The Journal of Neuroscience, January 1993, 13(1): 300–312

Directed Expression of an Oncogene to the Olfactory Neuronal Lineage in Transgenic Mice

Brian L. Largent,[1] Ronald G. Sosnowski,[2] and Randall R. Reed[3]

[1]Neuroscience Program, Department of Anatomy and Cell Biology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0616, [2]Department of Medicine, University of California at San Diego, La Jolla, California 92093-0636, and [3]The Howard Hughes Medical Institute, Department of Molecular Biology and Genetics and Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The mammalian olfactory system provides a useful model to understand the cellular and molecular mechanisms governing the development of the nervous system. The olfactory neuroepithelium undergoes continual turnover in the adult animal, resulting in a neural tissue containing cells at various stages of neurogenesis. We have generated a transgenic mouse line to examine the effects of directed expression of an oncogene within the olfactory neuronal lineage. A hybrid oncogene was constructed utilizing the regulatory elements for the olfactory marker protein gene to direct the olfactory neuronal-specific expression of simian virus 40 T-antigen, a potent oncogene. The resulting transgenic mouse line expressed T-antigen only in olfactory neurons. Ten-month-old transgenic mice displayed significant hypoplasia of the neuronal elements in the olfactory neuroepithelium. The transgenic mice developed neuroblastomas of olfactory neuronal origin at a low frequency. Distinct clonal lines were derived from the primary culture of the tumor. GAP-43, a growth-associated neuronal marker, was expressed by some of the cell lines. One of the cell lines, 2.2, appeared to be responsive to neurotrophic effects from the presumptive target tissue, the olfactory bulb.

[Key words: transgenic mice, targeted immortalization, oncogene, regeneration, neurodevelopment, olfactory system, olfactory neuron, olfactory neuroblastoma, tumorigenesis, cell culture]

The vertebrate olfactory system is uniquely amenable to studies of neurogenesis and signal transduction. This neural system is distinctive in that it retains, into adulthood of the animal, a stem cell population with the capacity to replenish primary sensory neurons. A continual turnover of the olfactory neuronal cell population is sustained by stem cell progeny that differentiate into functional olfactory neurons—replacing olfactory neurons that have degenerated and reestablishing synaptic connections within the CNS (Graziadei and Monti Graziadei, 1979, 1985). This process recapitulates many aspects of the neurodevelopmental process itself, that is, the commitment and maturation of neuroblasts to specific neuronal phenotypes, migration of neuronal precursors, axon extension to specific targets, and synapse formation with appropriate neuronal partners. The olfactory neuroepithelium has a relatively simple cellular composition consisting of three principal cell types—olfactory sensory neurons, sustentacular cells (support or glial-like cells), and basal cells (or stem cells). They reside in a pseudostratified columnar neuroepithelium that has well-defined synaptic projections to the olfactory bulb. Within the animal, the olfactory system is morphologically distinct, accessible, and manipulable.

The factors influencing cell proliferation, differentiation, synaptogenesis, and senescence in the olfactory neuroepithelium are mostly unknown. To gain insight into the cellular dynamics of this plastic neuroepithelium, laboratories have examined mitotic activity in the neuroepithelium (Graziadei and Monti Graziadei, 1979; Hinds et al., 1984; Mackay-Sim and Kittel, 1991), analyzed the effects of experimental lesions such as axotomy, target ablation, and naris occlusion (Monti Graziadei and Graziadei, 1979; Farbman et al., 1988; Maruniak et al., 1989; Stahl et al., 1990; Verhaagen et al., 1990), and developed *in vitro* cell culture systems (Goldstein et al., 1986; Calof and Chikaraishi, 1989; Coon et al., 1989; Pixley and Pun, 1990; Ronnett et al., 1991).

As a first step to examine the plastic nature of the olfactory neuroepithelium, we have assessed the consequences of ectopic oncogene expression in the olfactory neuronal lineage. The rationale for this approach was twofold. First, in actively dividing tissues, proto-oncogenic genes are expressed and likely serve a role in the regulation of cellular replication (Bishop, 1983). Perturbation of the neuroepithelium by the genetic targeting of oncogene expression can provide clues about the mechanisms regulating cell turnover and provides an interesting complement to the standard paradigm of negatively affecting the neuronal population by lesions of axons or neuronal targets. Second, transgenic targeting of oncogene expression to the neuronal lineage of the olfactory neuroepithelium may result in immortalized cell lines potentially useful for studies addressing gene expression during development.

Our experimental strategy utilizes germline transformation of mice to target oncogene expression to the olfactory neuronal lineage. A hybrid oncogene was constructed consisting of the early region of simian virus 40 (SV40), encoding the oncogenic large and small T-antigen (T-ag), fused to the cell-specific transcriptional regulatory elements of the rat gene for olfactory marker protein (OMP) (Rogers et al., 1987), a protein of unknown function that is selectively and abundantly expressed within mature

Received Nov. 25, 1991; revised July 16, 1992; accepted July 21, 1992.

We thank Paul Feinstein for expert technical assistance and Keith Peden for the wild-type and mutant SV40 T-antigen DNA and monoclonal antibodies to SV40 T-antigen protein. This work was supported by the NIH and the Howard Hughes Medical Institute. B.L.L. was a Glaxo Fellow of the Life Sciences Research Foundation.

Correspondence should be addressed to Randall R. Reed, Ph.D., at the above address.

olfactory neurons. The *cis*-acting regulatory elements of the OMP transcriptional promoter targeted expression of the oncogenic SV40 T-ag to primary olfactory neurons, thereby altering cell populations in a selective fashion within the transgenic animal. T-ag expression leads to the development of tumors and cells that proliferate in cell culture.

Materials and Methods

Isolation of genomic clones and construction of transgene. A bacteriophage lambda library (λ2001) was constructed with size-fractionated, BamHI-digested rat genomic DNA using standard methods (Berger and Kimmel, 1987; Sambrook et al., 1989). Genomic fragments containing OMP cDNA sequences were identified and mapped with restriction enzymes, and the DNA sequence of relevant regions was determined. A 3 kilobase (kb) fragment corresponding to the putative regulatory region of the OMP gene was joined to the coding region for SV40 large and small T-ag. The 5′ genomic fragment was prepared by a partial NcoI digest (the initiating ATG for the OMP gene is contained within an NcoI restriction site), blunting of the ends, and ligation of EcoRI linkers. This genomic fragment was ligated to the SV40 T-ag coding gene (encoding both large and small T-ag), which was prepared by an StuI digestion [29 base pairs (bp) 5′ of the initiating codon for the SV40 T-ag gene] and ligation of EcoRI linkers. This construct was linearized leaving intact the OMP regulatory region and the polyadenylation signal of the SV40 T-ag gene.

DNA sequencing and primer extension mapping. DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) (Sequenase, U.S. Biochemicals). For primer extension experiments, 25 μg of total RNA was annealed to approximately 5 ng of ^{32}P-labeled oligonucleotide primer. The annealed material was included in a reaction with reverse transcriptase (Muskavitch and Hogness, 1982) and resolved on an 8% polyacrylamide, 8 M urea denaturing gel (0.4 mm) and visualized by autoradiography.

Generation of transgenic mice. Fertilized eggs were harvested from B6D2 females (mated to B6D2 males) and approximately 200–400 copies of the hybrid OMP/SV40 T-ag oncogene were microinjected. Pups were screened at approximately 3 weeks for the presence of transgene sequences into genomic DNA. For screening of founder mice, DNA was extracted from tail tissue and analyzed by Southern blot with SV40 T-ag as a hybridization probe. Routine screening of litters of transgenic mice was accomplished by DNA extraction and a slot blot analysis of the DNA for the presence of SV40 T-ag sequences. During the generation of transgenic lines, the number of transgenic progeny (founder mice) obtained for each of the transgene constructs was similar to that seen for other constructs.

Histology and immunocytochemistry. For histological or immunocytochemical procedures, mice were anesthetized with pentobarbital and perfused with phosphate-buffered saline (PBS) followed by phosphate buffered 4% formaldehyde. Tissues were dissected, fixed by immersion overnight in phosphate buffered 4% formaldehyde, and, after decalcification with formic acid for tissues with bone or cartilage, paraffin imbedded. Sections (10–12 μm) were deparaffinated and rehydrated for staining with hematoxylin and eosin for immunohistochemistry.

OMP immunocytochemistry was performed with a rabbit serum raised against a synthetic 16 amino acid peptide (glutaraldehyde cross-linked to keyhole limpet hemocyanin) corresponding to the carboxyl end of OMP (Rogers et al., 1987). The serum was purified by affinity chromatography on a peptide-conjugated column. SV40 T-ag immunocytochemistry was performed with a mix of monoclonal antibodies (PAb 419, 402, 406, 423) (Harlow et al., 1981) that recognizes both large and small T-ag. Tissue sections were blocked for 20 min with the normal serum, incubated with primary antibody (anti-OMP at 1:400, and anti-T-ag at 1:100) for 2.5 hr at room temperature in standard immunoblot buffer (SIB) (Tris-buffered saline, 0.1% BSA, 0.05% Tween 20), washed 3 × 5 min (SIB, room temperature), incubated with the secondary antibody (AuroProbe LM, colloidal gold conjugated, Janssen) for 1 hr, and washed 3 × 5 min with Tris-buffered saline and 3 × 3 min with deionized water. Silver enhancement was performed according to the manufacturer's recommended procedure (Janssen) with a final wash of 3 × 5 min in deionized water.

Cell culture. Mouse tumor tissue, dissected under sterile conditions, was minced and placed in primary culture at several concentrations (≈15–75 mg tissue/10 cm^2) in Dulbecco's modified Eagle's medium/F12 media [15% fetal bovine serum, 5% horse serum, penicillin (500 U/ml)/streptomycin (500 μg/ml)] at 5% CO_2 and 37°C. Cellular debris was removed after overnight incubation of the primary cultures by washing with media. Cultures were passaged at least once every 7 d. Single-cell cloning was accomplished by plating cells at low density and the use of glass cloning cylinders. Limiting dilution into 96-well plates (Falcon Primaria) (≈0.1 cell/well) was used in some cases for a secondary round of cell cloning. The 2.2 cells required conditioned media for survival during single-cell cloning. Substrate experiments were done on tissue culture plastic coated with various substrates: fibronectin (GIBCO), 20 μg/ml, air dried, washed twice with PBS; laminin (GIBCO), 20 μg/ml in media for 45 min at room temperature; vitrogen (Collagen Corp.), 60 μg/ml water, air dried; poly-D-lysine (GIBCO), 60 μg/ml water, air dried, washed twice with PBS.

