Olf-1-Binding Site: Characterization of an Olfactory Neuron-Specific Promoter Motif

KATARZYNA KUDRYCKI, CATHY STEIN-IZSAK,† CHRISTIAN BEHN,‡ MARY GRILLO,
RICHARD AKESON,§ AND FRANK L. MARGOLIS*

*Department of Neurosciences, Roche Institute of Molecular Biology, Roche Research Center,
Nutley, New Jersey 07110*

We report characterization of several domains within the 5' flanking region of the olfactory marker protein (OMP) gene that may participate in regulating transcription of this and other olfactory neuron-specific genes. Analysis by electrophoretic mobility shift assay and DNase I footprinting identifies two regions that contain a novel sequence motif. Interactions between this motif and nuclear proteins were detected only with nuclear protein extracts derived from olfactory neuroepithelium, and this activity is more abundant in olfactory epithelium enriched in immature neurons. We have designated a factor(s) involved in this binding as Olf-1. The Olf-1-binding motif consensus sequence was defined as TCCCC(A/T)NGGAG. Studies with transgenic mice indicate that a 0.3-kb fragment of the OMP gene containing one Olf-1 motif is sufficient for olfactory tissue-specific expression of the reporter gene. Some of the other identified sequence motifs also interact specifically with olfactory nuclear protein extracts. We propose that Olf-1 is a novel, olfactory neuron-specific trans-acting factor involved in the cell-specific expression of OMP.

The complex and highly organized pattern of gene expression that leads to the determination of cellular phenotypes is mediated in part by the interplay between distinctive DNA sequence elements present in the promoter regions of different genes and the various transcription factors with which they interact. The transcription factors are capable of interacting with diverse DNA motifs and with each other in response to a variety of environmental and inter- and intracellular signals. These processes can be regulated by protein-protein interactions and by posttranslational modifications (for a review, see references 18, 26, and 36). Combinatorial effects of these interactions permit precise gene regulation in particular cell types at defined developmental stages.

Determination of cellular phenotype in the mammalian central nervous system must involve exceptionally complex patterns of transcriptional regulation. The central nervous system consists of as many as 10^{12} cells, many of which are distinct with respect to their morphological, biochemical, biophysical, and functional properties. In addition, patterns of gene expression in these cells undergo continuous changes during cell development (18, 42). A system that offers several advantages for studying neuron-specific gene expression is the peripheral olfactory system. The adult olfactory neuroepithelium has the unique ability to generate new neurons from precursor cells in a process essential for replacement of olfactory neurons lost in response to various environmental insults. This process can be experimentally stimulated by surgical or chemical damage to the mature receptor cells (8, 16, 20, 46, 56, 68). Furthermore, mature olfactory neurons are the sole or primary site of expression of several olfactory neuron-specific proteins which include novel gene products associated with signal transduction (1,

4, 11, 27, 34, 49). The first olfactory neuron-specific protein that was isolated (37) and cloned (9, 53) is the olfactory marker protein (OMP). OMP expression is highly restricted to mature olfactory neurons (2, 38, 39) in species as diverse as amphibia (31) and humans (7, 47), suggesting tight control of its expression by tissue- and development-specific factors. This 19-kDa cytoplasmic protein, of unknown physiological function, appears in the olfactory tissues at the beginning of the last trimester of gestation and is characteristic of olfactory neurons in their final stages of differentiation (58, 68).

Since OMP expression is tightly controlled in a spatial and temporal manner, identification of the specific elements responsible for this regulation should offer insight to the mechanisms regulating olfactory neuron-specific expression. In order to understand the mechanism of OMP transcriptional regulation, we have initiated a study to identify possible cis-acting elements within the OMP promoter that may be responsible for age- and tissue-specific expression of this protein. Analyses of regulatory elements within neuronal genes have been largely conducted in transgenic animals, because permanent differentiated cell lines are rarely available. Initial studies with transgenic mice demonstrated that 5.2 kb of the OMP promoter contained the information required for OMP-like tissue-specific reporter gene expression (9). Subsequent experiments conducted in our laboratory indicated that as little as 0.8 kb upstream of the OMP gene transcriptional start site is sufficient for correct cell-specific expression of reporter gene in vivo (17, 63). In this report we characterize several elements within the OMP gene promoter that may be responsible for tissue- and development-specific expression of this protein. Two of these contain a similar, novel sequence motif that interacts with nuclear proteins from olfactory neuroepithelium. We were unable to detect DNA-protein binding in the presence of nuclear extract from any other tissue that we have tested. The protein(s) that is involved in this olfactory neuron-specific binding was designated Olf-1. We propose that Olf-1 represents an olfactory neuron-specific trans-acting factor or a complex of factors capable of interacting with other

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* Corresponding author.
† Present address: Organon Inc., West Orange, NJ 07052.
‡ Present address: ABIMED, 4018 Langenfeld, Germany.
§ Present address: Children's Hospital Research Center, Cincinnati, OH 45229.

olfactory neuron-specific genes. As such, it may play a role in regulating the expression of genes associated with odor transduction or neuronal regeneration.

MATERIALS AND METHODS

Animals. Tissues for preparation of nuclear extracts were dissected from 10-day- and 3- and 6-week-old male and female CD rats and 3-week-old female CD-1 mice. Olfactory bulbectomies were performed on ZML-SD 4-week postnatal rats as previously described (68). Generation of transgenic mice is described below (see Transgenic mice). All animals were sacrificed by CO_2 narcosis and then were exsanguinated.

Preparation of nuclear extracts. Nuclear extracts from rat and mouse tissues were prepared essentially as previously described (12) with minor modifications. Briefly, the tissues were hand homogenized with 4 volumes of homogenization buffer [0.25 M sucrose, 15 mM Tris-HCl (pH 7.9), 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg of leupeptin per ml, 0.015 TIU of aprotinin per ml] in a Kontes all-glass homogenizer and centrifuged at 1,000 × g. The pellets were resuspended in 4 tissue volumes of buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05 mM PMSF, 1.25 μg of leupeptin per ml, 0.006 TIU of aprotinin per ml) and centrifuged at 2,500 × g. The pellets were resuspended in 4 volumes of buffer A and centrifuged at 25,000 × g. The pelleted nuclei were resuspended in 1 tissue volume of 1 M KCl mixed with 1 tissue volume of buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, and containing DTT, PMSF, leupeptin, and aprotinin at the same concentration as in homogenization buffer), homogenized as before, and lysed by 30 min of rocking. The lysate was centrifuged at 25,000 × g, and the supernatant was dialyzed against binding buffer (10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 3 mM DTT, 0.3 mM PMSF), and aliquots were stored at −80°C.

PCRs. An 11-kb EcoRI fragment containing the rat OMP locus subcloned into pBluescript (pGOMP [9]) was used as the template in all polymerase chain reactions (PCRs) creating DNA fragments that were used for subcloning or for synthesis of probes and competitors for electrophoretic mobility shift assay. Amplifications of 200 ng of the pGOMP were performed with 1 to 2 U of PYROSTASE DNA polymerase (Molecular Genetic Resources, Inc.) in the presence of 0.5 μg of each primer in 50 to 100 μl of PCR cocktail (PCR PYROSTASE DNA polymerase buffer; [Molecular Genetic Resources, Inc.] and 200 μM deoxynucleoside triphosphates [Pharmacia]). The initial denaturation (94°C, 7 min) was followed by 32 amplification cycles: 94°C, 2 min; 55°C, 2.5 min; 72°C, 3.5 min, and a final 10-min extension at 72°C. Reaction products were separated by electrophoresis on 1.6 to 2% agarose gels, and the amplified DNA fragments were excised and purified with a Mermaid kit (Bio 101).

Electrophoretic mobility shift assays (EMSAs). Reaction mixtures (50 or 75 μl total) containing 10 mM Tris-HCl (pH 7.9), 30 mM NaCl, 7% glycerol, 4.3 mM $MgCl_2$, 1 mM EDTA (pH 8), 0.18 mM PMSF, 1.8 mM DTT, 1.8 mg of aprotinin per ml, 1.2 mg of leupeptin per ml, 1 to 10 μg of poly(dI-dC), and 1 to 20 μg of protein extract were incubated on ice in the presence of radiolabeled DNA fragments. The probes were

labeled with [α-^{32}P]dCTP by the large fragment of the *Escherichia coli* DNA polymerase I (Klenow). All protein extracts were prepared from 3-week-old rats or mice except where noted. In the competition experiments, the appropriate DNA fragments were preincubated on ice with the binding reaction mixtures prior to addition of the ^{32}P-labeled probe. The reaction mixtures were mixed with the loading buffer, subjected to electrophoresis on 5% polyacrylamide gels (29:1, acrylamide-bisacrylamide), and analyzed by autoradiography.

