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Human gliomas and epileptic foci express high levels of a mRNA related to rat testicular sulfated glycoprotein 2, a purported marker of cell death

(TRPM-2/clusterin/*in situ* hybridization/brain)

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ABSTRACT **Clone pTB16 has been isolated by differential screening of a human glioma cDNA library. Northern blot analysis has shown that pTB16 expression is several times (>11-fold) higher in gliomas than in a primitive neuroectodermal tumor. This observation was supported by *in situ* hybridization and extended to nine other gliomas. Expression was virtually absent in adenocarcinoma cells metastasized to brain. Malignant gliomas showed stronger hybridization than benign gliomas, while blood capillaries did not show hybridization. pTB16 mRNA was also shown to be expressed in established glioma cell lines and at high levels in epileptic foci, indicating that expression of the gene may be limited to certain cell types and that its upregulation is not merely a consequence of cellular proliferation. Nucleotide sequence analysis identified pTB16 as the human counterpart for rat testicular sulfated glycoprotein 2 (SGP-2), whose function in the reproductive system remains unknown. Although SGP-2 transcripts, and hence pTB16, were recently shown to be increased in neurodegenerative diseases such as scrapie in hamsters and Alzheimer disease in humans, our observations with brain tumors and epilepsy are suggestive of a role for pTB16 in neuropathologies in general and support the hypothesis of its involvement in tissue remodeling and cell death.**

Gliomas or glia-derived neoplasms represent the largest group of human brain tumors. Most of these neoplasms stem from cells of astrocyte lineage. Astrocytomas are very aggressive tumors and they demonstrate a progression from benign (low grade) toward an increased malignant phenotype (high-grade astrocytoma, glioblastoma multiforme) associated with dedifferentiation and morphological changes. Astrocytoma of the highest grades (III and IV) are the most frequently encountered at the time of diagnosis and, as a rule, these types of gliomas have a poor prognosis. To gain insight into the molecular mechanisms underlying phenotypic expression in naturally occurring human gliomas, we have used a differential cDNA screening procedure to identify overexpressed genes in a high-grade astrocytoma. Such genes and/or their products may also be useful for early diagnosis or evaluation of tumor status (progression/regression) as well as potential targets for therapy.

Here we report the cloning and initial characterization of a human mRNA (pTB16) whose expression is increased in gliomas.‖ Nucleotide sequence analysis of pTB16 identified this cDNA as coding for the human counterpart of rat sulfated glycoprotein 2 (SGP-2), which is the major secretion product of Sertoli cells (1). Partial biochemical characterization (2) and immunohistochemical studies (3) on SGP-2 have been performed, but the function(s) of this glycoprotein is as yet unknown. Its cDNA was first isolated from cultured Sertoli cells by Collard and Griswold (4). Since that time, many cloned cDNAs from different tissues in various physiological or pathological states were found to be identical to, or a species counterpart of, SGP-2 (5–14). Thus, clusterin (8), testosterone-repressed message 2 (TRPM-2) (9), T64 (10), glycoprotein III (11), serum protein 40,40 (12), complement cytolysis inhibitor (13), and pADHC-9 (Alzheimer disease hippocampus clone 9) (14) are all designations for equivalent clones in rodents, birds, ungulates, and humans. In addition, we show that epileptic foci also express high levels of pTB16/SGP-2 mRNA, thereby broadening the spectrum of disorders of the central nervous system (CNS) in which the gene product is thought to play a fundamental role.

MATERIALS AND METHODS

Tissue Collection. For Northern blot analysis, surgical human brain samples and adult male (3 months old) Sprague–Dawley rat brain tissues were immediately snap frozen in liquid nitrogen before being stored at −80°C. Human glioma-derived cell lines Hs683 and H4 were purchased from American Type Culture Collection. For *in situ* hybridization studies, the same surgical specimens were used. G1 (astrocytoma, grade III), G2 (glioblastoma multiforme), and G3 (anaplastic astrocytoma) are all temporal lobe malignant gliomas. PNET is a primitive neuroectodermal tumor, while E1, E2, and E3 are temporal lobe epileptic foci from three different patients. Of the 10 other tumors (stereotaxic biopsies) used in this study, 4 were obtained from patients with glioblastoma multiforme, 2 with anaplastic astrocytoma, 3 with benign or mixed gliomas, and 1 with a brain metastasis of digestive origin. Nonmalignant postmortem (4 h) human temporal cortical tissues were obtained from an 81-year-old patient who died of cardiovascular disease who was devoid of major neuropathological deficits and had no history of neurological and/or psychiatric disorders (Douglas Hospital Brain Tissue Bank).

