic oligodeoxynucleotide probe demonstrated the presence of OMP poly(A)$^+$ mRNA at the 3-kilobase position (Fig. 5, lanes 1 and 2). The synthetic oligodeoxynucleotide probe did not hybridize to mRNA obtained from a fraction of the sucrose gradient that did not contain OMP mRNA (Fig. 5, lane 3).

DISCUSSION

We have shown that OMP, a M_r 18,500, developmentally regulated, neuron-specific protein is coded for by a large poly(A)$^+$ mRNA. Furthermore, in a cell-free protein synthesizing system, OMP is produced directly without the intermediate formation of a larger polypeptide precursor.

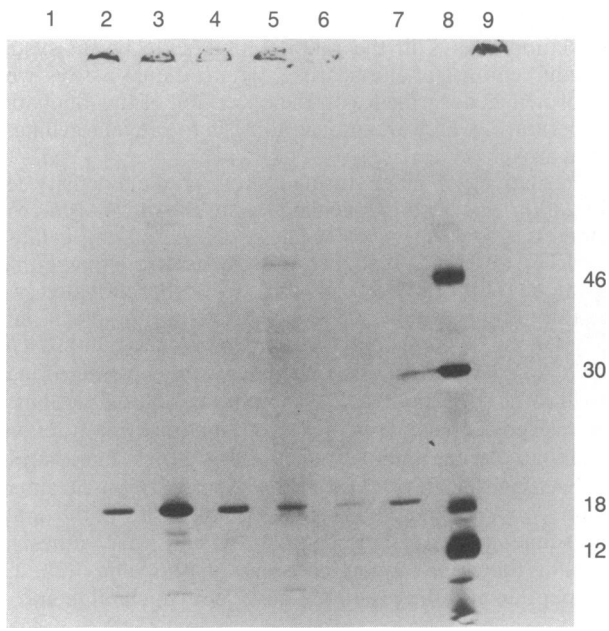

FIG. 3. Localization of OMP mRNA fractionated on a 1.2% methylmercury agarose gel. Poly(A)$^+$ mRNA was electrophoresed at 5 V/cm through a 0.3 × 4 × 11 cm gel; gel slices were translated and immunoprecipitated. Each lane represents immunoprecipitable products obtained from the translation of one 2-mm gel slice. Lane 1, >3800-nucleotide region. Lane 2, 3700-nucleotide region. Lane 3, 3400-nucleotide region. Lane 4, 3100-nucleotide region. Lane 5, 2800-nucleotide region. Lane 6, 2500-nucleotide region. Lane 7, immunoprecipitable product from translation of 1.5 μg of poly(A)$^+$ mRNA. Lane 8, ^{14}C size markers shown as $M_r \times 10^{-3}$. Lane 9, no exogenous mRNA added to translation assay.

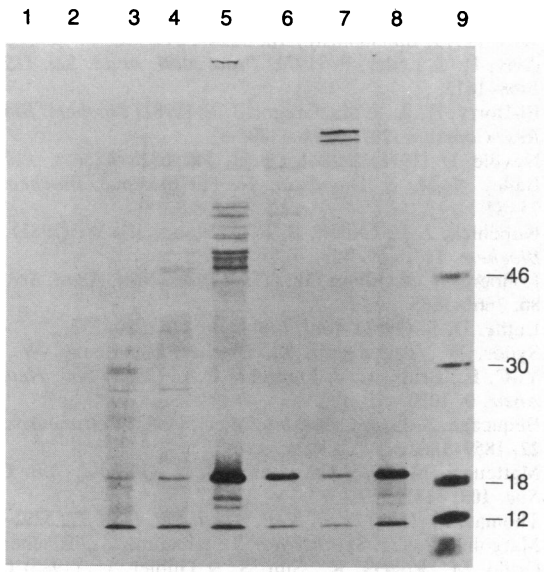

FIG. 4. Sucrose density gradient analysis of rat olfactory mucosa poly(A)$^+$ mRNA. mRNA was isolated from sequentially pooled fractions, translated, and immunoprecipitated. S values were determined from the sedimentation positions of *E. coli* 16S and 23S rRNAs in a companion sucrose density gradient as well as the internal rat 18S and 28S rRNAs. Lane 1, 9S fraction. Lane 2, 11S fraction. Lane 3, 13S fraction. Lane 4, 15S fraction. Lane 5, 18S fraction. Lane 6, 24S fraction. Lane 7, 27S fraction. Lane 8, immunoprecipitation from total poly(A)$^+$ mRNA. Lane 9, ^{14}C size markers shown as $M_r \times 10^{-3}$.