OMP gene fragments (see Fig. 1) and double-stranded oligonucleotides (see Table 1) that were employed in EMSA experiments as probes and competitors are listed below. The nucleotide numbers refer to positions within the OMP gene relative to the transcription initiation site (9). The DNA fragments and methods of their preparation were as follows: (i) F-1, nucleotides −131 to +55, was prepared by PCR amplification; and (ii) F-2, nucleotides −239 to −124, was prepared by HindIII and EcoRI restriction endonuclease digest of a PCR-amplified fragment of the OMP gene fragment (nucleotides −251 to −110). The amplification was performed with a 3′ primer in which nucleotides corresponding to the OMP gene residues −121 and −122 were modified in order to create an EcoRI site at position −124. The following fragments were created by restriction endonuclease digests of the F-2 or the PCR product from which this fragment was derived: (iii) F-3, AvaII, nucleotides −251 to −180; (iv) F-4, AvaII-EcoRI, nucleotides −180 to −124; (v) F-5, HphI, nucleotides −251 to −171; (vi) F-6, HphI-EcoRI, nucleotides −171 to −124; (vii) F-7, MnlI-MnlI, nucleotides −233 to −149; (viii) F-8, NlaIII-MnlI, nucleotides −185 to −149; and (ix) F-9, NlaIII-EcoRI, nucleotides −185 to −124. Another DNA fragment used in EMSA experiments was (x) βL, AvaII-AvaII restriction endonuclease fragment of β-lactamase open reading frame (pCATBasic [Promega], nucleotides 632 to 854).

Double-stranded synthetic oligonucleotides (Table 1) were as follows: (i) A, sense strand, nucleotides −190 to −158 of the OMP gene, and antisense strand, nucleotides −163 to −192; (ii) A*, sense strand, nucleotides −192 to −163, and antisense strand, nucleotides −189 to −160; (iii) A*m1, identical to A* except that residue −179 was altered from G to A with complementary change in the antisense strand; (iv) A*m2, identical to A* except that residues −175 and −176 were altered from CC to AG with complementary change in the antisense strand; (v) A*m3, identical to A* except that residue −178 was altered from T to A with complementary change in the antisense strand; (vi) B, sense strand, nucleotides −715 to −685, and antisense strand, nucleotides −713 to −683; (vii) C, sense strand, nucleotides −734 to −705, and antisense strand, nucleotides −731 to −702; (viii) D, sense strand, nucleotides −105 to −76, and antisense strand, nucleotides −108 to −72; (ix) E, sense strand, nucleotides −435 to −406, and antisense strand, nucleotides −438 to −409; (x) F, sense strand 5′-ATGTGCCTCTGGTCCCCGC CGTGTGTGT-3′, nucleotides −994 to −965, and antisense strand 3′-ACGGAGACCAGGGGCGGCACACACACACA C-5′, nucleotides −990 to −962 (sequence of the oligonucleotide F is given in full, since this part of the rat OMP gene was not previously reported); (xi) G, sense strand, nucleotides +2162 to +2193, and antisense strand, nucleotides +2160 to +2195; (xii) H, sense strand, nucleotides +2107 to +2138, and antisense strand, nucleotides +2109 to +2140; (xii) Hm1, identical to H except that residue +2123 was altered from A to T with complementary change in the

TABLE 1. Effects of modifications within the Olf-1-binding site on its binding activity

Oligonucleotide[a]	Position of oligonucleotide (mutated nucleotide)[b]	Sequence[c]	Olf-1-binding activity[d]
A (proximal Olf-1 site)	−190 to −158	5'-CCATGCTCTGG TCCCCAAGGAG CCTGTCACCCT-3'	+++
B (distal Olf-1 site)	−683 to −713	5'-GATCCTCCACC TCCCCTGGGAG ATGTGAGGC-3'	+++
C	−734 to −705	5'-AGATCTCCAGCGTCC TCCCCGGCCTC ACAT-3'	−
D	−105 to −76	5'-TCATGTG TCCCCTGTTCT GACAACTGGGTG-3'	−
E	−409 to −438	5'-GGTTGCTTCC TCCCCACCTCA TTCTCTCGA-3'	−
F	−994 to −965	5'-ATGTGCCTCTGG TCCCCGCCGTG TGTGTGT-3'	−
G	+2162 to +2193	5'-AAAGACTGTATGCCC TCCCCTCTGTG GTGTGG-3'	−
H	+2140 to +2109	5'-AGACACAACTG TCCCCATAGGG CAAATGGAGT-3'	+
Hm1	+2140 to +2109 (+2123)	5'-AGACACAACTG TCCCCAaAGGG CAAATGGAGT-3'	+
A* (proximal Olf-1 site)	−192 to −163	5'-ACCCATGCTCTGG TCCCCAAGGAG CCTGTC-3'	+++
A*m1	−192 to −163 (−179)	5'-ACCCATGCTCTGa TCCCCAAGGAG CCTGTC-3'	+++
A*m2	−192 to −163 (−175-6)	5'-ACCCATGCTCTGG TCagCAAGGAG CCTGTC-3'	−
A*m3	−192 to −163 (−178)	5'-ACCCATGCTCTGG aCCCCAAGGAG CCTGTC-3'	+

$$\text{TCCCC}_T^A\text{NGGAG}^e$$

I (UBE)	−563 to −592	5'-AGATGGCTGGCTCGGCTCCCAGAGGCTGTG-3'	—[f]

[a] In some experiments oligonucleotide A was replaced by oligonucleotide A* (see text).

[b] Numbers indicate positions of the sequence within the OMP gene that corresponds to the indicated strand of the double-stranded oligonucleotides used in EMSA experiments. Locations of mutated residues are shown in brackets.

[c] Double-underlined capital letters indicate residues which differ from the consensus sequence of the Olf-1-binding motif. Double-underlined lowercase letters indicate mutated residues. The GGAG element of the Olf-1-binding site that is repeated in the oligonucleotides H and Hm1 is indicated with a single underline.

[d] +++, strong Olf-1-specific binding; +, weak Olf-1-specific binding; −, no binding (Fig. 4).

[e] Consensus sequence of the Olf-1-binding motif.

[f] Oligonucleotide I (UBE) does not show Olf-1-specific binding; however, it interacts with other nuclear proteins from olfactory neuroepithelium and other tissues (Fig. 3).

antisense strand; (xiii) I, sense strand, nucleotides −590 to −561, and antisense strand, nucleotides −592 to −563.

DNase I footprinting analyses. DNase I footprinting analyses were performed with a Hotfoot Footprinting Kit (Stratagene). In order to prepare a sense probe for footprinting of the proximal Olf-1-binding site, the F-2 fragment (nucleotides −239 to −124, see Electrophoretic mobility shift assays) was subcloned in pBluescript (Stratagene), excised from the vector with KpnI and XbaI restriction endonucleases, and ^{32}P labeled at the XbaI site. The antisense probe was prepared by amplifying a fragment of the OMP gene spanning nucleotides −251 to +55, digestion of the PCR product with restriction endonucleases HindIII and PvuII, and subcloning the resulting fragment (nucleotides −239 to +40) into pSP73 (Promega). This clone was cleaved with BglI (position −66) and BglII (site in the polylinker), and the antisense probe was labeled at the BglII site. In order to footprint the distal Olf-1 site, the OMP gene fragment spanning nucleotides −810 to −534 was amplified by PCR and subcloned into PCR 1000 vector (Invitrogen). Sequence of the 5' primer used in this amplification (5'-AGCTCTCAA ATCCCAATCATC-3') is given in full, since only part of this OMP promoter region was previously reported (9). The subcloned DNA fragment was then excised in separate reactions by KpnI and EcoRI or SpeI and EcoRI restriction endonucleases. The KpnI-EcoRI fragment was ^{32}P labeled at the EcoRI site and used as a sense strand probe. The SpeI-EcoRI fragment, which was used as antisense probe, was ^{32}P labeled at the SpeI site after the EcoRI site had been filled in with cold dATP and dTTP by the large fragment of E. coli DNA polymerase II (Klenow). The sense strand probe for footprinting of the initiation region was prepared by PstI-XhoI excision of the HindIII-PvuII fragment subcloned into pSP73 (see above) and labeling it with ^{32}P at the

XhoI site. The antisense strand probe for footprinting of the OMP gene initiation region was created by PCR amplification of an OMP gene fragment, which contains sequences adjacent to the transcriptional start site (nucleotides −133 to +55), with 5' primer in which nucleotides corresponding to the OMP gene residues −121 and −122 were modified to create an EcoRI site at position −124. The PCR product was then digested by EcoRI and PvuII restriction endonucleases and subcloned into pSP73. This clone was cleaved with BglII (site in polylinker) and PvuII (position +40) and ^{32}P labeled at the BglII site. Probes for DNase I footprinting were labeled by Superscript mouse mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories) with appropriate [α-^{32}P]deoxynucleoside triphosphates. All protein extracts used were from 3-week-old rats or mice except where noted. The footprinting reactions were electrophoresed on 6 or 8% sequencing polyacrylamide gels and analyzed by autoradiography.