RNA Isolation and Northern Blot Analysis. Total RNA and poly(A)$^+$ mRNA were isolated (15, 16) from frozen tissues or cultured cells, size fractionated on a denaturing gel, blotted,

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Abbreviations: SGP-2, sulfated glycoprotein 2; PNET, primitive neuroectodermal tumor; TRPM-2, testosterone repressed message 2; CNS, central nervous system.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M74816).

and hybridized (16) to nick-translated DNA probes (17) by standard procedures. Densitometric quantification and measurements of autoradiograms were performed by computer-assisted LKB Ultrascan densitometer (Pharmacia).

cDNA Cloning and Differential Screening. A human glioma (see above) cDNA library was constructed in pGEM-1 (Promega) by using a vector-primed cloning procedure (18), except that the RNA·DNA hybrid free end was ligated to the blunt termini of vector primer before replacement (19) of the RNA strand by DNA, thus avoiding the need of linker molecules. Approximately 20,000 recombinants were initially screened by differential hybridization of replicate colony lifts to [^{32}P]cDNA probes (16) derived from astrocytoma and control poly(A)$^+$ mRNA. Control mRNA was isolated from a normal peripheral region of a surgically removed benign oligodendroglioma (Basile Pasquier, neuropathologist; Centre Hospitalier Régional et Universitaire de Grenoble, France). Tertiary differential screening by hybridization onto dot-blotted plasmid DNA allowed the isolation of several potential clones. These double-stranded cDNA clones were in turn used as nick-translated [^{32}P]DNA probes to assay for transcript prevalence by Northern blotting.

***In Situ* Hybridization.** For detection of pTB16 mRNA by *in situ* hybridization, a ^{35}S-labeled antisense complementary RNA probe was synthesized. For control experiments, the complementary ^{35}S-labeled sense strand was used. These probes were generated from a 149-base-pair *Sst* I subfragment of pTB16 [91% homologous to rat SGP-2 cDNA (4)] cloned into pGEM-1, essentially as described by the manufacturer (Promega).

Frozen sections (20 μm thick) thaw-mounted on poly(L-lysine)-coated slides were warmed to room temperature and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. *In situ* hybridization was carried out as described (20) except for minor modifications. They included pretreatment of tissue sections with proteinase K for only 10 min, hybridization at 60°C with 10 μl of probe-containing buffer per section, posthybridization removal of coverslips in 4× standard saline citrate/14 mM 2-mercaptoethanol/1% sodium thiosulfate, and a washing in hybridization buffer at 65°C. Exposure time of slides dipped into Kodak NTB-2 nuclear track emulsion was first estimated by direct autoradiography with Hyperfilm-^{3}H (Amersham). Slides were developed and stained through the emulsion with 0.5% cresyl violet for microscope examination. Because of the digestion of RNAs by ribonuclease, only cell nuclei were stained.

RESULTS

Identification and Characterization of Clone pTB16. Three successive screenings by differential hybridization to glioma and control cDNA probes led to the isolation of over a dozen clones from the human glioma cDNA library we have constructed in pGEM-1. Clone pTB16 (others will be described elsewhere) was ^{32}P-labeled by nick-translation and used to hybridize equal amounts of poly(A)$^+$ mRNA from starting control brain tissue or glioma transferred to nylon (Fig. 1). The probe detected a single ≈1.8-kilobase (kb) RNA in both samples, albeit at much lower abundance in the control (21 times less). Sequence analysis (21) revealed that pTB16 shares an overall 76% homology with a previously described rat testicular SGP-2 cDNA (4). Our sequence has a poly(A) homopolymer stretch preceded by 1416 nucleotides (data not shown) identical to the 3′ end of the recently published sequence for human serum protein 40,40 cDNA (12) except that we did not find thymidine residues 25 and 38 nucleotides downstream from the stop codon [in agreement with the data from Jenne and Tschopp (13) for human cytolysis inhibitor].

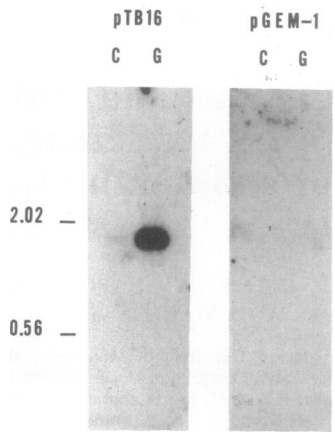

FIG. 1. Increased expression of pTB16-related mRNA in glioma G1 (G) compared to control tissue (C). Poly(A)$^+$ mRNA isolated from frozen specimens (450 ng each) was loaded in the lanes indicated. Northern blots were probed with ^{32}P-labeled nick-translated pTB16 or pGEM-1. Final washings were done at 55°C in 0.1× standard saline citrate/0.5% SDS. Exposure time to XRP-O-Mat film was 1 week with intensifying screens. Numbers on the left refer to molecular size markers in kb.

