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Activation of Retrovirus in Transgenic Mice: Association with Development of Olfactory Neuroblastoma

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A line of transgenic mice that express the human adenovirus type 12 E1A and E1B genes under the regulatory control of the mouse mammary tumor virus long terminal repeat was studied. Mice from this line develop olfactory neuroblastomas at approximately 6 months of age. Large numbers of type C retrovirus (ecotropic murine leukemia virus) particles were found in the tumor rosettes. No similar examples of virus activation were identified in tumors from other transgenic experiments. Examination of spontaneous olfactory neuroblastomas from three domestic cats also demonstrated retrovirus in tumor rosettes.

Endogenous retroviral sequences are present in the genomes of many vertebrates, including humans, cats, and mice (1, 3, 16). These viruses may be activated by a variety of agents, including radiation and carcinogens (12). The role of endogenous sequences in humans is unknown. However, in inbred mice they may be associated with induction of disease through recombinational events which generate tumorigenic viruses (7). We demonstrated that the development of olfactory neuroblastoma in transgenic mice is accompanied by activation of endogenous retroviruses and that this association also occurs in domestic cats.

As part of our study of mechanisms of viral pathogenesis, we have generated transgenic mice that carry the entire early 1 (E1) region of human adenovirus type 12 (Ad12). The E1 region genes, E1A and E1B, are known to cooperate in the neoplastic transformation of cells in culture (6). The product of the E1A gene has been shown to regulate transcription of not only E1B and other early adenovirus genes but also of cellular genes, including those that encode β-tubulin and the heat shock protein (8, 18). Since the Ad12 E1 region under its own regulatory control element was found to induce embryonic lethality in transgenic mice, we chose to substitute the Ad12 E1A enhancer and promoter with the mouse mammary tumor virus (MMTV) long terminal repeat, which does not appear to be active during early embryonic development. Eleven transgenic founder mice identified by Southern blot analysis developed neoplasms of the stomach (11). The appearance of stomach tumors in multiple founder transgenic mice (9 of 11 males and 2 of 12 females) was consistent with the hypothesis that expression of the transgene, Ad12 E1A and/or E1B, is responsible for the development of this malignancy.

One of the female transgenic founder animals (designated G5) that did not develop gastric carcinoma gave rise to progeny mice with a different disease phenotype. At approximately 6 months of age, the G5 progeny mice develop progressive lethargy with bulging anteriorly of the cranium. In the first mouse examined, a tumor was found occupying the frontal lobes of the brain. Examination of early lesions from subsequent mice indicated that the tumors originated from the nasal sinuses and extended through the cribriform plate to involve the olfactory bulbs of the anterior brain. After derivation of homozygous animals, tumors were found in 12 of 12 mice whose olfactory mucosae, nasal cavities, and anterior brains were examined. The tumor phenotype in the G5 line was faithfully transmitted to the F_5 generation. None of these mice had stomach tumors, indicating that the enhancer sequences of the transgene were most likely not active. Detection of neural tumors in mice from only one founder line may be consistent with either expression of the transgene in the brain by a cellular promoter at the site of integration or disruption of an essential cellular gene resulting from integration of the transgene.

To examine the role of the transgenes in the development of the observed phenotype, we searched for expression of Ad12 E1A and E1B transcripts by Northern (RNA) blot hybridization. Total RNAs were isolated from selected tissues of several mice, including brain and brain tumor, ovary, kidney, spleen, intestine, stomach, liver, heart, lung, and salivary gland tissues. Figure 1 shows the results from an F_1 mouse, G5A1. Both E1A and E1B transcripts were detected in the brain tumor but not in other tissues. In addition, the frontal lobe from an animal from the G5 transgenic line examined before a tumor developed was also found to be negative. Therefore, it appears that expression of the transgene and tumor development in mouse line G5 are tightly associated and that the malignancy arises as a consequence of tissue-specific expression of the transgene by a brain-specific regulatory element.

Histologic examination was performed on tissues from necropsied animals. All 12 homozygous animals examined developed tumors between the ages of 6 and 9 months. Neoplastic features were found only in the olfactory sinuses and brains. The tumors were composed of sheets of neoplastic cells which varied from round to moderately elongate in appearance. After recognition of the potential primitive neural derivation of the lesions, extensive sections were taken through the cribriform plate and the olfactory mucosa. Neoplastic cells extended through the sensory nasal mucosa and connective tissue in solid sheets (Fig. 2a) and occasionally formed characteristic neuroepithelial rosettes with basally oriented nuclei and elongate apical cytoplasm (Fig. 2b).