Transgenic mice. The OMP-lacZ transgene was constructed as follows. The HindIII-NcoI restriction fragment of the OMP gene (nucleotides −239 to +55) was subcloned into pSDKlacZpA, generously provided by S. Potter, to place the OMP gene fragment directly in front of the bacterial β-galactosidase (lacZ) coding region, which is followed by a simian virus 40 polyadenylation signal. For injections, the transgene was excised from the vector by digestion with HindIII and BamHI and was purified by agarose gel electrophoresis. The DNA was dissolved in 10 mM Tris-HCl (pH 7.5) and 0.2 mM EDTA at a concentration of 10 μg/ml and microinjected into fertilized oocytes of B6C3 F1 mice as previously described (9, 22). Transgenic mice were identified by PCR analysis of tail tip DNA with synthetic oligonucleotides essentially as previously described (7). The conditions for DNA amplification were as described above (see PCRs).

Exogenous β-galactosidase activity was quantified by a solid-phase enzyme-linked immunosorbent assay with a mouse anti-β-galactosidase monoclonal antibody (Boehringer) that specifically recognizes the bacterial but not the murine enzyme with CPRG (chlorophenol red-β-D-galactopyranoside SQ) as a substrate (14).

RESULTS

Identification of putative regulatory *cis* elements within the OMP promoter. Two elements that interact with an olfactory neuron-specific factor(s) have been identified within the OMP promoter by EMSA and DNase I footprinting analyses. Initial EMSA experiments indicated that a fragment of the OMP promoter located between nucleotides −239 and −124 (fragment F-2 [see Fig. 1A and Materials and Methods]) upstream of the transcription start site formed a complex with a component(s) of the nuclear protein extract prepared from rat olfactory epithelium. The extent of nucleotide sequence involved in this binding was further defined by EMSA analysis of several ^{32}P-labeled DNA fragments derived from this OMP promoter region. The probes were prepared by restriction enzyme digestion of a PCR-amplified DNA fragment (nucleotides −251 to −110 [see Materials and Methods]) either directly after its synthesis or following cleavage with *Hin*dIII and *Eco*RI (nucleotides −239 to −124 [see Materials and Methods]). The results of these experiments are summarized in Fig. 1A. A bandshift identical to that observed in the initial experiments was present when probe F-7, F-8, or F-9 was used. Formation of this complex was completely eliminated by prior digestion of the F-2 fragment with either *Ava*II or *Hph*I restriction endonucleases (fragments F-3 through F-6). These data demonstrate that the DNA sequence that binds with the component(s) of the olfactory neuroepithelium nuclear protein extract is located within the 36-bp *Nla*III-*Mnl*I restriction endonuclease fragment (F-9) of the OMP promoter.

The specificity of this DNA-protein interaction was evaluated by EMSAs performed with nuclear protein extracts prepared from a variety of tissues and in the presence of specific and nonspecific competitors (Fig. 1B). Preincubation of the olfactory epithelium nuclear protein extract with a 20-fold molar excess of the unlabeled F-2 fragment competitively inhibited binding with the ^{32}P-labeled F-8 probe. In contrast, neither the fragment F-1 nor a fragment of β-lactamase cDNA (Fig. 1A and Materials and Methods) exhibited such competition with F-8. Identical results were obtained when F-2 and β-lactamase cDNA fragments were used in competition with the ^{32}P-labeled F-9 probe (data not shown). The specific bandshift observed in the presence of a nuclear protein extract prepared from olfactory neuroepithelium was not detected with protein extracts prepared from cerebellum, cerebral hemispheres, thymus, lungs, or liver (Fig. 1B) or with heat-inactivated olfactory neuroepithelium extract (data not shown). Thus, this binding must involve specific interactions between a nuclear protein(s) present in olfactory neuroepithelium and a specific nucleotide sequence motif. A more slowly migrating complex of lower intensity was also formed between F-8, and other OMP gene fragments containing this binding site, and nuclear proteins from the cerebellum (Fig. 1B, and data not shown). However, this interaction was reduced when 10 μg instead of 2 μg of poly(dI-dC) was used in the binding reactions, and it was not inhibited by preincubation of the probe with either the specific (oligonucleotide A; Materials and Methods and Table 1) or the nonspecific (double-stranded oligonucleotide,
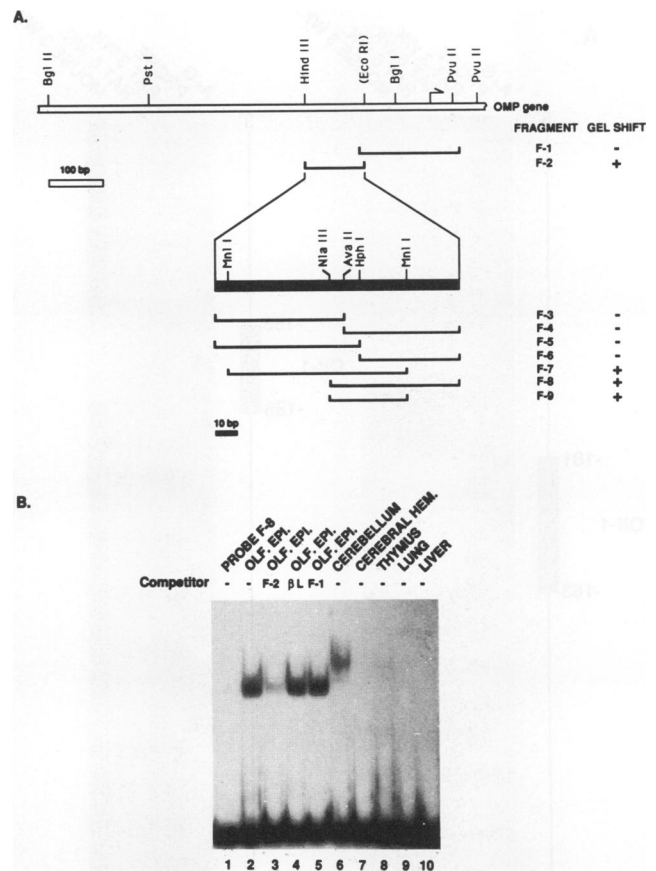

FIG. 1. Identification of a region within the OMP promoter that specifically binds with the olfactory neuron-specific nuclear factor Olf-1. (A) Summary of the results of the initial EMSA experiments. The restriction enzyme map of a portion of the OMP gene is shown at the top. Only selected restriction sites are shown. The *Eco*RI site was created in fragment F-2 by PCR and is shown in parentheses. The arrow represents the transcription initiation site. DNA fragments that were used as probes and/or competitors in EMSAs are identified as brackets. Shaded bar represents an OMP gene region that was first identified as binding to Olf-1 (nucleotides −239 to −124). Binding activity of the radiolabeled DNA fragments F-1 through F-9 in the presence of nuclear proteins from olfactory neuroepithelium is shown on the right. +, Olf-1-specific shift; −, no Olf-1 binding. EMSA analysis of one of these fragments is shown in panel B. The F-8 (*Nla*III-*Eco*RI) fragment was analyzed in the presence of 5 μg of nuclear protein-enriched extract from olfactory neuroepithelium (lanes 2 to 5) and from other neural (lanes 6 and 7) and nonneural tissues (lanes 8 to 10). The specificity of the DNA-protein interaction was determined in the presence of at least a 10-fold molar excess of competitors that are specific (F-2, lane 3) or nonspecific (F-1, lane 4; βL, fragment of the β-lactamase coding region, lane 5) for Olf-1 binding. Migration of the free probe is also shown (PROBE F-8). For details on the preparation of probes and competitors, see Materials and Methods.

nucleotides −50 to −9) competitors (data not shown), indicating that it is unrelated to the olfactory neuron-specific complex.