RNA isolation and Northern blot analyses. For total RNA isolation (Chirgwin et al., 1979), rat and mouse tissues (from adults between 1 and 8 months of age) were rapidly dissected, frozen in liquid nitrogen or placed on dry ice, and homogenized (Brinkman polytron) in a buffered solution of guanidinium thiocyanate. Tissue culture cells were washed once on the plate with phosphate-buffered saline (PBS) and scraped from the plate in the buffered solution of guanidinium thiocyanate. Total RNA was isolated by centrifugation through a CsCl cushion. PolyA$^+$-enriched RNA was isolated from total RNA by chromatography over oligo(dT)-cellulose (Aviv and Leder, 1972). Northern blot analysis was performed by electrophoresis of glyoxal-denatured RNA in 1% agarose (Sambrook et al., 1989) and transfer to a nylon membrane (Nytran, S&S), and the nylon membrane baked in a vacuum oven at 80°C for 2 hr. Filters were prehybridized and then hybridized [5× saline-sodium phosphate-EDTA (SSPE), 5× Denhardt's solution, 25 μg/ml denatured salmon sperm DNA, 0.5% sodium dodecyl sulfate (SDS), 50% deionized formamide] with probes labeled with α-^{32}P-dCTP. Hybridizations were performed at ≈1 × 10^6 cpm/ml (≈1 × 10^9 dpm/μg probe) for 36 hr at 42°C. Typical washes of membranes hybridized with cognate probes were at 0.2× saline–sodium citrate (SSC), 0.5% SDS for 1 × 20 min at room temperature and 2 × 20 min at 65°C. Lower-stringency washes were at 0.5 or 1× SSC, 0.5% SDS.

Results

Construction of transgene and characterization of transgenic mice

The construction of a transgene required the isolation of the 5′ flanking region of the OMP gene and the associated *cis*-acting regulatory sequences. Southern hybridization analysis of BamHI-digested rat genomic DNA revealed that the 5′ end of the OMP cDNA hybridized to a fragment of 3.8 kb. Restriction analyses indicated that the 5′ fragment contained approximately 3.0 kb of sequence upstream of the initiating methionine for OMP. The DNA sequence of the adjacent 1 kb of 5′ flanking region was determined to identify known regulatory elements (Fig. 1). Examination of the sequence, identical to that previously reported (Danciger et al., 1989), demonstrated an absence of the classical regulatory elements, TATA and CAAT boxes, but revealed a number of GC-rich regions closely resembling binding sites for SP1 (Kadonaga et al., 1987; Kadonaga et al., 1988). A consensus match was found for the 5 bp core sequence (CCAGG) of a putative *cis*-acting regulatory element found in the type II sodium channel gene and several other neuronal specific genes (Maue et al., 1990).

The sites of transcription initiation were determined by primer extension experiments (Fig. 2) and corresponded closely to those previously described (Danciger et al., 1989). Nuclease protection assays corroborated the position of putative transcription start sites identified by primer extension experiments (data not shown). Comparing the primer extension products with sequence reaction products of the same genomic DNA clone identified the nucleotide position of the putative transcription start sites within the cloned DNA (corresponding to 56, 57, 60, and 61 bp upstream of the initiating codon). A consensus sequence found at this site, termed the "initiator

```
-787 TCTCCAGCGTCCTCCCCGGC CTCACATCTCCCAGGGGAGG
-747 TGGAGGATCAGTTTAGGTGA AATACCTGGTAGGATTTTGG
-707 TGGCAGTGAGAGCCAGGCCC ATAGCTATGTGGGGCAGTAT
-667 GCGGTTGGATCAATCAGACC ACAGCCTCTGGGAGCCGAGC
-627 CAGCCATCTGTCTGGCAGAT GATTGGGATTTGAGAGCTGC
-587 AGGTTCAGATGGGAGGTGAC AGCTGGGCTGGGTCCTGGTG
-547 ATGATAAAGGAGAGGGAGAA ACCCAGGGCATCTGACGGGA
-507 CCTGGCAGGGACTTCGAGAG AATGAGGTGGGGAGGAAGCA
-467 ACCATGGTAAGTCGGCTTGG CTGACTACAGAGAACGAATG
-427 TATGCTACTGGTGCTGCCCC TCCCTGCCCCCTCTTCCTGG
-387 GCAGTCTCCAGTTACCTCCA TGTGTCTGTCACCCCACCTG
-347 TCTCCCAACAGCCCTGTGGA GTATTCTGTTCTTCACAAAC
-307 AAGCAAACCTCAAGCTTGCC ACTAGCACTGTAGTCAAGGT
-267 GGTTGCCACAGCAGTTGATA CCCATGCTCTGGTCCCCAAG
-227 GAGCCTGTCACCCTCCAGCC TGCCTACGGCACGGCTTTGC
-187 CACTTAGAAGGCAGTTGGAC ACACACTCATGTGTCCCCTG
-147 TTCTGAGAACTGGGTGGGGC CAGGAAGGCTGGAAAGGGAG
-107 GCGGGCCTTCAGGTGGCCTC TTCTCGTGGCACCTGAGGCT
 -67 CCAGCCCACTTGATTCCCTG ACGTCTGTGGCAGTGGTGGC
 -27 AGTGGCAACAGCTGTAGCAC TTGGGCCATGGCAGAGGACG
```

Figure 1. Nucleotide sequence of the regulatory region of the OMP gene. Nucleotide positions are numbered in reference to the first nucleotide of the ATG initiating codon (*underlined boldface*). The transcription start sites are marked with *arrowheads*, as determined by primer extension experiments. The consensus sequence 5 bp core sequence (CCAGG) of a putative *cis*-acting regulatory element found in the type II sodium channel gene and several other neuronal-specific genes is noted by *underlined italic letters.*

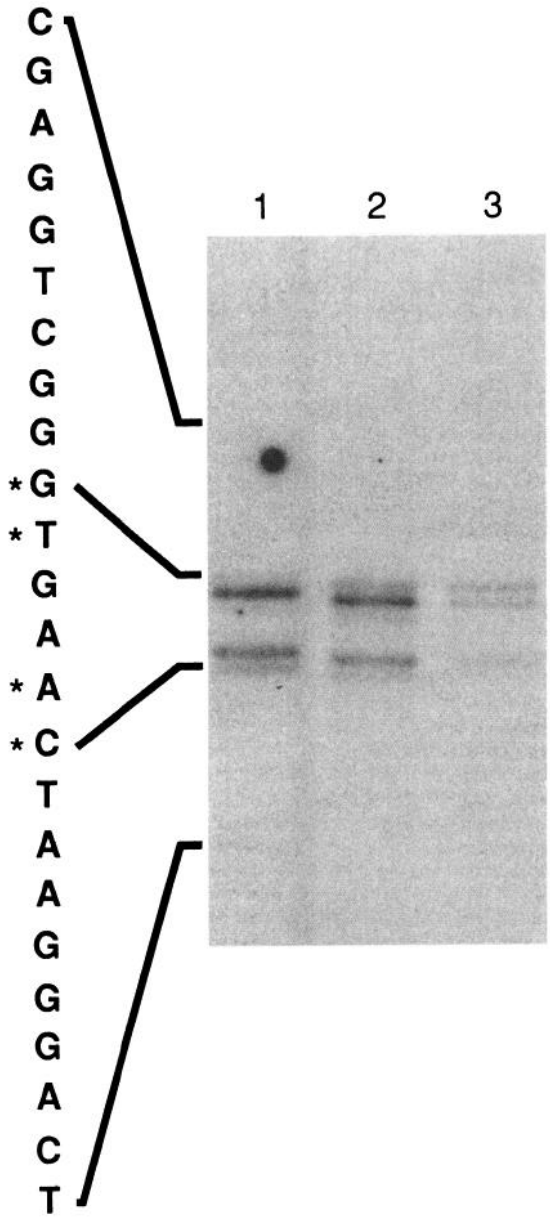


Figure 2. Primer extension analysis of OMP and transgene transcription start sites. Several start sites are noted for transcription of the OMP gene. Each start site is denoted by an *asterisk* next to the genomic sequence (antisense-strand sequence is shown). The start sites did not vary for OMP transcription in rat (*lane 1*) or mouse olfactory tissue (*lane 2*) and were identical to the transcriptional start sites for the OMP–SV40 T-ag transgene in 11.2 transgenic mouse olfactory tissue (*lane 3*). No extension products were observed for brain RNA or in the absence of olfactory RNA (data not shown).

sequence," is present at the transcription start sites in many eukaryotic genes (Smale and Baltimore, 1989).

A 3 kb DNA fragment, containing presumptive 5′ OMP regulatory elements, was isolated and fused with the viral oncogene SV40 T-ag containing coding sequences for both large and small T-ag (Fig. 3). This construction placed the initiating methionine for the oncogene within a few bases of the original location of the initiation codon for OMP. A mouse line was generated that carried the integrated hybrid OMP wild-type SV40 T-ag transgene DNA. In addition to the wild-type T-ag gene, several transgenic lines were constructed with mutant alleles of T-ag: tsA58, a temperature-sensitive allele, and 5041, a transformation-defective allele retaining the ability to immortalize cells (K. Peden, unpublished results). Transgenic mouse lines containing these two alternative constructs were also obtained and have been partially characterized.

The structure of the transgene in progeny of the founder mouse was examined by Southern DNA blot analysis, which revealed the presence of an essentially intact transgene with a copy number of 1 for the 11.2 line (Fig. 3). Specifically, one 2.6 kb PstI fragment contained most of the coding region of the transgene. A second PstI fragment represented a junctional fragment containing the major portion of the 5′ putative regulatory sequence. In the 11.2 transgenic line, the junctional fragment migrated at approximately 3 kb. A PstI/XbaI digest of 11.2 genomic DNA and hybridization with the transgene revealed an intact 1.6 kb PstI/XbaI fragment, indicating that the integration site lies upstream of the XbaI restriction site at the most 5′ sequence of the transgene construct. Thus, the majority of the regulatory sequences of the transgene were intact in the 11.2 transgenic line. The 11.2 mouse line has been bred to homozygosity and maintained as such with no apparent substantive deleterious effects other than those associated with olfactory specific transgene expression.