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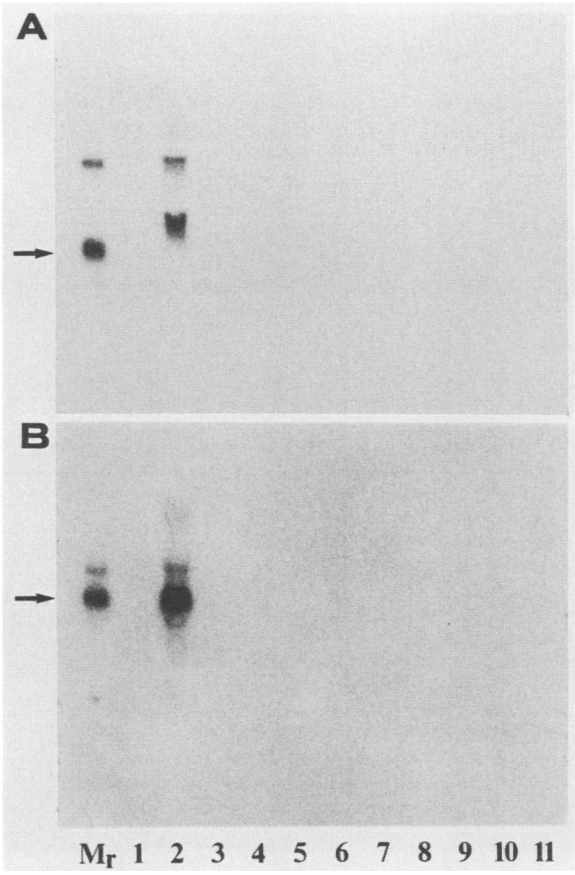


FIG. 1. Tissue-specific expression of the MMTV-Ad12 E1A and E1B genes. Total RNAs from tissues of transgenic mouse G5A1 (lanes 2 to 11) were examined by Northern blot hybridization using ^{32}P-labeled DNA probes specific for either the E1A (A) or the E1B (B) gene. Lanes: M_r, a control from Ad12-transformed mouse fibroblast cell line C57AT1; 1, negative control animal brain tissue; 2, forebrain; 3, ovary; 4, kidney; 5, spleen; 6, intestine; 7, muscle; 8, liver; 9, heart; 10, lung; 11, salivary gland. The arrows indicate the authentic E1A and E1B transcripts from the Ad12-transformed mouse line. The transgenic E1A transcript is somewhat larger than the wild-type mRNA because it is initiated within the MMTV long terminal repeat, which provides 270 additional nucleotides in the 5'-untranslated region. The transgenic E1B transcript is identical to the wild-type mRNA.

These features are consistent with a diagnosis of olfactory neuroblastoma.

Olfactory neuroblastoma is a general diagnostic term referring to tumors that arise from primitive neural precursors in the nasal sinuses and cranium (5). The specific cells of origin are uncertain and may be from submucosal glands. This rare neoplasm occurs in humans, monkeys, dogs, cats, rodents, fish, and cattle (2, 13, 17). The tumors are locally aggressive and can metastasize. In all 12 of the homozygous mice examined, tumors were seen either extending from the olfactory mucosa or infiltrating the olfactory bulb. Electron microscopic examination was performed on three of the tumors from separate animals to identify characteristic features of neuroblastoma, including neurosecretory granules and neurofilaments. However, the tumor cells were poorly differentiated and only membrane junctions were seen between cells and at the apical portion of the rosette (Fig. 2c). Immunoperoxidase staining for neuron-specific enolase and S-100 were also negative (data not shown). Surprisingly, large numbers of mature retrovirus particles were found within the rosette lumen, as well as between adjacent neuroepithelial cells (Fig. 2c and d). Virus particles with type C morphology were found budding from the membranes of tumor cells (Fig. 2d, inset). Retrovirus particles have not been previously observed in transgenic mice derived in our laboratory from B6D2 × CD-1 mice, including those with the Ad12 E1 region genes which developed gastric carcinoma, and have not been detected in tumors that occurred in transgenic animals reported by others.

To characterize the virus further, extracts of the tumors were prepared and tested for infectious type C retroviruses (Table 1). Of three tumor extracts prepared from F_1 or F_2 progeny derived by backcrossing the G5 transgenic founder to CD-1 mice, all demonstrated high titers by XC plaque assay for mouse ecotropic retrovirus. Extracts prepared from the olfactory bulbs of age-matched CD-1 mice, as well as from normal brain tissue of mouse G5F4H9, which had an olfactory tumor, showed no detectable virus. The G5F4H9 mouse with no virus in the brain but virus in the tumor also contained virus in extracts from the salivary gland and spleen. In addition, xenotropic viruses were detected in olfactory tumor extracts from two of the three ecotropic virus-positive animals. Recovery of both ecotropic and xenotropic viruses is consistent with the known possibility of induction of both virus classes in vitro (4). Restriction enzyme analysis of the virus grown in culture and probed by Southern blot hybridization showed no evidence of envelope gene recombination (data not shown). The relationship between virus expression and tumor development is unknown and must be studied further.