The nucleotide sequence of the binding site was characterized by DNase I footprinting experiments. Nucleotides −181 to −163 of the sense strand and nucleotides −186 to −166 of the antisense strand were protected from DNase I digestion in the presence of nuclear protein extracts from rat olfactory epithelia. Slightly larger footprints of the same
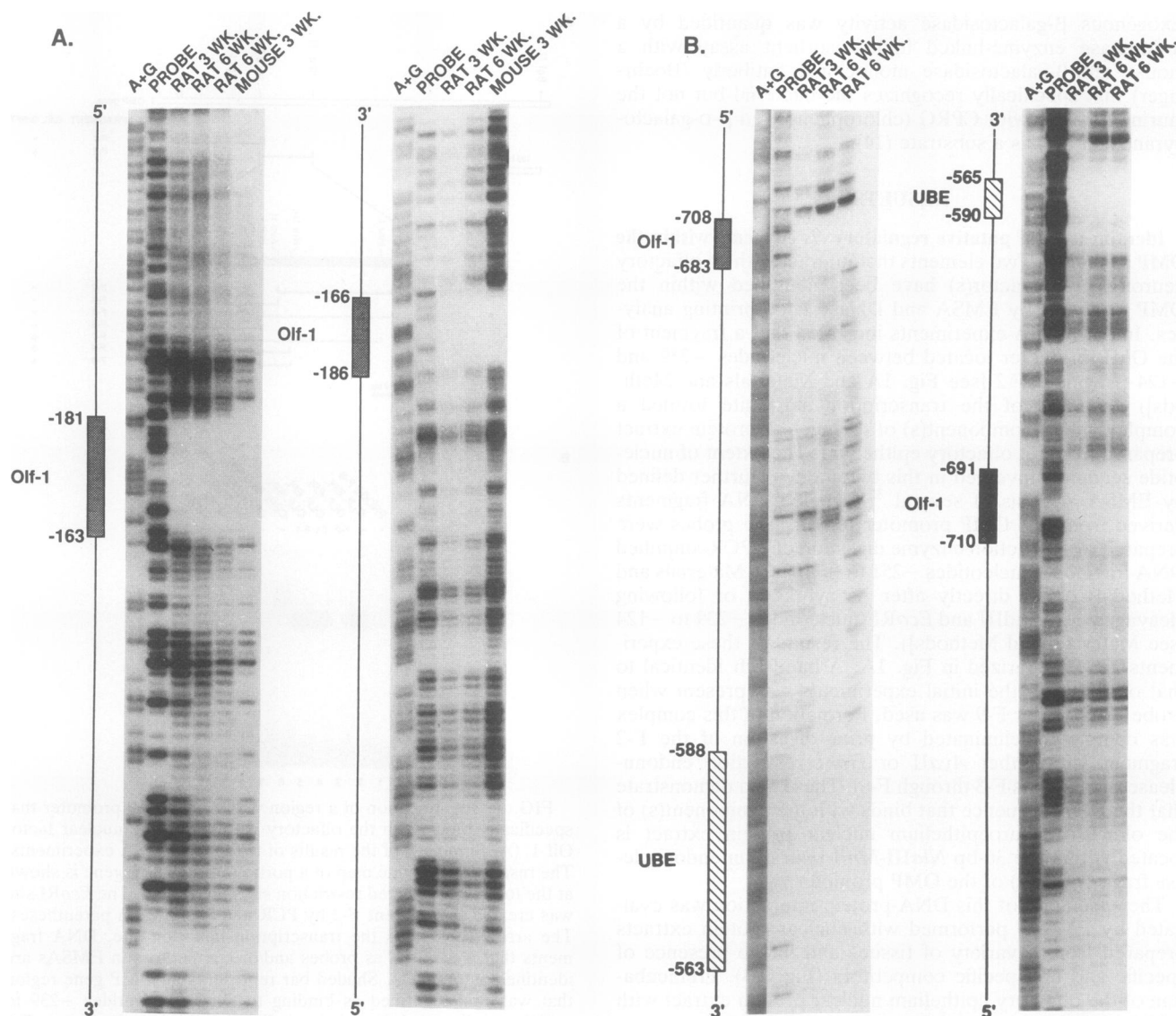
FIG. 2. DNase I footprint analysis of the Olf-1 and the UBE sequence motifs. The OMP 5' flanking region was analyzed by DNase I footprinting with 25 μg of nuclear protein extract from olfactory neuroepithelia of 3- and 6-week-old rats and 20 μg of nuclear protein extract from olfactory neuroepithelia of 3-week-old mice. Shaded bars outline regions protected by interaction with Olf-1. Striped bars outline UBE. Positions of binding sites relative to the transcription initiation start site in the OMP gene are indicated by numbers. Probes treated with DNase I in the absence of any protein extract are shown in lanes labeled PROBE. Chemically cleaved probes (A+G reactions [41]) were used as sequence markers (A+G). (A) Olf-1 binding to its proximal binding site on antisense (left) and sense (right) strands. (B) Olf-1 binding to its distal binding site on antisense (left) and sense (right) strands. Footprints of UBE are also shown.

region were obtained with mouse olfactory nuclear protein extract (Fig. 2A). Over 2.3 kb of the 5' flanking region of the rat OMP gene (9, 64) was searched for similarities with the protected sequence, and several regions with some degree of homology were identified. One such region, located approximately 700 bp upstream of the OMP gene transcription initiation site, contains the sequence 5'-CTCCCAGGGGA GG-3', in which 9 of 13 nucleotides are complementary to the motif 5'-GGTCCCCAAGGAG-3' that forms the core of the footprint described above (see Fig. 8B and Table 1). This region was analyzed by DNase I footprinting with the same protein extracts as used before. A prominent footprint that includes the 5'-CTCCCAGGGGAG-3' sequence was observed between nucleotides −708 to −683 on the sense strand and nucleotides −710 to −691 on the antisense

strand. Since further analysis showed that both these sequences apparently bind the same component that is present only in olfactory nuclear extracts, we have named these sites the proximal (sense strand, nucleotides −181 to −163) and distal (sense strand, nucleotides −708 to −683) Olf-1-binding sites (see the next section).

An additional, unrelated footprint was observed between nucleotides −588 to −563 on the sense strand and nucleotides −590 to −565 on the antisense strand (Fig. 2B) and will be referred to as the upstream binding element (UBE). The nucleotide sequences of all three footprints (see Fig. 8B) were compared with sequences of cis-acting elements present in various data banks, but no exact matches were found (see Discussion).

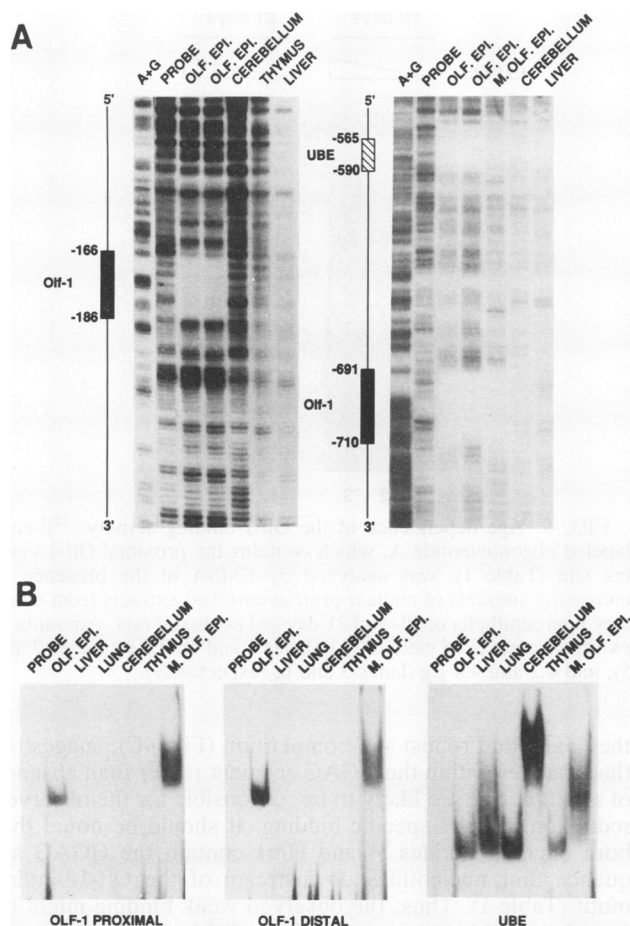Tissue specificity of the Olf-1 and UBE motifs. The protein-

FIG. 3. Tissue specificity of the Olf-1 and the UBE sequence motifs. (A) DNase I footprint analyses of ^{32}P-labeled sense strand probes containing the proximal (left) and distal (right) Olf-1-binding sites in the presence of 25 μg of nuclear protein extract from neuroepithelia (OLF. EPI.), cerebellum, and thymus, 19 μg of liver extract of 3-week postnatal rat, and 16 μg of nuclear protein extract from olfactory neuroepithelia of 3-week-old mice (M. OLF. EPI.). Positions of binding sites relative to the transcription initiation site in the OMP gene are indicated by numbers. Chemically cleaved footprinting probes (A+G reactions [41]) were used as sequence markers (A+G). Probes treated with DNase I in the absence of protein extract are also shown (PROBE). (B) Tissue-specific binding of nuclear proteins with ^{32}P-end-labeled oligonucleotides, which contain the proximal Olf-1-binding site (oligonucleotide A* [left panel]), the distal Olf-1-binding site (oligonucleotide B [central panel]), and the UBE (oligonucleotide I [right panel; see Materials and Methods and Table 1]) were examined by EMSA in the presence of 2.6 μg of nuclear protein-enriched extracts from 3-week-old rat olfactory neuroepithelia (OLF. EPI.), 9.2 μg of liver, 1.9 μg of lung, 7.5 μg of cerebellum, and 7 μg of thymus extracts, and 4 μg of protein extract from olfactory neuroepithelia of 3-week-old mice (M. OLF. EPI.). Binding reactions performed in the absence of protein extract are shown in lanes labeled PROBE.