Northern blot analysis demonstrated that expression of T-ag message was confined to olfactory tissue from the 11.2 line and paralleled the expression of the endogenous mouse OMP gene (Fig. 4). Hybridization with a G_s probe (G-protein) revealed detectable levels of RNA in each tissue with signal intensities approximating expected levels. The exposure time for the T-ag blot was 30 times that for OMP, indicating the relatively low

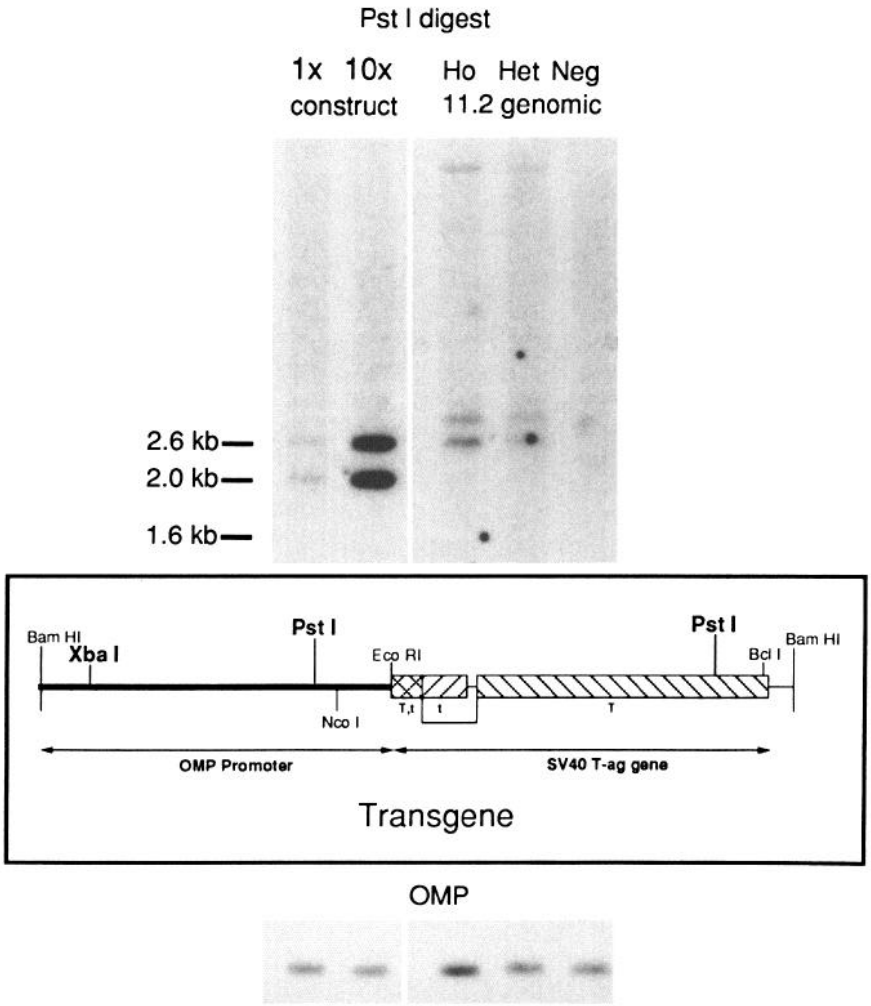


Figure 3. Southern hybridization analysis of structural transgene in 11.2 transgenic mouse line. Genomic DNA (10 μg mouse) from 11.2 homozygotic (*Ho*), hemizygotic (*Het*), and negative (*Neg*) mice was digested with Pst I and size fractionated by electrophoresis in 0.9% agarose. Fragments corresponding to the transgene DNA were visualized by hybridization with a probe of the entire transgene construct. The transgene construct (at amounts corresponding to 1× and 10× copy number equivalents for 10 μg mouse genomic DNA) was used as quantitative control. A restriction map of the transgene is shown for reference. The control blot shown at the *bottom* demonstrates levels of genomic DNA loaded within each lane as seen by hybridization to the endogenous gene for OMP. Hybridization to the 3′ fragment junctional between the transgene construct and the vector, pGEM2, was noted (appropriately sized at ≈1.1 kb) on the original blot but is too small to be seen in this figure. Several higher-molecular-weight bands are seen in the experimental lanes for the homozygote (*Ho*) and hemizygote (*Het*) genomic DNA. The higher and more intensely hybridized band is likely the 3′ junctional fragment for the integrated transgene construct. The slightly lower and less intense band could be a DNA band corresponding to the endogenous mouse OMP promoter region that would likely exhibit very weak homology to the rat OMP regulatory sequences present in the hybridization probe.

level of expression (≈3%) for the transgene compared to the endogenous single copy OMP gene. Primer extension experiments revealed the same transcription start sites for the transgene in the 11.2 mouse line as for the endogenous OMP gene in both rat and mouse (Fig. 2).

Immunocytochemical experiments using monoclonal antibodies to SV40 T-ag revealed the presence of T-ag protein within the nuclei of the few remaining olfactory neuronal cells of a 10-month-old 11.2 transgenic mouse (Fig. 5; see also below). These cells were routinely observed in isolated pairs and might therefore represent two-cell clones of T-ag–positive cells. These observations would support previous studies (Calof and Chikaraishi, 1989; Mackay-Sim and Kittel, 1991) suggesting that olfactory stem cell progeny undergo an additional round of mitosis before terminal differentiation.

Histological consequences of oncogene expression in the olfactory neuroepithelium

When the olfactory neuroepithelium was examined for the presence of T-ag, the neuroepithelium from 10-month-old 11.2 mice was noted to have undergone a remarkable hypoplasia (Fig. 5). The number of apparently mature olfactory receptor neurons in these mice was greatly diminished compared to negative littermates. The number of immature neuronal elements appeared to be similarly diminished. The aggregate loss of neuronal cells was also reflected in the atrophied appearance of the nerve fascicles in the submucosa. The basal cells and sustentacular cells appeared normal upon histological examination. Alterations in neuron number were not restricted to tumor-bearing mice. Every mouse of the 11.2 line that was examined demonstrated this phenotype with some variability in the severity of the hypoplasia. Variability could also be observed among different areas of neuroepithelium within the olfactory turbinates of an individual mouse.

The cellular organization in the olfactory bulb, the synaptic target of olfactory receptor neurons, was also markedly abnormal. In the normal olfactory bulb, innervation by the primary olfactory neurons occurs in discrete synaptic units referred to as glomeruli. In the olfactory bulbs of 10-month-old 11.2 mice with severe neuroepithelial hypoplasia, the glomeruli were virtually absent, although the mitral cells (the predominant second-order neurons of the olfactory system) appeared normal (Fig. 5). The absence of glomerular structures is likely a response to the hypoplasia in the olfactory neuroepithelium. Transgenic 11.2 mice at 2 months of age did not demonstrate the neuronal hypoplasia noted in olfactory neuroepithelium from older animals and, likewise, did not demonstrate a loss of glomeruli in the olfactory bulb.

Tumorigenesis

For a number of reported transgenic mouse lines, the presence of SV40 T-ag resulted in a robust tumorigenesis in the expressing tissue (Brinster et al., 1984; Hanahan, 1985; Mahon et al., 1987;

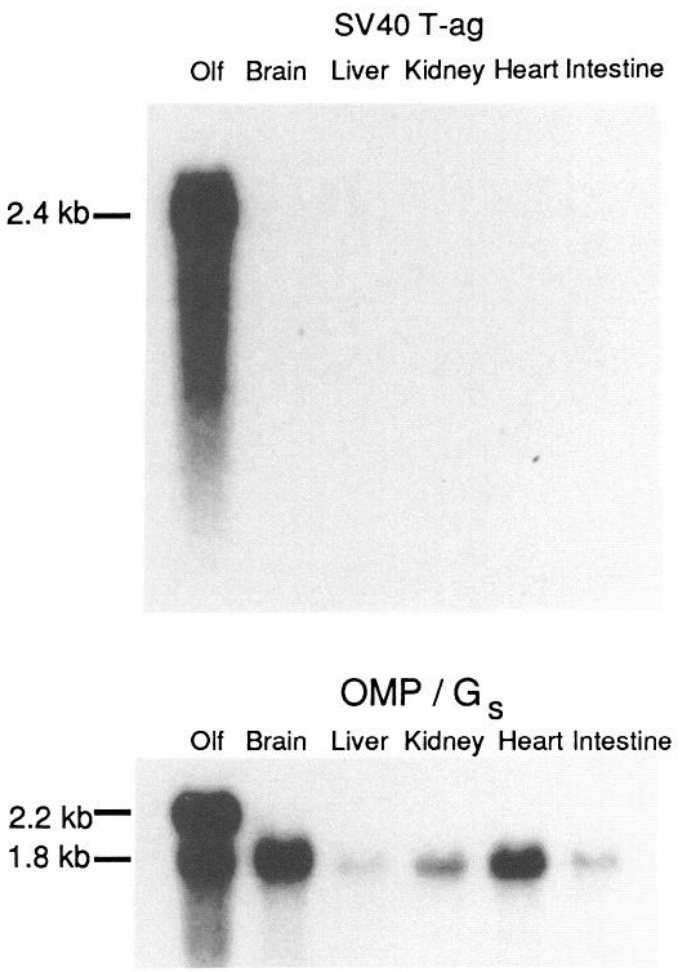


Figure 4. RNA hybridization analysis of transgene expression in various tissues. PolyA-enriched RNA (12.5 μg) for each of the indicated tissues was glyoxylated and size fractionated through 1% agarose. Visualization of SV40 T-ag message is shown at the *top*. A control hybridization of the identical blot is shown at the *bottom*. The OMP probe detects message (2.2 kb) only in olfactory tissue, while G_s hybridization shows intact RNA (1.8 kb) for each lane at the expected size and abundance. The *top panel* shows an exposure 30 times that for the control blot. Even at this overexposure, no detectable T-ag expression is seen in the other tissues.