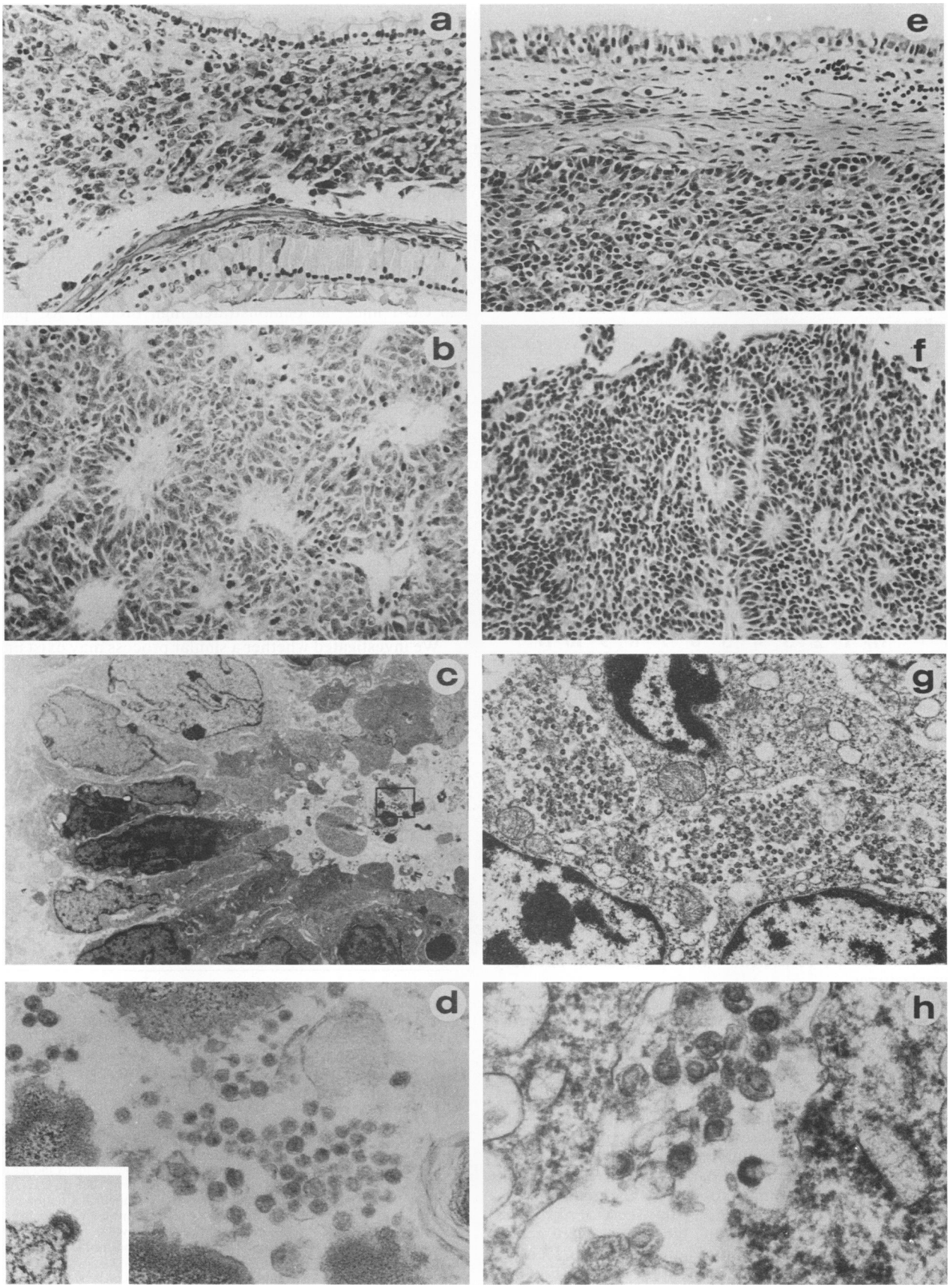
We investigated whether a similar process may exist under natural conditions in other animals, such as domestic cats, in which endogenous viral sequences are common. Tissues from three domestic cats with olfactory neuroblastoma were available for examination. Each of these animals had been referred from the community for treatment. Testing for antibody to feline leukemia virus had been performed on two of the cats, and the results were positive. Histologically, the tumors showed features similar to those seen in mice with

TABLE 1. Biological characterization of the type C retrovirus in mouse olfactory neuroblastoma[a]