To determine the size of the OMP mRNA, total poly(A)$^+$ mRNA was fractionated in two systems and the region containing OMP mRNA was located by translation and subsequent immunoprecipitation of the product. In denaturing agarose gels, OMP mRNA was located in a region corresponding in size to ≈3400 nucleotides. On sucrose density gradients, the OMP mRNA was located in the range of 18–21 S. Fractions from the gradient subjected to electrophoresis, RNA transfer blotted, and probed with the synthetic oligodeoxynucleotide indicated once again an mRNA of 3000 nucleotides. These results were unexpected since the predicted size of the coding region for OMP is about 500 nucleotides or one-sixth the size of the mRNA found. If one considers an additional 100–200 nucleotides to account for the poly(A) tail, it becomes evident that there is at least four times as much noncoding material at the 5′ and 3′ ends of this mRNA as there is coding information. The function of these sequences is unknown but they may play a regulatory role. Alternatively, this large mRNA may encode more than one polypeptide, although as yet no polycistronic mRNAs have been reported in eukaryotes.

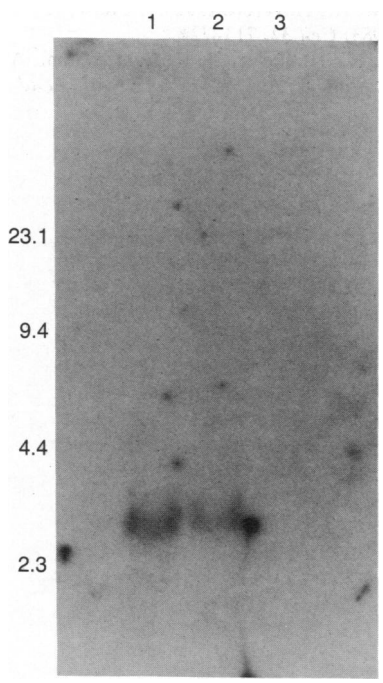

FIG. 5. RNA transfer blot hybridization of synthetic probe to sucrose gradient-fractionated mRNA. Poly(A)$^+$ mRNA from sucrose gradient fractions (5 μg) was subjected to electrophoresis on a 1.2% agarose gel in the presence of 10 mM methylmercury hydroxide, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled synthetic oligonucleotide. Size markers (shown in kilobases) were λ DNA digested with *Hin*dIII. Lane 1, 18S fraction. Lane 2, 22S fraction. Lane 3, 34S fraction.

Evidence for the direct synthesis of OMP derives from the detection of a single immunoprecipitated radiolabeled polypeptide that comigrates with authentic OMP on both reversed-phase HPLC and on NaDodSO$_4$/PAGE. To confirm the identity of the immunoprecipitated product, it was shown that an excess of unlabeled authentic OMP could displace the translational product from the immunoprecipitate. Furthermore, an extract of cerebellum proteins devoid of OMP (4) did not displace the radiolabeled immunoprecipitated product. It is unlikely that a protein precursor was initially synthesized since the OMP produced translationally is indistinguishable in size from the native protein. If a larger protein precursor had been synthesized, OMP with properties of the native protein would not have been produced since the required processing enzymes are absent from the rabbit reticulocyte lysate system. It is important to note that the elution position of the *in vitro* synthesized OMP on HPLC coincides with the major native OMP peak and not with the preceding minor peak. The minor peak has been demonstrated to be closely related to OMP (25) and is probably a partially deamidated product generated by isolation procedures. Thus, it follows that the translational products would not exhibit a peak corresponding to a product produced solely upon isolation. However, HPLC profiles of the translational products do contain a shoulder peak (Fig. 2*B*) that is not present in native OMP. Since OMP as isolated has been shown to possess a blocked amino terminus (25), it is possible that the shoulder peak represents the free amino-terminal form of OMP. This is reasonable since the enzyme activity most commonly responsible for the addition of amino-terminal blocking groups to proteins (acetyltransferase) (26–28) is present and active in the reticulocyte lysate (29).

In the preceding studies, poly(A)$^+$ mRNA was used exclusively for the translation and immunoprecipitation of OMP. When poly(A)$^-$ mRNA was translated and immunoprecipitated in an identical manner, various proteins were produced but an OMP immunoprecipitate was not detected (data not shown). Thus, one can conclude that OMP mRNA is polyadenylylated. Investigators have demonstrated that brain mRNAs contain a highly complex set of poly(A)$^-$ mRNAs (30–33). This observation appears to be a neuronal characteristic since the sequence complexity of tissues and cells of nonneuronal origin is present in the poly(A)$^+$ mRNA. In addition, Chaudhari and Hahn (34) have suggested that neuronal proteins that are developmentally regulated would be encoded by poly(A)$^-$ mRNAs. However, OMP clearly represents an example of a developmentally regulated, neuron-specific protein whose mRNA is polyadenylylated and, therefore, cannot be categorized in this manner.

It is of interest to note that in general the population of mRNAs isolated from neural tissues is larger in size than that

isolated from nonneural tissues (35). OMP mRNA definitely can be categorized in this manner. In addition, other examples of tissue-specific proteins that are encoded by unusually large mRNAs have been reported (36, 37). The significance of this observation is unclear but OMP mRNA represents a neuronal-specific example of this phenomenon.

The cloning of OMP mRNA awaits further investigation. Coupled with our knowledge of the biology of its cell of origin and the biochemical properties of OMP, the molecular biological aspects should enable us to extend our studies of the function of the olfactory neuron.

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