Hanahan, 1988; Hammang et al., 1990; Mellon et al., 1990; Windle et al., 1990). In contrast, we have found the frequency of tumor appearance to be low in the 11.2 mouse line. Of those mice reaching an age of 1 year, approximately 3% of hemizygotes and 7% of homozygotes exhibited tumor formation, again suggesting a dosage effect for the transgene. The youngest mouse noted to develop a tumor was an 8-month-old homozygote, emphasizing the slow process of tumorigenesis in the 11.2 mouse line. There was no apparent gender difference for tumorigenesis.

A total of 16 mice from the wild-type T-ag transgenic mouse line have developed tumors. The tumor-bearing animals were first noted by the appearance of conspicuous neurological deficits indicative of CNS involvement, for example, circling behavior, postural tilt, and locomotor inactivity. In each animal, the tumor presented as a large, solid mass apparently originating from the nasal cavity and invading maxillary and cranial structures through the palate and cribriform plate of the ethmoid bone. These neurological deficits as well as noticeable swelling of the mandibular area were apparently caused by the expansion of the tumor from the primary site within the olfactory epithelium. Histological examination of these tumors revealed characteristics of a neuroblastoma suggesting a neural origin. Small, darkly staining cells with rounded, T-ag–immunopositive nuclei predominated within the tumor mass arranged as columns or rosettes within a fibrillar matrix. RNA blot analysis confirmed the existence of mRNA for T-ag within the tumor.

Generation of cell lines

Seven of the transgenic mouse neuroblastomas were placed in primary cell cultures. Primary cultures of minced tumor tissue resulted in a heterogeneous population of cell types that were maintained for multiple passages and then placed in cryostorage. The appearance of the cultures from each of the tumors was essentially identical with respect to distinct cellular morphologies. Cell lines derived from primary plating of tumor tissue were subcloned to ensure clonal consistency. During amplification of the individual foci, the clones appear to retain their morphology and characteristics. Several clones, referred to as 2.2, 7.17, 5.4, and suspension cells, were selected on the basis of morphology and preliminary expression data. The cellular morphology within each of these clonal cell lines is consistent, uniform, and distinct.

The 2.2 clone of cells displayed a spindle shape with a small phase-bright elliptical cell body and two thin, long processes (Fig. 6). Under some conditions the 2.2 cells formed long fasciculated arrays of a few to tens of cells. The 2.2 cells did not achieve a high cell density, and the processes of 2.2 cells often appeared to end in growth cone-like structures. Cells of the 7.17 clone were more variable in shape with generally a spindle appearance but without the long processes noted for 2.2 cells (Fig. 6). The 7.17 cells grew more quickly than the 2.2 cells and could achieve very high densities (e.g., were not contact inhibited). The 5.4 clone of cells represented the slowest-growing cells from the olfactory neuroblastoma, doubling every 2–4 d. These cells were large (40–150 μm), flat, polygonal cells that adhered tightly to the tissue culture plastic substrate (Fig. 6). The final cell type, suspension cells, were large (10–50 μm), phase-bright round cells that, while doubling about every 12–24 hr, demonstrated little adhesion to the substratum and often clustered into large floating spheres of cells. These cells were quite apparent in the initial stages of the primary culture, constituting a large percentage of the cells in the culture. After suspension cells were allowed to expand and left in culture undisturbed for a period of several days to a week, a small percentage of the cells attached to the substrate and formed foci of clonal growth. When growing as clones of attached cells, the suspension cells changed or differentiated into each of the other cell types described above.

Interestingly, from foci of the other cloned cell lines (e.g., 7.17 cells) cells could arise that grew in suspension. These clonally derived suspension cells were able, in turn, to give rise to each of the other cloned cell types when allowed to form attached foci. Thus, suspension cells derived from a single cell clone were not restricted to that cell type. The suspension cells may represent an undifferentiated, multipotent cell.

The various cell clones were analyzed for the expression of the neuronal markers GAP-43 and 5008. GAP-43 is a transiently expressed neuronal marker present predominantly in neurons actively extending neurites (Karns et al., 1987; Verhaagen et al., 1989). 5008 is one of four cDNAs initially identified in a differential screen of an olfactory cDNA library for neuronally associated cDNAs (E. Barbosa and R. Reed, unpub-

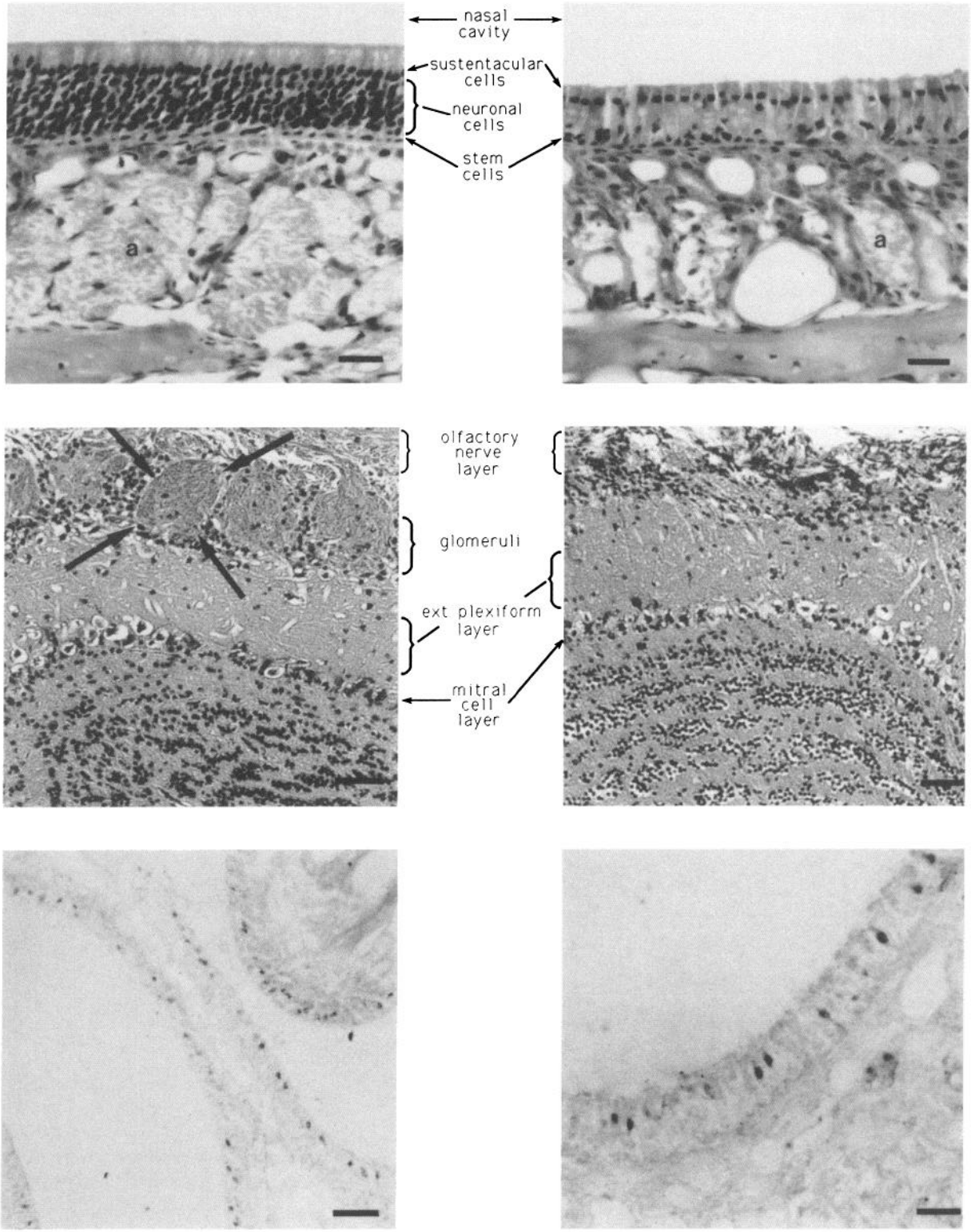
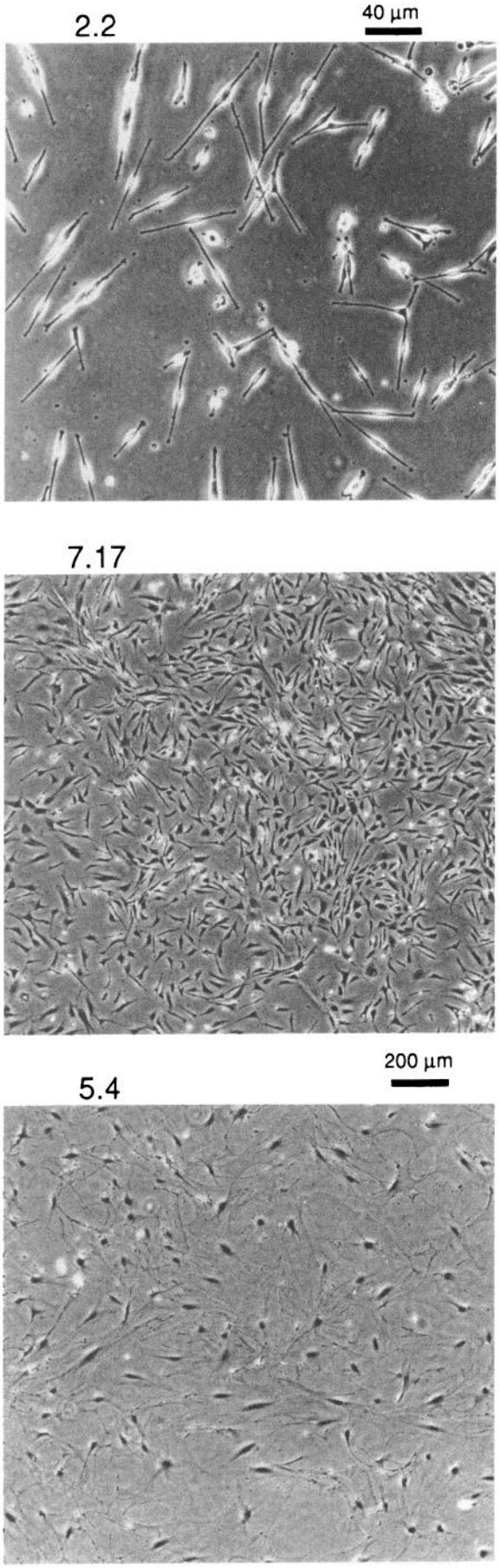