binding regions of the OMP promoter identified by footprinting (see above) were analyzed for their abilities to interact with nuclear proteins from a number of neuronal and non-neuronal tissues by DNase I footprinting and EMSAs (Fig. 3, and data not shown). The footprints of the proximal and distal Olf-1-binding sites were observed only in the presence of nuclear extracts from olfactory neuroepithelia. Furthermore, synthetic double-stranded oligonucleotides A and B

representing the proximal and distal sites, respectively (see Materials and Methods and Table 1), bound nuclear proteins derived only from olfactory neuroepithelium (Fig. 3B, left and central panel). Each oligonucleotide formed complexes with nuclear proteins from rat olfactory tissue that migrated at the same rate on mobility shift gels; in the presence of proteins from mouse olfactory neuroepithelium, these oligonucleotides formed complexes identical to each other but slightly larger than those observed with the rat nuclear proteins (Fig. 3B, left and central panels). This finding is consistent with the results of the DNase I footprinting analysis of the proximal binding region, in which a larger footprint was observed in the presence of mouse olfactory nuclear extract (Fig. 2A and 3A). Identical results were obtained in multiple independent experiments. They represent the first example of molecular analysis of an interaction between the promoter region of an olfactory neuron-specific gene with a putative tissue-specific regulatory nuclear protein(s) from olfactory neuroepithelium. We have named the olfactory neuron-specific factor (or complex of factors) Olf-1.

In contrast to the tissue specificity of the Olf-1-binding site, the UBE interacted with nuclear protein extracts from every tissue tested. The boundaries of the protected areas differed slightly when extracts from various tissues were used in DNase I footprinting (Fig. 3A, right panel, and data not shown). Consistent with this observation, the complexes formed between a double-stranded oligonucleotide spanning this region (oligonucleotide I [see Materials and Methods and Table 1]) and nuclear proteins from various tissues migrated with different rates on the mobility shift gels (Fig. 3B, right panel). These results indicate that the properties of the factors binding to this segment of the OMP promoter are different in various tissues. The UBE sequence exhibits partial homology with the Olf-1 consensus motif (Table 1). However, since interaction between UBE and a component(s) of the olfactory neuroepithelium nuclear extract is not inhibited in the presence of a double-stranded oligonucleotide competitor containing the Olf-1-binding site, and interaction between Olf-1 and its binding site is not inhibited in the presence of competitors containing the UBE (data not shown), it is clear that the factor(s) binding to UBE is distinct from Olf-1.

Analysis of the Olf-1-binding site. The consensus sequence of the proximal and distal Olf-1-binding sites has been defined by analysis of Olf-1 binding with double-stranded synthetic oligonucleotides containing nucleotide substitutions within the Olf-1 sites and with sequences resembling the Olf-1-binding site that occur elsewhere within the OMP gene (see Table 1 and Materials and Methods). The 13-nucleotide imperfect inverse repeats at the center of the Olf-1-specific footprints consist of the sequence (G/C)(G/C)TCCCC(A/T)(A/G)GGAG (Table 1). Six double-stranded oligonucleotides spanning OMP gene regions that exhibit partial homology to this motif (oligonucleotides C, D, E, F, G, and H) and four double-stranded oligonucleotides with substitutions within the Olf-1-binding sequences (oligonucleotides A*m1, A*m2, A*m3, and Hm1), were examined by EMSA for their ability to bind Olf-1 and to compete for its binding with oligonucleotides which contain the proximal Olf-1-binding site (oligonucleotides A and A*). Results of these experiments are presented in Fig. 4 and summarized in Table 1. Five oligonucleotides carrying sites partially homologous to the Olf-1 motif (oligonucleotides C, D, E, F, and G) were incapable of either binding to or competing for the olfactory neuron-specific factor (Fig. 4B, and data not
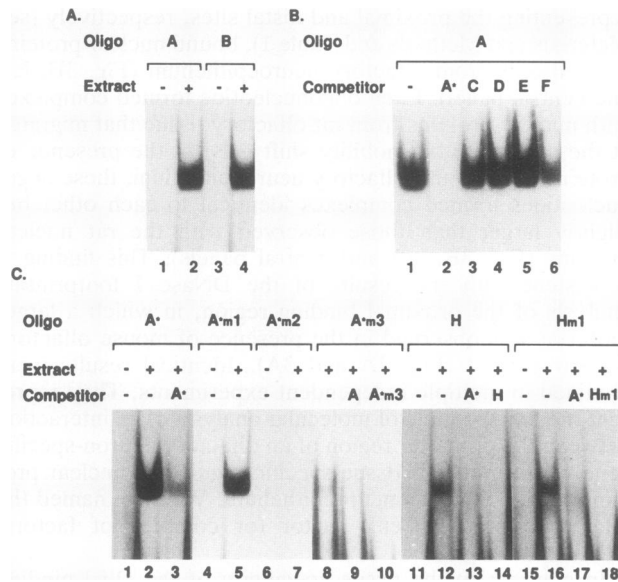
FIG. 4. Identification of a consensus sequence of the Olf-1-binding motifs. Double-stranded oligonucleotides used as probes and competitors in EMSA analyses are described in Table 1 and Materials and Methods. (A) Olf-1-specific binding activity of the proximal and distal Olf-1 sites (oligonucleotides A and B, respectively) in the presence (lanes 2 and 4) and in the absence (lanes 1 and 3) of 5.6 μg of nuclear protein extract from olfactory neuroepithelium. (B) Olf-1-binding motif (oligonucleotide A) in the presence of a 200-fold molar excess of competitors carrying OMP gene sequences partially homologous with the Olf-1 motif (C, D, E, and F, lanes 3, 4, 5, and 6, respectively). Self-competition of the proximal Olf-1 site is shown in lane 2. No competitor was used in reaction 1 (−). The binding reactions were performed in the presence of 5.5 μg of nuclear protein-enriched extract from olfactory neuroepithelium. (C) Oligonucleotides carrying nucleotide substitutions within the Olf-1 motif (A*m1, A*m2, and A*m3) and the downstream Olf-1-like-binding site (oligonucleotide H) containing altered sequence (oligonucleotide Hm1). The EMSAs were performed in the presence (+) or in the absence (−) of nuclear protein-enriched olfactory neuroepithelium extract (2.6 μg, lanes 2 and 3, 12 to 14, and 16 to 18; 5.6 μg, lanes 5 and 7 to 10) and 200 ng of the following competitors: oligonucleotide A* (lanes 3, 13, and 17); oligonucleotide A (lane 9); oligonucleotide A*m3 (lane 10); oligonucleotide H (lane 14); and oligonucleotide Hm1 (lane 18). Reactions performed in the absence of protein are shown in lanes 1, 4, 6, 8, 11, and 14.

FIG. 5. Age dependence of the Olf-1-binding activity. ^{32}P-end-labeled oligonucleotide A, which contains the proximal Olf-1-binding site (Table 1), was analyzed by EMSA in the presence of increasing amounts of nuclear protein-enriched extracts from olfactory neuroepithelia of 10- and 21-day-old postnatal rats. Amounts of extract protein used were 0.9 μg (lanes 1 and 4), 2.2 μg (lanes 2 and 5), and 4.2 and 4.4 μg (lanes 3 and 6, respectively).