Mouse strain (tissue)	Virus titer[b] ($\log_{10}$ 0.1 ml^{-1})		
	Ecotropic virus	MCF virus	Xenotropic virus
G5A4 (tumor)	3.6	−	+
G5F4A2 (tumor)	2.7	−	−
G5F4H9 (tumor)	4.0	−	+
G5F4H9 (normal brain)	−	−	−
G5F4H9 (salivary gland)	2.6	−	−
G5F4H9 (spleen)	1.4	NT	NT
CD-1 (olfactory bulb)	−	−	−
CD-1 (olfactory bulb)	−	−	−

[a] Two percent extracts of tumor or normal tissue were tested for (i) ecotropic virus by XC plaque assay in SC-1 cells, (ii) mink cell focus-forming virus (MCF) virus by immunofluorescence and cytopathic focus induction in mink lung cells and SC-1 cells by the UV-mink assay, and (iii) xenotropic virus by immunofluorescence in mink lung cells. Monoclonal antibodies for immunofluorescence assay (514 for mink cell focus-forming virus and 19-1 for xenotropic murine leukemia virus) were kindly provided by M. W. Cloyd (University of Texas, Galveston). The assays were performed as previously described (10).

[b] NT, Not tested; +, positive by immunofluorescence with monoclonal antibody 19-1 after one passage of infected cells; −, no virus detected.


a
e
b
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c
g
d
h

FIG. 2. Morphologic evaluation of tumors that arose in the nasal olfactory mucosa of transgenic mice (a to d) and domestic cats (e to h). Panels: a, tumor that replaced mucous glands between nasal respiratory mucosa (top) and olfactory sensory epithelium (bottom); b, sheetlike proliferation of tumor cells peripherally arranged around a central lumen; c, electron microscopic appearance of a rosette formed by pleomorphic tumor cells (the boxed area is shown in panel d); d, large number of retrovirus particles with type C morphology (budding virus shown in inset); e, proliferating primitive cells adjacent to nasal mucosa; f, characteristic neural rosettes formed by tumor cells; g, large number of retrovirus particles in extracellular space between tumor cells; h, detail of C-type particles in a cat tumor with central dense cores; some show tailing. Paraffin-embedded tissues were stained with hematoxylin and eosin (a, b, e, and f) or epon-embedded tissues (c, d, g, and h) were prepared for electron microscopy. Magnifications: a, b, e, and f, ×240; c, ×3,000; g, ×10,000; d, ×60,000; h (inset), ×80,000.

primitive neoplastic cells extending from the olfactory mucosa (Fig. 2e) and forming characteristic neuroepithelial rosettes (Fig. 2f). Electron microscopic examination demonstrated the presence of type C particles within the tumor rosettes, as well as in intercellular spaces (Fig. 2g and h). Additional studies are in progress to determine whether the virus found in the tumors is exogenous feline leukemia virus or an endogenous retrovirus. As in the transgenic mice, the tumors from the cats were poorly differentiated, with very few neurosecretory granules or neurofilaments. Immunoperoxidase stains were positive for neuron-specific enolase and S-100 (data not shown), confirming the neural derivation of the tumors.

The G5 transgenic mouse line appears to be useful for the study of the mechanisms responsible for the development of olfactory neuroblastoma, a malignancy for which the cells of origin remain unknown. The finding that G5 progeny mice do not express the Ad12 E1 transcripts in the stomach or develop gastric carcinoma, characteristics of mice carrying the MMTV long terminal repeat-Ad12 E1 region construct, suggests that the MMTV long terminal repeat is somehow made nonfunctional at the G5 integration site. Instead, selective expression of Ad12 E1 transcripts in olfactory neuroblastoma is consistent with integration of the transgenes at or near a *cis*-regulatory element that can confer tissue-specific expression and with involvement of the product(s) of the E1A and/or E1B gene(s) in the manifestation of disease.

Integration of the transgene may disrupt events required for normal development. Proposed mechanisms for the development of a specific phenotype through insertional mutagenesis may involve disruption of a gene essential for normal growth or activation of a gene at a developmentally inappropriate time (9, 14, 15, 19). Detection of murine ecotropic virus selectively in tumor tissues of G5 mice is consistent with activation of an endogenous retrovirus by Ad12 *trans*-activator genes. This is the first example in transgenic mice. Although we cannot exclude the possibility that insertion of the transgene in the G5 strain resulted in inactivation of an essential cellular gene required for suppression of tumorigenicity, it is also possible that activation of the endogenous retrovirus played a role as a cofactor in the development of olfactory neuroblastoma. Our ability to observe a similar correlation of the presence of retroviruses and the development of olfactory neuroblastoma in domestic cats strongly supports but by no means proves the contention that activation of type C retroviruses is an important step in the development of this form of malignancy. Whether a similar correlation also exists in human olfactory neuroblastoma is unknown and warrants investigation.

We are now seeking to determine whether inoculation of the cloned ecotropic virus from G5 tumors can induce olfactory neuroblastoma in recipient nontransgenic mice and to molecularly clone and characterize this virus to better define its potential pathogenicity.

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