Figure 5. Comparative histology of olfactory tissues in 11.2 transgenic and normal mice. In the *top two panels* are shown comparative coronal sections (hematoxylin and eosin stained) through the nasal cavity of a negative littermate (*left*) and a 10-month-old homozygote 11.2 mouse (*right*). The *top panels* display a significant hypoplasia in the olfactory neuroepithelium of the 11.2 mouse. Note the loss of neuronal cells. Sustentacular cells and stem cells appear unperturbed. The loss of neuronal cells is reflected in the degenerative appearance of the axon fiber bundles (*a*). These photomicrographs represent the most extreme examples of the abnormal phenotype. In the *middle panels*, a coronal section through the olfactory bulbs demonstrates the obvious loss of glomerular structures in the 11.2 mouse. A glomerular structure is outlined by *arrows* in the normal mouse olfactory bulb. These examples represent an extreme case of cellular aberrations found in the 11.2 transgenic line. The *bottom panels* display immunostaining for SV40 T-ag in olfactory neuroepithelium of the 11.2 transgenic mouse, shown at two different magnifications. Note nuclear staining for T-ag throughout the extent of the neuroepithelium. This staining seems to coincide with the few remaining neuronal elements seen in the *top panel* for the 11.2 mouse. The SV40 T-ag staining is often seen in clusters of two cells. Scale bars: *top, middle, and bottom right*, 40 μ; *bottom left*, 80 μm.



lished observations). 5008 is abundantly expressed in olfactory neurons (B. L. Largent, R. G. Sosnowski, and R. R. Reed, unpublished observations), and sequence analysis suggests that it is the rat homolog of mouse chromogranin A, an acidic glycoprotein associated with endocrine and neural cells (Iacangelo et al., 1988).

For the neuronal marker GAP-43, RNA blots revealed the expression of this gene in suspension cells and 7.17 cells (Fig. 7). Expression of GAP-43 in 2.2 or 5.4 cells was not detected by RNA blot analysis. Expression of 5008 was seen in primary cultures derived from the neuroblastoma. While 5008 message was seen in the aggregate of primary culture cells after 5 d, the neuroblastoma tumor did not contain detectable 5008 mRNA. The 2.2 cells and suspension cells expressed 5008, while 5.4 cells lacked detectable expression for this gene. Since the suspension cells constitute a large proportion of the primary culture, most or all of the 5008 expression seen in the primary culture is likely derived from suspension cells.

While the initial primary culture showed high levels of expression of T-ag message, many of the cloned cell lines expressed T-ag message at levels below the detection limit for RNA blots. Suspension cells maintain an easily detectable level of T-ag expression yet do not express message for OMP, a gene presumably under similar regulatory control. OMP message is not observed in any of the cloned cell lines when examined by RNA blot analysis.

Since T-ag expression in suspension cells occurred in the absence of OMP expression, DNA blots were performed on genomic DNA obtained from suspension cells to detect possible rearrangement events. Three separate restriction enzyme digests were performed that would generate various combinations of junctional fragments. Each of these individual digests (HindIII, BglII, or BamHI) produced identical patterns of sized fragments for 11.2 transgenic mouse genomic DNA compared to suspension cell genomic DNA revealing an absence of transgene rearrangement. Furthermore, primer extension experiments demonstrate that T-ag expression in suspension cells utilized the same transcriptional start sites as OMP in mouse (or rat) olfactory neuroepithelium (data not shown).

The paradoxical nature of the differential regulation of T-ag and OMP expression led to an immunocytochemical examination of the olfactory neuroepithelium of 11.2 transgenic mice. In mice at 2–4 months of age, OMP immunoreactivity was observed in the mature population of olfactory neurons while T-ag was localized to the nuclei of a population of neurons subjacent to and overlapping with the mature sensory olfactory neurons (Fig. 8). These immunocytochemical staining patterns suggest that expression of T-ag preceded detectable OMP expression in this cell lineage. However, primer extension experiments demonstrate that T-ag expression (in 11.2 transgenic mouse olfactory tissue or cell lines) utilized the same transcription start sites as the endogenous OMP gene.

Response of 2.2 cells to various stimuli

The substrate specificity of 2.2 cells was examined on a variety of substances constituting or mimicking components of the extracellular matrix (poly-D-lysine, vitrogen, fibronectin, and lam-

←

Figure 6. Cell clones derived from olfactory neuroblastoma. Phase-contrast photomicrographs of cells 2.2, 7.17, and 5.4 are shown. Scale bar: 40 μm for 2.2 cells; 200 μm for 7.17 and 5.4 cells.

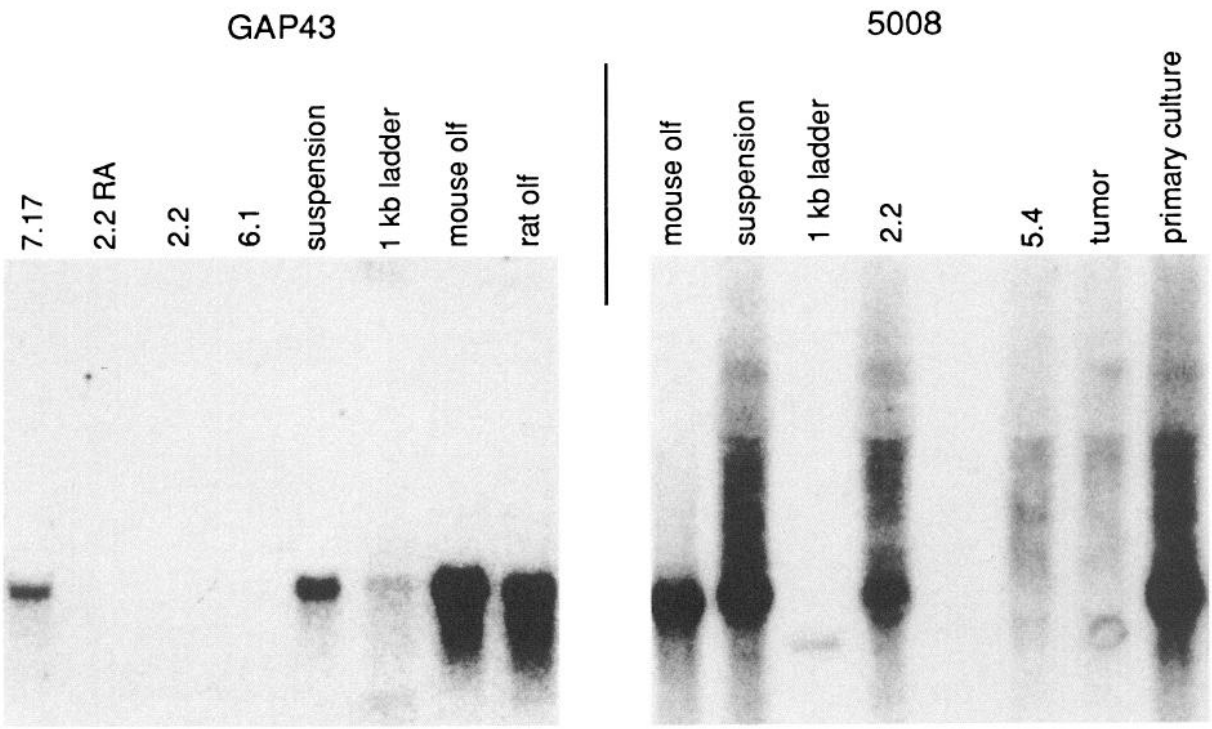


Figure 7. Northern hybridization analysis of expression of GAP-43 and 5008 in clonal cell lines. Hybridization experiments were performed as described in Materials and Methods with purified cDNA inserts radiolabeled as hybridization probes (wash stringency was 1 × 20 min at room temperature and 2 × 20 min at 65°C with 0.2× SSC, 0.5% SDS). The size of the primary band noted in the GAP-43 blot is 1.5 kb, the same size expected for GAP-43 message. The size of the primary band seen in the 5008 blot is 1.9 kb (the size of 5008 message). The higher-molecular-weight hybridization seen with 5008 probe is due to a repeated element within the cDNA for the 5008 gene.

inin) (Fig. 9). The 2.2 clone of cells displayed a marked preference for vitrogen-coated surfaces, exhibiting a faster growth rate, an ability to grow to higher densities, and an attachment to the substrate that required trypsinization for passage. Morphologically, the cells differed only slightly, being less refractile as a result, perhaps, of the tighter cell attachment to the plate surface. Interestingly, laminin and fibronectin failed to support substantial growth of 2.2 cells. Laminin is expressed in various components of the olfactory system, including the olfactory fascicles, and is thought to play a role in the ability of this neuronal system to regenerate (Liesi, 1985).

A number of agents are known to cause differentiation responses in cultured cells. In particular, dibutyryl cAMP has been widely used to induce differentiation in cultured neuronal cells (Goldstein et al., 1986; Johnson et al., 1988; Lang et al., 1989). Dibutyryl cAMP caused marked changes in the 2.2 cells, slowing the division of the cultured cells and inducing a morphological change characteristic of differentiation in neurons. These changes included the cell body becoming more refractile and the substantial extension and thinning of the neuritic-like processes (Fig. 9).