shown). In all these oligonucleotides, at least two purines within the GGAG-segment of the Olf-1-binding site are replaced by pyrimidines. Purine substitutions within this segment (oligonucleotide H) weaken but do not eliminate the Olf-1 binding (Fig. 4C). This result was confirmed by DNase I footprinting of the OMP gene fragment containing the sequence of oligonucleotide H, which showed weak protection of the Olf-1-like motif (data not shown). Since the GGAG sequence is preceded by another purine in both the proximal and distal Olf-1-binding sites, we have considered the possibility that the weak Olf-1 binding with oligonucleotide H was due to the presence of a pyrimidine at the position of that purine and that Olf-1 binding requires the presence of a purine pentamer rather than their exact sequence. However, oligonucleotide Hm1, which contains a purine pentamer without the GGAG sequence, bound Olf-1 as weakly as did oligonucleotide H. Both oligonucleotides H and Hm1 were capable of only weak competition with the proximal Olf-1-binding site (oligonucleotide A*), although
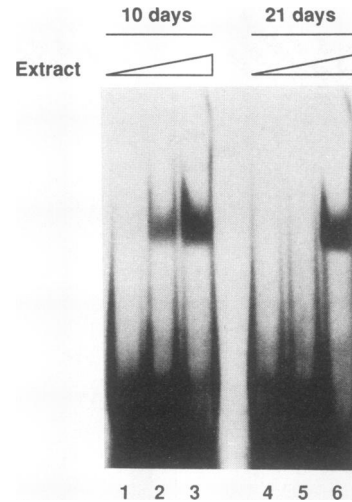
they exhibited robust self-competition (Fig. 4C), suggesting that changes within the GGAG segment rather than absence of a fifth purine are likely to be responsible for the observed reduction in Olf-1-specific binding. It should be noted that both oligonucleotides H and Hm1 contain the GGAG sequence four nucleotides downstream of the Olf-1-binding motif (Table 1). Thus, the observed weak binding might be associated with interactions of the Olf-1 components with the TCCCC element and the downstream GGAG element. However, this possibility seems to be unlikely, since no footprint covering the downstream GGAG element was detected. Next, we have examined the importance of other nucleotides of the Olf-1-binding motif (Fig. 4C). Although replacement of a single residue preceding the TCCCC segment from G to A had no effect on Olf-1-specific binding (oligonucleotide A*m1), substitution of the T within this sequence by A (oligonucleotide A*m3) significantly reduced the ability of the altered site to bind Olf-1 and to compete for its binding with the intact Olf-1-binding site. Olf-1-specific binding was completely abolished when the third and fourth residues within the TCCCC segment were replaced by A and G (oligonucleotide A*m2). On the basis of these results we define the consensus sequence for Olf-1 binding as TCCC C(A/T)NGGAG.

Age dependence of the Olf-1-binding activity. In olfactory tissue OMP is expressed exclusively in mature olfactory receptor neurons (for a review, see reference 39) and appears 1 week after precursor cell mitosis (43). One day after birth, neurons expressing OMP are confined to a single layer in the olfactory neuroepithelium. The thickness of the cell layer expressing OMP increases progressively until by 3.5 weeks of age most of the cells of the neuroepithelium express OMP except for those in the basal cell region (67). To identify a possible correlation between the Olf-1-binding activity and the expression of OMP at different stages of olfactory neuron maturation we have measured Olf-1-binding activity of nuclear protein extracts prepared from olfactory neuroepithelium of 10-day-, 21-day-, and 6-week-old rats (Fig. 2 and 5, and data not shown).
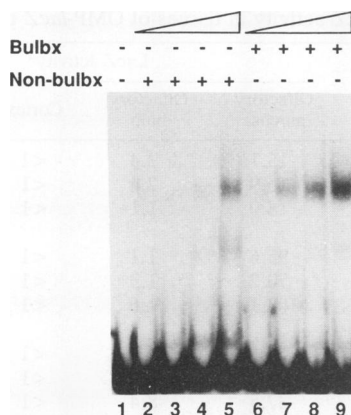
FIG. 6. EMSA analysis of Olf-1-binding activity in olfactory neuroepithelium of bulbectomized rats. Rats were olfactory bulbectomized at 4 weeks of age and sacrificed 4 days after surgery, and their olfactory neuroepithelia were used to prepare nuclear protein-enriched extracts (see Materials and Methods). A ^{32}P-end-labeled PCR-amplified fragment of the OMP promoter (nucleotides −241 to −113), which contains the proximal Olf-1-binding site (see Materials and Methods), was incubated with increasing amounts of protein extracts from bulbectomized animals (Bulbx) and from age-matched littermate controls (Non-bulbx). Amounts of extract protein used were 3 μg (lanes 2 and 6), 5 μg (lanes 3 and 7), 10 μg (lanes 4 and 8), and 20 μg (lanes 5 and 9). Migration of free probe is also shown (lane 1).

Multiple EMSA titration experiments with several independently prepared nuclear protein extracts from olfactory neuroepithelia of 10- and 21-day-old rats demonstrate greater binding activity of Olf-1 in the tissues of younger animals (Fig. 5). Consistent with this finding are results of DNase I footprint titration which demonstrate that Olf-1-binding activity is approximately twofold higher in extracts of younger animals (data not shown). In contrast, we did not detect decreases in binding activity of nuclear proteins present in extracts from younger and older animals when oligonucleotides containing the UBE motif (oligonucleotide I [Table 1]) or carrying the binding sites for NF-κB, AP1, or Oct-1 (Hotfoot Footprinting Kit) were used as probes. To confirm the finding that Olf-1-binding activity was higher in olfactory tissues enriched in immature olfactory neurons, we also measured its activity in actively regenerating olfactory tissues of animals that had undergone bilateral olfactory bulbectomy. In two separate experiments olfactory neuroepithelia of 4-week-old surgically treated rats were allowed to regenerate for periods of 4 or 14 days and then were used for preparation of nuclear protein extracts (see Materials and Methods). The binding activity of Olf-1 in these extracts was tested by EMSA experiments, using as a probe a PCR-amplified fragment of the OMP gene promoter (nucleotides −241 to −113) which contains the proximal Olf-1-binding site, and compared with the binding activity of nuclear protein extracts prepared from olfactory tissues of age-matched control rats. The Olf-1 activity of a nuclear protein extract from rats sacrificed 4 days after bulbectomy was greater than that of the extract obtained from untreated age-matched control animals (Fig. 6) or from rats whose neuroepithelium was allowed to regenerate for 2 weeks (data not shown).

Footprint analysis of the transcription initiation region. Although the 5′ flanking region of the OMP gene does not contain canonical TATA or CCAAT boxes, it is GC rich and

contains several GGCGGG motifs (9). There are three imperfect Sp1-binding sites (15) within the first 100 nucleotides upstream of the transcription initiation site. These sites are located at positions −54, −66, and −79 (see Fig. 8B). In addition, a pyrimidine-rich sequence 5′-GATTCCCT-3′ that resembles the initiator (Inr) consensus sequence (54) occurs at position +1. In order to identify DNA-protein interactions occurring within the region adjacent to the OMP transcriptional start site and to test whether any of the identified initiator or Sp1 motifs are involved in the binding with nuclear proteins from the olfactory neuroepithelium, we have analyzed the OMP gene fragment spanning nucleotides −239 to +41 by DNase I footprinting. Several protected areas stretching across 83 nucleotides (position −81 to +2) were readily visible when olfactory neuroepithelium nuclear protein extracts were used (Fig. 7, left panel); in additional experiments the footprints extended as far as position +14 (data not shown). Furthermore, in the presence of olfactory neuroepithelium nuclear protein extracts a PCR-amplified fragment of the OMP gene spanning nucleotides −113 to +41 forms a complex that migrates as a broad, diffuse band on mobility shift gels (data not shown). The footprint observed between nucleotides −14 to +14 contains the Inr-like motif; those between nucleotides −81 to −72 and −59 to −44 contain sequences that are 89% identical to Sp1-binding sites. The footprint between nucleotides −67 to −64 overlaps a sequence that is 79% identical to an Sp1-binding site. An OMP gene fragment containing all three Sp1 sites (nucleotides −113 to +41) showed a typical Sp1 gel mobility shift pattern with purified Sp1 (25) that was generously provided by S. Jackson (data not shown). This demonstrates that at least one of the potential Sp1 sites residing within the first 100 nucleotides of the OMP 5′ flanking region is capable of binding with authentic Sp1. Despite this finding, we were unable to detect complex formation characteristic for Sp1 binding between a double-stranded oligonucleotide containing the Sp1-binding site (Hotfoot Footprinting Kit) and components of nuclear protein extracts prepared from rat or mouse olfactory neuroepithelium in the mobility shift assay. In contrast, a characteristic Sp1 gel shift was observed when purified Sp1 was added to the olfactory nuclear extract or when nuclear extract prepared from rat cerebellum was used (data not shown).

To determine whether there is any tissue selectivity in binding of nuclear protein to the transcription initiation region, DNase I footprint analyses were carried out in the presence of nuclear protein extracts from several nonolfactory tissues. Experiments with the sense strand probe showed a diffuse pattern of protection spanning nucleotides −84 to −41 when nuclear extracts from thymus or liver were used. This pattern varied slightly with different extract preparations (data not shown). Since this area contains the potential Sp1-binding sites, it is possible that these footprints are due to Sp1 interactions with their binding motifs. A footprint between nucleotides −67 to −87 was readily visible when the radiolabeled antisense probe was tested in the presence of nuclear protein extracts obtained from olfactory neuroepithelium, cerebellum, or thymus but not with those from liver or lung.