If 2.2 cells represent olfactory neuronal cell precursors, one

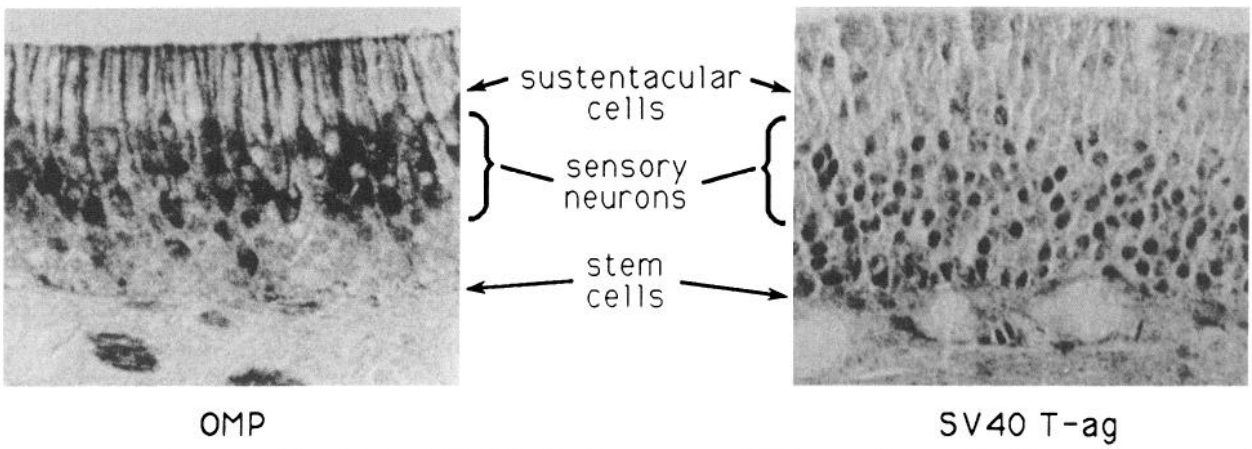


Figure 8. Comparative immunostaining of OMP and SV40 T-ag in olfactory system of 11.2 transgenic mouse line. Mouse olfactory neuroepithelium from a 2-month-old mouse was prepared for immunohistochemistry as described in Materials and Methods. Note the presence of T-ag immunoreactivity (*right*) in nuclei of cells in the lower half of the neuroepithelium, while OMP immunoreactivity is most apparent in the cytoplasm of the mature olfactory neurons residing in the middle third of the neuroepithelium (*left*).

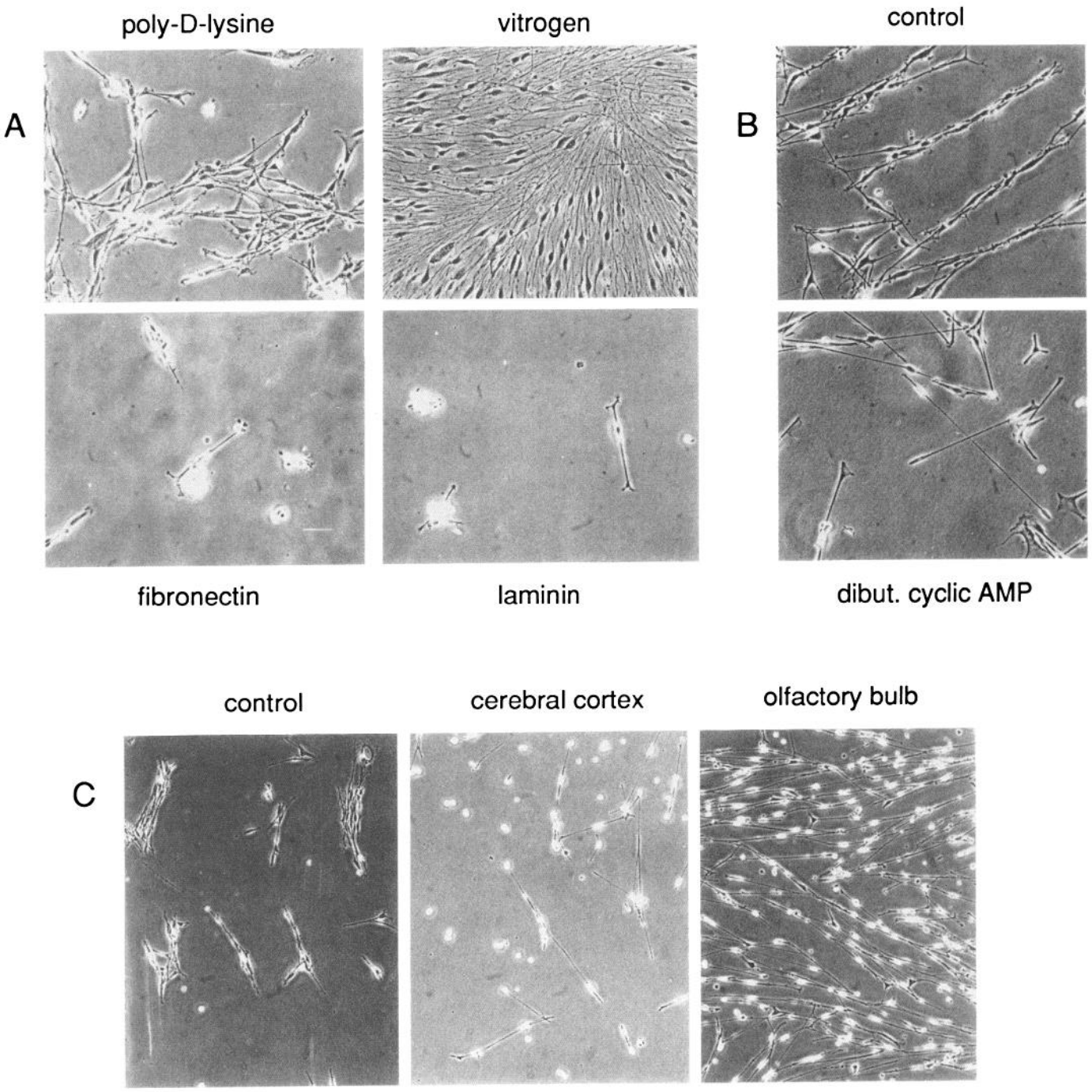


Figure 9. Response of 2.2 cell line to various conditions. *A*, The effect of substrate was examined by culturing 2.2 cells on substrates coated with poly-D-lysine, vitrogen (a bovine dermal collagen), fibronectin, or laminin. *B*, The response of 2.2 cells to dibutyryl cAMP exposure. The 2.2 cells were grown in the presence of 1 mM dibutyryl cAMP for 48 hr. The changes in cell morphology included the soma becoming more phase bright and a thinning and substantial lengthening of the cell processes. Note the asymmetrically bipolar appearance of the cells and the presence of growth cone-like structures at the distal tip of the long process. These cell morphologies are stable at least for several days after removal of the dibutyryl cAMP in the culture media. In the presence of dibutyryl cAMP, 2.2 cell doubling rates diminished. *C*, The response of 2.2 cells to soluble coculture with cerebral cortex and olfactory bulb. 2.2 cells were grown as a coculture for 48 hr. Coculture with cerebral cortex causes changes in 2.2 cells similar to those observed with dibutyryl cAMP, that is, more refractile cell bodies and a thinning and lengthening of the cell processes. Coculture with olfactory bulb, the presumptive target tissue for these cells, also causes a morphological response similar to that seen for dibutyryl cAMP but serves an additional, supportive role for the culturing of 2.2 cells as noted by the evident increase in cell number compared to coculture with cerebral cortex.

might expect them to respond to the presence of the appropriate target tissue, the olfactory bulb. For other neuronal systems, target tissues have been shown to have neurotrophic and/or neurotropic effects on their respective afferent neurons (Maisonpierre et al., 1990). To examine the possible effects of neuronal targets on the growth of 2.2 cells in culture, a coculture experiment was performed using plate inserts containing a Millipore membrane to allow diffusion of soluble factors in the

media between each culture but prevent physical contact. The 2.2 cells were cocultured with either mouse olfactory bulb or mouse cerebral cortex (Fig. 9). When 2.2 cells were cultured in the presence of mouse cerebral cortex, the cells underwent a morphological change quite similar to that observed for treatment with dibutyryl cAMP. This response could be a result of the release of endogenous cAMP in brain tissue. Likewise, during coculture with olfactory bulb, 2.2 cells underwent a similar morphological response, but additionally these cells demonstrated an increase in cell number (increased mitotic activity/decreased cell death), suggesting the presence of a supportive (neurotrophic) factor present in olfactory bulb, the presumptive target tissue. The change in morphology of 2.2 cells after coculture with either cerebral cortex or olfactory bulb did not reverse after cessation of the coculture and repeated changing of the culture media.

Discussion

A major effort in developmental neurobiology is directed toward understanding the molecular and cellular phenomena associated with neurogenesis, cell migration, axonal guidance, and cell death. The interesting biological attributes of the olfactory system make it a useful experimental system to study these processes. This study demonstrates a genetically targeted perturbation of the neuronal lineage in olfactory neuroepithelium. The specific objectives of this work were twofold: (1) to assess the cellular consequences of targeted oncogenic expression to the olfactory neuronal lineage and (2) to generate cloned, immortalized cell lines of the olfactory neuronal lineage that may be useful for the identification and molecular analysis of genes expressed in neuronal precursor cells.

Penetration of the tumor phenotype

Past studies utilizing SV40 T-ag in transgenic mice paradigms illustrate the remarkable effectiveness of this oncogene for transforming various cell types with virtually 100% efficiency over very short time courses of weeks to several months (Brinster et al., 1984; Hanahan, 1985; Mahon et al., 1987; Hanahan, 1988; Hammang et al., 1990; Mellon et al., 1990; Windle et al., 1990). This study contrasts with previous reports in that the observed tumorigenicity with SV40 T-ag was quite low. This result could be specific to the 11.2 transgenic line—an explanation that cannot be excluded since only one mouse line was available for study. However, the bulk of the data would suggest that the experimental observations in this line were biological responses to the expression of oncogene within the olfactory neuroepithelium. First, the expression of the hybrid oncogene was cell type specific as designed. Second, transgenic homozygotic mice demonstrated gene dosage effects; that is, there were higher levels of SV40 T-ag message, relatively greater levels of histological aberrations within the olfactory neuroepithelium, and higher incidence of tumor formation in homozygotes as compared to hemizygotes.

Several other factors could contribute to the weak tumorigenicity observed in the 11.2 transgenic mouse line. The continual transit of olfactory neurons across the neuroepithelium (as they are born, mature, and die) constrains the time frame for the successful "seeding" of a tumorigenic cell and may play a role in the low percentage of mice developing neuroblastomas. The observed hypoplasia in the olfactory neuroepithelium could contribute to the reduced levels of tumor formation. However, due to the timing of these events, it seems likely that the hypoplastic response occurred some time after tumor initiation (in those animals forming tumors) since these tumors appeared relatively slow growing. In addition, in spite of several recent reports of success in generating neuronal cell lines from transgenic mice (Hammang et al., 1990; Mellon et al., 1990) and retroviral transfection (Frederiksen et al., 1988), neuronal cells are thought to be more resistant to transformation than many other cell types. A study of a transgenic line carrying SV40 T-ag driven by the insulin gene demonstrated an unexpected transient expression of SV40 T-ag in cells of the neural tube and neural crest, yet no evidence of transformation of CNS neurons was noted (Alpert et al., 1988).