Tissue specific *cis*-regulatory elements reside within 0.3 kb upstream of the OMP gene translation start site. To begin assessment of the biological significance of the Olf-1 motifs, we have examined the ability of a fragment of the rat OMP gene spanning nucleotides −239 to +55, where the translation start site is located at position +56 (9), to drive expression of *lacZ* in transgenic mice. In three strains of
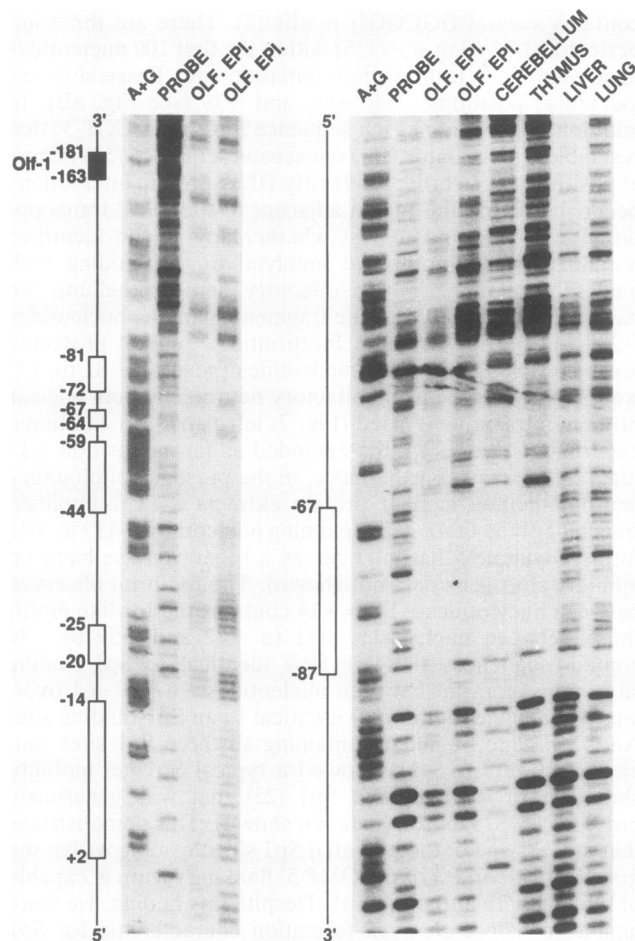
FIG. 7. DNase I footprint analysis of the OMP transcription initiation region. Several areas protected from DNase I digestion (outlined as open boxes) are visible within a stretch of 83 nucleotides adjacent to the transcription initiation site on both the sense (left panel) and antisense strand (right panel) probes (for description of the probes, see Materials and Methods). The shaded box outlines the proximal Olf-1-binding site. Tissue selectivity of nuclear protein(s) binding to the antisense strand is demonstrated on the right panel. Positions of binding sites relative to the transcription initiation start site in the OMP gene are indicated by numbers. DNase I footprint analyses were performed with 25 and 34 μg (right panel, lanes 3 and 4, respectively) of nuclear protein extract from 3-week-old rat neuroepithelia (OLF. EPI.), 25 μg of cerebellum, thymus, and lung extract, and 19 μg of liver extract. Probes treated with DNase I in the absence of any protein extract are also shown (PROBE). Chemically cleaved footprinting probes (A+G reactions [41]) were used as sequence markers (A+G).

TABLE 2. LacZ activity in tissues of OMP-*lacZ* transgenic mice

| Strain | LacZ activity[a] | | | |
	Olfactory mucosa	Olfactory bulb	Cortex	Cerebellum
H-OMP-*lacZ*-1	15.1	2.4	<1	<1
	16.9	2.4	<1	1.7
	8.0	1.1	<1	<1
H-OMP-*lacZ*-3	95.6	1.1	<1	<1
	70.2	1.3	<1	<1
	103.1	1.6	<1	<1
H-OMP-*lacZ*-6	27.7	14.3	<1	<1
	29.7	12.8	<1	<1
	37.5	8.4	<1	<1

[a] Picograms of LacZ detected per milligram of tissue are presented as means of duplicate assays for individual 9-week-old male mice. The observed LacZ activity in control mice is routinely below 1 pg/mg of tissue.

specific regulation of the OMP gene. No expression was observed when a reporter gene was placed under the control of a smaller fragment of the OMP promoter from which the Olf-1 motif was deleted (17). More-detailed analysis of the biological role of the Olf-1 motif is currently under investigation.

DISCUSSION

We have identified several sequence motifs within 800 bp of the 5' flanking region of the OMP gene that may be involved in tissue- and development-specific transcriptional regulation of OMP expression (Fig. 8). Two of the identified sequences are located approximately 0.5 kb apart and share a common motif that binds a novel, olfactory neuron-specific factor(s), Olf-1. Another region involved in binding of nuclear proteins, the UBE, interacts with components of nuclear extracts from all tissues that we have tested. We have also identified several areas adjacent to the transcription initiation start site that are protected from DNase I digestion in the presence of nuclear proteins of the olfactory neuroepithelium. One of these exhibits tissue selectivity in binding nuclear proteins derived from some but not all nonolfactory tissues.

One possible role of Olf-1 is to act as a part of the mechanism that restricts OMP expression to the mature receptor neurons of the olfactory epithelium. Both the proximal and the distal Olf-1-binding sites reside within the 0.8-kb fragment of the OMP gene 5' flanking region (Fig. 2 and 8), which must contain elements responsible for tissue-specific OMP expression since, like the 5.2-kb upstream fragment (9), it was shown to be capable of directing the expression of a reporter gene to olfactory neurons in transgenic mice (17, 63). Furthermore, our data indicate that an OMP gene fragment that contains 239 bp upstream of the transcription initiation site, including the proximal Olf-1-binding site, is sufficient to confer olfactory tissue-specific expression of a reporter gene in transgenic mice (Table 2). In addition, expression was not observed when a reporter gene was placed under the control of an OMP promoter fragment lacking the Olf-1 motif (17). Interestingly, EMSA experiments with nuclear extracts prepared from olfactory neuro-epithelium at different stages of maturation (Fig. 5 and 6) suggest that Olf-1-binding activity is higher in tissues enriched in immature olfactory neurons. Thus, the expression

mice expressing the OMP-*lacZ* transgene, high levels of exogenous β-galactosidase activity were observed in olfactory neuroepithelium but not in cortex or cerebellum (Table 2). In one of these strains some β-galactosidase activity was detected in the olfactory bulb; however, even in this strain this activity in the olfactory mucosa exceeded that in the olfactory bulb by at least twofold. The β-galactosidase activity observed in the olfactory bulb may reflect variable amounts of the enzyme transported along the olfactory axons. Since the Olf-1 motif is located within the transgene, approximately 180 nucleotides upstream of the OMP gene transcription initiation site, these results are consistent with the hypothesis that this motif is involved in olfactory neuron-
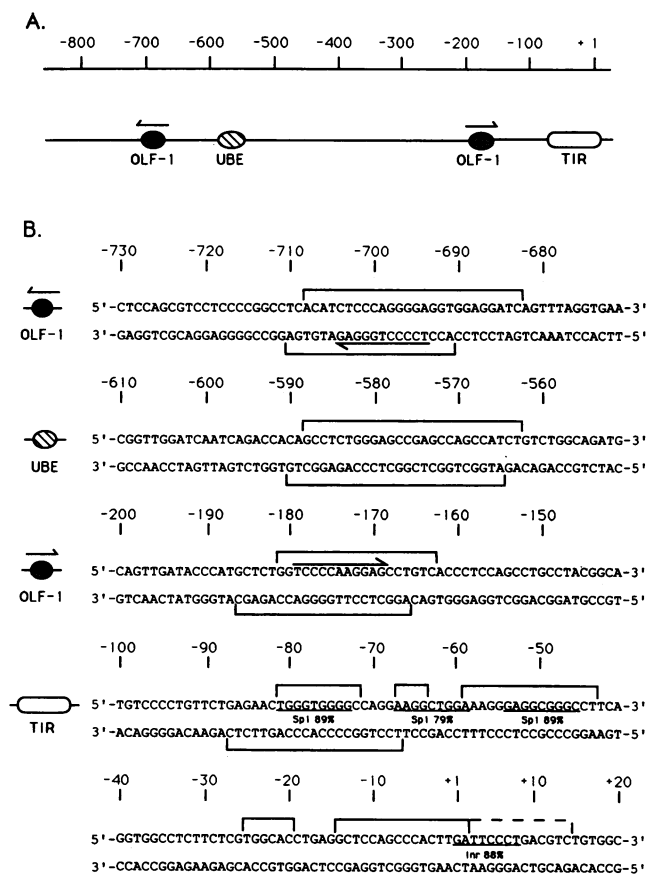
FIG. 8. Putative *cis*-acting elements identified within the OMP gene by EMSA and DNase I footprinting analyses. (A) Diagram of the sequence motifs involved in binding of nuclear proteins relative to the OMP transcription initiation start (+1). The shaded ovals with right- and left-pointing arrows represent proximal and distal Olf-1-binding sites, respectively. The striped oval represents the UBE. The open oval represents regions located in proximity to the transcription initiation start site (TIR). (B) Partial nucleotide sequence of the OMP gene 5' flanking regions that contain the putative *cis*-acting elements. Nucleotides are numbered relative to the transcription start site as indicated above the sequence. The sequences corresponding to DNase I footprints identified on both DNA strands are marked by brackets; the core elements of the Olf-1-binding sites are marked by arrows. The dashed-line bracket indicates a frequently observed extension of a footprint (data not shown). Potential Sp1-binding sites and the pyrimidine-rich initiator-like (Inr) region are underlined on the sense strand and marked accordingly. Similarity between the sites and their respective consensus sequences [Sp1, (G/T)(G/A)GGC(G/T)(G/A)(G/T) (15); Inr motif, YAYTCYYY, where Y denotes a pyrimidine (54)] is given as a percentage of identical residues.