Aberrations in the olfactory system result from SV40 T-ag expression in neuronal lineage

The loss of neuronal elements in the olfactory neuroepithelium was a peculiar and interesting consequence of the expression of SV40 T-ag in olfactory neurons. The usual result of T-ag expression is typically one of hyperplasia or overt tumorigenesis (Brinster et al., 1984; Hanahan, 1985; Mahon et al., 1987; Hanahan, 1988; Hammang et al., 1990; Mellon et al., 1990; Windle et al., 1990). In this instance, however, in the long-term a decrease or loss of cellular elements was observed when SV40 T-ag was expressed in the neuronal lineage of the olfactory neuroepithelium.

Studies measuring ^{3}H-thymidine incorporation indicate that under normal conditions the population of olfactory neurons undergoes a continual process of turnover in the neuroepithelium as mature cells degenerate and are replaced by differentiating precursors. Lesions to the olfactory system that deplete the neuronal population seem to increase the mitotic rate of the basal cells responsible for repopulating the neuroepithelium. Current reasoning on the population dynamics of the olfactory system, based on data from several labs, suggests that mature olfactory neurons are longer lived (perhaps 90 d or more) than originally predicted from the cellular half-life of 30–60 d observed in ^{3}H-thymidine incorporation experiments in mammals (Hinds et al., 1984) as well as amphibians (Mackay-Simm and Kittel, 1991). Much of the observed cellular turnover is likely to occur in the population of immature neuronal cells available to replace functioning olfactory neurons that have died, perhaps through environmental insult (Graziadei and Monti Graziadei, 1985; Stahl et al., 1990; Mackay-Sim and Kittel, 1991). Thus, effectively two populations of neuronal cells could exist in the olfactory neuroepithelium: a longer-lived mature population of neurons functioning as sensory neurons, and a shorter-lived population of precursor cells that is maintained to provide a ready reserve of cells to replace efficiently the mature sensory cells that have degenerated.

In neurodevelopment, the idea of neuronal pruning involves the generation of excess neurons that are lost as they fail to make viable synapses. This process may be somewhat analogous to the cellular turnover seen in the olfactory neuroepithelium as immature cells die if they fail to establish a functional synapse within a specified period of time. In this context, the presence of the oncogene SV40 T-ag may affect the population dynamics of this neuroepithelium in a number of fashions. SV40 T-ag may arrest cells in immature states by suppression of neuronal precursor differentiation. In this manner, a proportion of neuronal cells may have difficulty in fully completing differentiation and establishing viable synapses—processes that normally would lead to a mature, functioning olfactory neuron. In the long term,

this response could result in an epithelium depleted of neuronal elements. External cellular cues could also play a role in the hypoplastic response. Expression of SV40 T-ag may suppress the expression of specific genes required for the temporal stabilization of neuronal cells within the epithelium, for example, surface adhesion proteins required for the positional establishment of that cell within the neuroepithelium. This might lead to cell loss beyond the capacity of the stem cells to maintain a steady state population of precursor and mature neurons. Since younger mice (≈2 months of age) do not demonstrate the dramatic loss of neuronal elements in the olfactory neuroepithelium seen in the older mice (10 months old), the effect of SV40 T-ag is likely a modest one requiring a substantial period of time to manifest its effects.

It is not known if the basal cells of the olfactory neuroepithelium continue to divide (or what the relative rate of division might be) in the older mice of the 11.2 line. A suppression of the division rate could account for our experimental observations since the steady state level of cell number would be directly affected by decreased cell production. The signals that affect the division rate of the stem cells are unknown. Although the direct effect of SV40 T-ag, as an oncogene, would be to increase cell mitosis, it is conceivable that expression of SV40 T-ag has disrupted crucial signals within the olfactory neuroepithelium affecting stem cell division.

In the 11.2 transgenic mouse line, the olfactory bulb is affected in the long term by a significant deafferentation (loss of afferent neuronal input) resulting from the loss of mature neurons in the olfactory neuroepithelium. Various experimental results suggest glomerular development in the olfactory bulb is dependent upon innervation by olfactory neurons; that is, development occurs coincident with or immediately after innervation by axons of olfactory neurons (Graziadei and Monti Graziadei, 1986). Development of glomeruli may be a relatively plastic event than can continue to occur postnatally (Pomeroy et al., 1990). Arrival of olfactory neurons in insects has been shown to allow glomerular development by providing a framework, a "protoglomerular template," to support the formation of the olfactory glomerulus (Oland et al., 1990). The presence of glomeruli in younger animals and decrease in glomeruli size and loss of structure seen in older mice presumably results from loss of sensory input. Interestingly, older 11.2 transgenic mice appeared to suffer from a significant deficit in the ability to detect odorants (i.e., anosmia) as tested by measuring the time taken by an animal deprived of food for 1 d to find hidden food in its cage. We have not yet examined the age of onset of this phenomenon. While not eliminating their ability to mate, this deficit may have affected the eating habits of these mice, as the older members of the 11.2 line were significantly less obese than negative littermates, mice of the other transgenic lines, and control CD1 mice.

Transgene expression

The cellular regulation of transgene expression was similar but not identical to that of the endogenous OMP gene. Slight differences in expression between transgenes and their endogenous homologs are not uncommon. In this case, the discrepancies may be attributable to effects of the transgene's chromosomal location or to the use of a rat promoter sequence for the transgene in mouse cells. Alternatively, the 3 kb of sequence utilized as a regulatory region for the transgene, while containing the primary regulatory sequences to direct expression in a cell-specific fashion, may lack certain elements that restrict OMP expression to the fully mature (differentiated) phenotype. Examples of the roles of "silencer" elements have been reported recently, that is, the suppression of neuronal genes in non-neuronal cells (Maue et al., 1990; Mori et al., 1990; Wuenschell et al., 1990). Analogous silencing elements might restrict normal OMP gene expression to the fully differentiated olfactory neuron. The precise mechanisms controlling temporal regulation of OMP expression are not known, but clearly, interactions with the olfactory bulb, perhaps functional synaptic contacts, play a role in olfactory-specific gene control as well as neuronal maturation (Verhaagen et al., 1990).

A previous study examining regulation of the OMP promoter in transgenic mice (Danciger et al., 1989) utilized 5.5 kb of 5′ OMP flanking sequence, the Thy 1.1 coding region, and 3.2 kb of the 3′ end of the OMP gene containing 200 bp of the 3′ untranslated region and the polyadenylation signal. The OMP gene is unusual in that it contains a relatively large (1630 bp) 3′ untranslated region (Rogers et al., 1987). The inclusion of additional presumptive OMP regulatory sequences notwithstanding, the results of that and the present studies are largely corroborative.

Origin of immortalized cells

A number of points support the contention that the cloned cell lines presented in this study are of olfactory neuronal origin. RNA blot analyses and immunocytochemical localizations of SV40 T-ag demonstrated the successful targeting of oncogene expression to the olfactory neuronal lineage. Primer extension experiments indicated that SV40 T-ag transcripts from olfactory neuroepithelium of the 11.2 line and cultured cells derived from the olfactory neuroblastoma utilized the same transcription initiation sites as the mouse OMP gene.

While T-ag expression in the 11.2 line of transgenic mice appeared to be restricted to the olfactory neuronal lineage and the tumors were apparently T-ag induced neuroblastomas, a critical question remains concerning the cellular origin of the tumor. Given the relatively low sensitivity of gross tissue analysis by RNA gel blots, the possibility remained that a small population of cells within the CNS might express the transgene in adequate amounts to stimulate tumorigenesis. Autopsy and histological examination of tumor-bearing mice revealed the tumor mass to be merely compressed against the CNS tissue, with no physical attachments to any tissue within the cranium but with distinct continuity of the tumor mass with the posterior nasal cavity. The consistent presentation of tumors would indicate a tumorigenic response to the expression of the transgene in the olfactory epithelium rather than a spontaneous tumorigenic event.

These mouse neuroblastomas are histologically similar to human esthesioneuroblastomas, which are thought to be of olfactory neuronal origin (Elkon et al., 1979). Both types of tumor present as slow-growing small-cell neuroblastomas with few mitotic figures. Human esthesioneuroblastomas characteristically invade the CNS through the cribriform plate and compress the brain, analogous to the presentation of the mouse tumors. A recent report describes a line of transgenic mice in which activation of a retrovirus has unexpectedly induced formation of apparent olfactory neuroblastomas (Koike et al., 1990). Extensive parallels exist between the neuroblastomas described in that study and the tumors presented here, including the histological presentation, the time course of tumorigenesis, and the obser-

vation that these tumors in mice, without exception, invade the cranium apparently through the cribriform plate.

Gene expression in cell lines and responses to stimuli

The cloned cell lines derived from the olfactory neuroblastoma are unlikely to be responsive to odorants, as they are relatively immature cells that do not express mRNAs encoding proteins thought to mediate the olfactory transduction cascade such as the G-protein G_{olf} (Jones and Reed, 1989) or the type III adenylyl cyclase (Bakalyar and Reed, 1990). Currently, there are no available markers specific for the olfactory system that are expressed by immature cells in the neuroepithelium. These cloned cell lines, representing precursors to the fully differentiated neurons, would not therefore be expected to express any of the available markers. Placing cells in tissue culture—in the absence of the microenvironment of the neuroepithelium—would likely have negative effects on the expression of olfactory markers unique to the fully differentiated neuron. As noted earlier, the olfactory neuron expresses high levels of OMP at the same time that axonal contact with the olfactory bulb is established.

These immortalized cells are proposed to represent clones at various states of differentiation along the olfactory neuronal lineage. That notion is supported by the observation that various of these cell lines express several genes characteristically expressed by olfactory neuronal precursors within the neuroepithelium—either 5008 (Largent, Sosnowski, and Reed, unpublished observations) or GAP-43 (Verhaagen et al., 1989; Verhaagen et al., 1990). While the patterns of expression of the 5008 and GAP-43 genes do not define the differentiation state of each of these cell clones, they do provide clues as to the origin of these cell clones and their respective lineages. The expression of 5008 (chromogranin A) is consistent with an olfactory neuronal origin since it is highly expressed in olfactory neurons. In addition, the expression of GAP-43 strongly suggests a neuronal lineage and emphasizes the immature nature of these cells. GAP-43 is a "growth-associated protein" seen as a transient neuronal marker in neurons actively extending neurites, that is, immature neurons. GAP-43 has been shown by immunocytochemistry in the olfactory system to be present in a population of immature neuronal precursors, the developing neurons in this system (Verhaagen et al., 1989, 1990). Finally, the differential expression of these genes would imply that each of these cells, deriving from a common source (a clonal tumor), have stabilized in cell culture at varying differentiation states.

In addition to dibutyryl cAMP, we tested the effects of retinoic acid and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate on the 2.2 cell line. Each of these "differentiation agents" induced morphological changes in 2.2 cells within 24 hr, with subtle differences among their effects. The neurite extension response of 2.2 cells to treatment with dibutyryl cAMP is relevant to a previous report describing the positive effect of cAMP on neurite outgrowth from explant cultures of chick olfactory epithelium (Johnson et al., 1988).

Use of cell lines for molecular analysis of neurodevelopment

The identification of the genes involved in the process of neurogenesis would be facilitated by access to neuronal precursors at various stages of neurogenesis and in sufficient quantities to perform biochemical and molecular analyses. Immortalized cell lines representing neuronal precursors at various stages in a neuronal lineage could serve as a biological source for such experiments (Frederiksen et al., 1988; Birren and Anderson, 1990; Lendahl and MacKay, 1990). The use of cell lines to study differentiation and cell-specific gene expression has been particularly successful in areas of hematopoiesis, immunology, and muscle cell biology—systems with accessible and stable stem cell populations. In contrast, most neuronal precursors are present transiently within the embryonic nervous system and are not readily accessible. Some cell lines, such as the PC12 pheochromocytoma cell line and embryonal carcinoma cells, have proven useful for a number of neurobiological studies (Lendahl and MacKay, 1990).

In this report, we have directed the immortalization of olfactory neuronal cells through targeted tumorigenesis in transgenic mice. The data support the contention that the clonal cell lines represent stable derivatives of neuronal precursors at various states of differentiation. One should be cautious in drawing conclusions concerning biological phenomena via experimentation with immortalized cell lines, particularly transformed lines. Nonetheless, we are hopeful that the clonal cell lines described in this study will be useful toward the molecular identification and characterization of developmentally significant genes. Further, the observed perturbations of the olfactory neuroepithelium and target tissue, the olfactory bulb, induced by the directed expression of oncogene to the olfactory neuronal lineage provide additional insight into the complex control of the neuronal turnover in this system and suggest avenues for future experimentation.

References

Alpert S, Hanahan D, Teitelman G (1988) Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 53:295–308.

Aviv H, Leder P (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69:1408–1412.

Bakalyar HA, Reed RR (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science 250:1403–1406.

Berger SL, Kimmel AR (1987) Methods in enzymology: guide to molecular cloning techniques. Orlando, FL: Academic.

Birren SJ, Anderson J (1990) A v-*myc*-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 4:189–201.

Bishop JM (1983) Cellular oncogenes and retroviruses. Annu Rev Biochem 52:301–354.

Brinster RL, Chen HY, Messing A, van Dyke T, Levine AJ, Palmiter RD (1984) Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. Cell 37:367–379.

Calof AL, Chikaraishi DM (1989) Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor *in vitro*. Neuron 3:115–127.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5304.

Coon HG, Curcio F, Sakaguchi K, Brandi ML (1989) Cell cultures of neuroblasts from rat olfactory epithelium that show odorant responses. Proc Natl Acad Sci USA 86:1703–1707.

Danciger E, Mettling C, Vidal M, Morris R, Margolis F (1989) Olfactory marker protein gene: its structure and olfactory neuron-specific expression in transgenic mice. Proc Natl Acad Sci USA 86: 8565–8569.

Elkon D, Hightower SI, Meng LL, Cantrell RW, Constable WC (1979) Esthesioneuroblastoma. Cancer 44:1087–1094.

Farbman AI, Brunjes PC, Rentfro L, Michas J, Ritz S (1988) The effect of unilateral naris occlusion on cell dynamics in the developing rat olfactory epithelium. J Neurosci 8:3290–3295.

Frederiksen K, Jat PS, Valtz N, Levy D, McKay R (1988) Immortalization of precursor cells from the mammalian cns. Neuron 1:439–448.

Goldstein NI, Quinn MR, Teeter JH (1986) Differentiation of a cell line from olfactory epithelium is induced by dibutyryladenosine 3′,5′-

cyclic monophosphate and 12-*O*-tetradecanoylphorbol-13-acetate. Brain Res 379:205–214.
Graziadei PPC, Monti Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1–18.
Graziadei PPC, Monti Graziadei GA (1985) Neurogenesis and plasticity of the olfactory sensory neurons. Ann NY Acad Sci 457:127–142.
Graziadei PPC, Monti Graziadei GA (1986) Principles of organization of the vertebrate olfactory glomerulus: an hypothesis. Neuroscience 19:1025–1035.
Hammang JP, Baetge EE, Behringer RR, Brinster RL, Palmiter RD, Messing A (1990) Immortalized retinal neurons derived from SV40 T-antigen-induced tumors in transgenic mice. Neuron 4:775–782.
Hanahan D (1985) Heritable formation of pancreatic b-cell tumours in transgenic mice expressing recombinant insulin-simian virus 40 oncogenes. Nature 315:115–122.
Hanahan D (1988) Dissecting multistep tumorigenesis in transgenic mice. Annu Rev Genet 22:479–519.
Harlow E, Crawford LV, Pim DC, Williamson NM (1981) Monoclonal antibodies specific for simian virus 40 tumor antigens. J Virol 39: 861–869.
Hinds JW, Hinds PL, McNelly NA (1984) An autoradiographic study of the mouse olfactory epithelium: evidence for long-lived receptors. Anat Rec 210:375–383.
Iacangelo A, Okayama H, Eiden, LE (1988) Primary structure of rat chromogranin A and distribution of its mRNA. FEBS Lett 227: 115–121.
Johnson RR, Farbman AI, Gonzales F (1988) The effect of cyclic Amp on neuritic outgrowth in explant cultures of developing chick olfactory epithelium. J Neurobiol 19:681–693.
Jones DT, Reed RR (1989) G_{olf}: an olfactory neuron specific-G protein involved in odorant signal transduction. Science 244:790–795.
Kadonaga JT, Carner KR, Masiarz FR, Tijan R (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51:1079–1090.
Kadonaga JT, Courey AJ, Ladika J, Tjian R (1988) Distinct regions of Ap1 modulate DNA binding and transcriptional activation. Science 242:1566–1570.
Karns LR, Ng S-C, Freeman JA, Fishman MC (1987) Cloning of complementary DNA for GAP-43, a neuronal growth-related protein. Science 236:597–600.
Koike K, Jay G, Hartley JW, Schrenzel MD, Higgins RJ, Hinrichs SH (1990) Activation of retrovirus in transgenic mice: association with development of olfactory neuroblastoma. J Virol 64:3988–3991.
Lang E, Mazauric-Stüker M-L, Maelicke A (1989) States of developmental commitment of a mouse embryonal carcinoma cell line differentiating along a neural pathway. J Cell Biol 109:2481–2493.
Lendahl U, McKay RDG (1990) The use of cell lines in neurobiology. Trends Neurosci 13:132–137.
Liesi P (1985) Laminin-immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones. EMBO J 4:2505–2511.
Mackay-Sim A, Kittel P (1991) Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. J Neurosci 11:979–984.
Mahon KA, Chepelinsky AB, Khillan JS, Overbeek PA, Piatigorsky J, Westphal H (1987) Oncogenesis of the lens in transgenic mice. Science 235:1622–1628.
Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446–1451.
Maruniak JA, Lin PJ, Henegar JR (1989) Effects of unilateral naris closure on the olfactory epithelia of adult mice. Brain Res 490:212–218.
Maue RA, Kraner SD, Goodman RH, Mandel G (1990) Neuron-specific expression of the rat brain type II sodium channel gene is directed by upstream regulatory elements. Neuron 4:223–231.
Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI (1990) Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1–10.
Monti Graziadei GA, Graziadei PPC (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. II. Degeneration and reconstitution of the olfactory sensory neurons after axotomy. J Neurocytol 8:197–213.
Mori N, Stein R, Sigmund O, Anderson DJ (1990) A cell type-preferred silencer element that controls the neural-specific expression of the SCG10 gene. Neuron 4:583–594.
Muskavitch MAT, Hogness DS (1982) An expandable gene that encodes a *Drosophila* glue protein is not expressed in variants lacking remote upstream sequences. Cell 29:1041–1051.
Oland LA, Orr G, Tolbert LP (1990) Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. J Neurosci 10:2096–2112.
Pixley S, Pun RYK (1990) Cultured rat olfactory neurons are excitable and respond to odors. Dev Brain Res 53:125–130.
Pomeroy SL, LaMantia A-S, Purves D (1990) Postnatal construction of neural circuitry in the mouse olfactory bulb. J Neurosci 10: 1952–1966.
Rogers KE, Dasgupta P, Gubler U, Grillo M, Khew-Goodall YS, Margolis FL (1987) Molecular cloning and sequencing of a cDNA for olfactory marker protein. Proc Natl Acad Sci USA 84:1704–1708.
Ronnett GV, Hester LD, Snyder SH (1991) Primary culture of neonatal rat olfactory neurons. J Neurosci 11:1243–1255.
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.
Smale ST, Baltimore D (1989) The "initiator" as a transcription control element. Cell 57:103–113.
Stahl B, Distel H, Hudson R (1990) Effects of reversible nare occlusion on the development of the olfactory epithelium in the rabbit nasal septum. Cell Tissue Res 259:275–281.
Verhaagen J, Oestreicher AB, Gispen WH, Margolis FL (1989) The expression of the growth associated protein B50/GAP43 in the olfactory system of neonatal and adult rats. J Neurosci 9:683–691.
Verhaagen J, Oestreicher AB, Grillo M, Khew-Goodall Y-S, Gispen WH, Margolis FL (1990) Neuroplasticity in the olfactory system: differential effects of central and peripheral lesions of the primary olfactory pathway on the expression of B-50/GAP43 and the olfactory marker protein. J Neurosci Res 26:31–44.
Windle JJ, Weiner RI, Mellon PL (1990) Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol 4:597–603.
Wuenschell CW, Mori N, Anderson DJ (1990) Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is achieved through selective derepression. Neuron 4:595–602.