of Olf-1 may itself be developmentally regulated with a time course that differs from that seen for OMP. This suggests that Olf-1 may initially be expressed in a population of cells more immature than those that express OMP. It is conceivable that Olf-1 activity is modified during different stages of olfactory neuronal differentiation by interactions with other transcriptional factors or by posttranslational modifications (3, 24, 35, 36, 50), resulting in suppression of OMP expression in immature cells and activation in fully differentiated olfactory neurons. An example of modifications to *cis*-acting element binding activities during postnatal brain development was recently reported (55).

Immunochemical studies have demonstrated the presence of OMP in olfactory tissues of virtually all vertebrates (for a review, see references 38 and 39). Furthermore, OMP has been immunocytochemically localized to mature olfactory neurons in species as diverse as amphibia (31), marsupials (33), and humans (7, 47). Assuming that Olf-1 functions as a transcriptional regulator of the OMP gene, it is possible that this factor is similarly phylogenetically conserved. Our studies with nuclear extracts from mouse olfactory neuroepithelium demonstrate the presence of Olf-1 activity in this species (Fig. 2 and 3). Comparison of the mouse and rat olfactory extracts on mobility shift gels and by DNase I footprinting indicates that the mouse and rat Olf-1 are very similar but not identical (Fig. 2 and 3). Despite these differences, which may be a result of posttranscriptional modifications, it is likely that these factors play a similar role in both species, since the rat Olf-1 consensus motif is conserved in the OMP gene of both mice and humans (5). Olf-1 may also be involved in the regulation of transcription of other olfactory neuron-specific genes. Although the consensus motif [TCCCC(A/G)NGGAG] of the proximal and distal Olf-1-binding sites reported here is unique compared with sequences of other *cis*-acting elements from various computer data bases, a sequence 90% identical to the Olf-1 motif is present in the 5' flanking region of another olfactory neuron-specific gene, the olfactory cyclic nucleotide-activated channel (52), suggesting that Olf-1 may be of general importance in regulating olfactory neuron-specific gene expression.

Several other neuron-specific genes contain in their 5' flanking regions motifs that exhibit partial homology to the purine tetramer or pyrimidine pentamer of the Olf-1-binding site. These motifs include (i) CCAGGAG, which occurs, for example, in the rat genes encoding GAP-43 (48), type II sodium channel (40), SCG10 (44) and periferin (66); and (ii) CCTTCGCCCCCGC, which is common to the rat and human synapsin I genes (57) and human nerve growth factor receptor gene (59). Another putative *cis*-acting element within the nerve growth factor promoter contains a sequence identical to the first six nucleotides of the Olf-1 motif (13). In vitro studies showed that the CCAGGAG and CCTTCGCC CCCGC motifs are not sufficient by themselves for neuron-specific gene expression (40, 48, 65); however, these sequences, possibly in cooperation with other *cis*-acting elements, might be involved in this process at some stage of neural differentiation. Recently, interactions between two distinct *cis*-acting motifs (AP-1 and E box) were shown to be responsible for neuron-specific expression of the tyrosine hydroxylase gene (69). Thus, one could speculate that the factors that interact with sequences showing partial homology with the Olf-1-binding motif may be components of the Olf-1 factor that are responsible for ensuring general expression of neuron-specific genes.

Another sequence within the 5' flanking region of the OMP gene that binds to nuclear proteins and may be involved in regulation of OMP gene expression is UBE. In contrast to Olf-1, UBE interacts with components of nuclear extracts from a variety of neural and nonneural tissues, forming DNA-protein complexes whose mobilities vary with the tissue origin of the nuclear extract used in the binding reaction (Fig. 3B). This indicates that the factors from various tissues that interact with UBE are not identical. Although the UBE sequence exhibits partial homology with the Olf-1 motif, the EMSA competition experiments demonstrate that factors binding to UBE are different from Olf-1.

The function of UBE in the regulation of OMP gene expression will be determined by future investigations.

The OMP gene promoter does not contain a canonical TATA box within 2 kb of its 5' flanking region (9, 64). It has been shown in recent years that transcriptional initiation in TATA-less genes can be activated through initiator sequences, which typically contain the transcription initiation site (51, 54, 62, 70). A few factors that bind to the initiator sequence and support basal transcription have been isolated (54, 60). Interestingly, an Inr-like sequence motif was identified at position +1 in the OMP gene (Fig. 8). This site is also conserved in the sequence of the mouse OMP gene (5). Furthermore, our footprinting analysis demonstrated that the Inr-like sequence in the rat OMP gene is protected from DNase I digestion by the presence of olfactory nuclear protein extracts. Transcription initiation from an Inr element can be mediated by interactions of the TFIID complex with the Sp1 factor (51, 61). The first 100 bp of the 5' flanking region of the OMP gene contains Sp1-like-binding sites that are at least partially protected from DNase I digestion in the presence of protein extracts from olfactory neuroepithelium (Fig. 7 and 8). EMSA experiments demonstrated that at least one of these sites is capable of binding authentic Sp1 (data not shown); however, we did not observe a typical Sp1 gel shift in the presence of nuclear extracts from the olfactory neuroepithelia. Our inability to detect Sp1 activity in the nuclear protein extracts from olfactory neuroepithelia indicates that Sp1 is either absent or present at very low concentrations in this tissue. The latter would be, perhaps, not surprising since the level of expression of this transcription factor varies considerably in different tissues and is reported to be restricted to a few cell types in the mammalian brain. For example, Sp1 was detected in the human brain only in glial cells (19) and in the mouse nervous system in oligodendrocytes and motor neurons (10). Therefore, the DNA-protein interactions detected by the DNase I footprinting analysis within the transcription initiation region might be attributed to binding with factors other than endogenous Sp1, such as, for example, ETF, which binds to various GC-rich regions and stimulates transcription from TATA-less promoters (28). The ability of brain-specific factors to bind to sequence motifs that are recognized by other transcriptional regulators has been described previously (29). Interestingly, our footprinting data indicate that the area containing two of the potential Sp1-binding sites (Fig. 8, nucleotides −87 to −77) is capable of binding nuclear proteins with some degree of tissue selectivity (Fig. 7).

The sequences that we have identified within the 5' flanking region of the OMP promoter are likely to function as regulatory elements responsible for tissue-specific expression of OMP within the olfactory receptor neurons. Selective gene expression in neuronal cells can be achieved by more than one mechanism. For example, a silencer can impose tissue-specific expression on a relatively neutral core promoter, as in the case of the rat brain type II sodium channel gene (30, 40) and the SCG10 gene (44, 45), or the core promoter can exhibit substantial tissue specificity, as was observed for the GAP-43 gene (48). Both positively and negatively acting promoter elements have been described for an increasing number of neuronal genes including SCG10 (45), neural cell adhesion molecule (21), type II sodium channel (40), β-amyloid precursor protein (32), synapsin I (23), GAP-43 (48), and nerve growth factor (13). The high level of tissue- and development-specific control of OMP gene expression implies a complex pattern of regulation, in which both positive and negative regulatory elements may

be involved. Data presented in this report suggest that the sequence motif interacting with the newly defined olfactory neuron-specific binding factor(s) Olf-1 plays a role in establishing the specific pattern of OMP gene expression. It is conceivable that the *cis*- and *trans*-acting factors involved in DNA–Olf-1 complex formation also participate in regulating transcriptional activation of other olfactory neuron-specific genes.

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