Northern Blot Analysis. Several brain tumors were first investigated for the expression of pTB16-related transcripts by Northern blotting. While the level of expression was relatively low in a PNET, variable but higher levels were observed in all gliomas (11- to 25-fold when normalized with actin) (Fig. 2*A*). A transcript was also detected in the two glioma-derived cell lines we have examined. The lack of tissue and RNA from the control initially used in the differ-

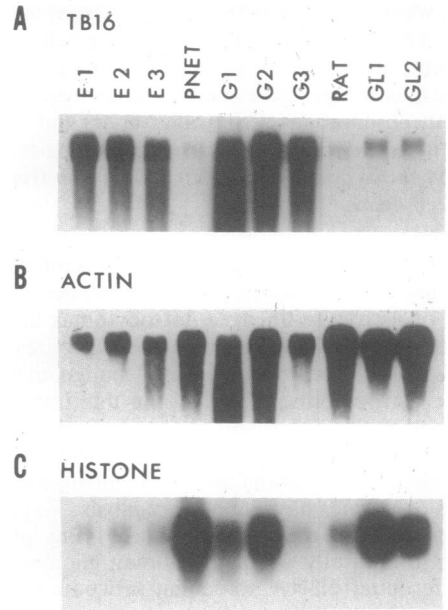

FIG. 2. Northern blot analysis of total RNA from surgically removed brain specimens, established glioma cell lines, and whole normal rat brain. (*A*) Total RNA (10 μg) was electrophoresed on a 1.4% agarose/formaldehyde gel, transferred to nylon, and hybridized to a 0.93-kb *Pst* I fragment from a pTB16 coding sequence followed by washings to a final stringency in 0.1× standard saline citrate at 55°. (*B* and *C*) Rehybridization of the same membrane to probes encoding β-actin and histone H4. Probes were eluted from membrane between each hybridization (22). The sources of the RNA in the various lanes are as follows: lanes E1, E2, and E3, epileptics; lane PNET, primitive neuroectodermal tumor; lanes G1, G2, and G3, gliomas; lane RAT, rat total brain; lanes GL1 and GL2, glioma cell lines.

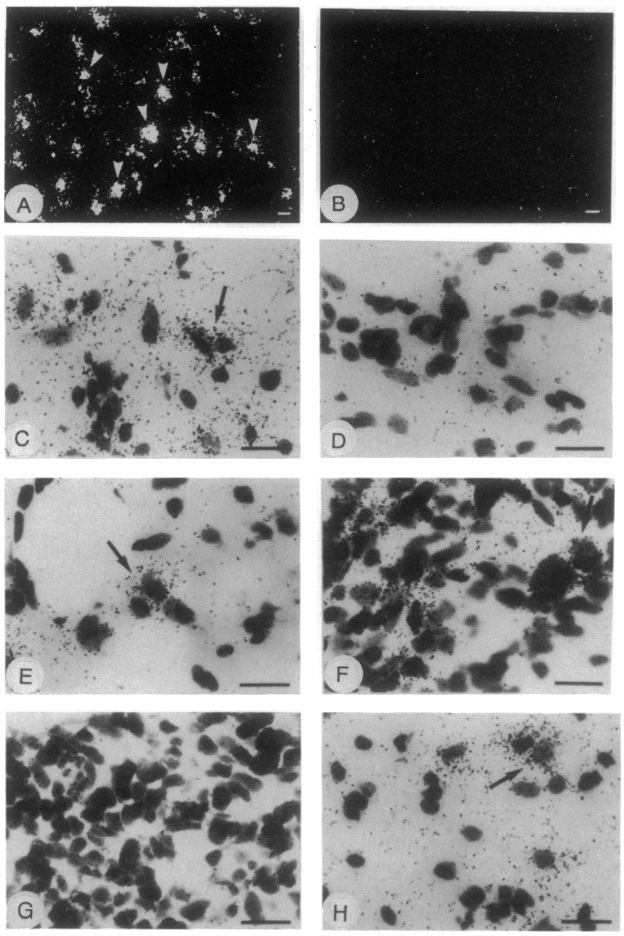

FIG. 3. *In situ* hybridization with pTB16 antisense RNA probe in the same surgical specimens that were used for Northern blot analysis. Low-power darkfield (*A*) and high-power brightfield (*C*) photomicrographs of glioma tissue corresponding to G1 in Fig. 2. A very strong labeling was seen over numerous cells (white arrowheads, black arrows). (*B* and *D*) Views of control hybridization experiments using sense RNA probe. (*E* and *F*) Views of sections from gliomas corresponding to G3 and G2 in Fig. 2, respectively; intensively labeled cells were also found (see black arrows). (*G* and *H*) Hybridization to a PNET and to epileptic brain tissues, respectively. All sections were counterstained with cresyl violet. (Bars = 20 μm.)

ential screening steps made quantitations of pTB16 transcript overexpression in our tumor samples difficult to assess. RNA from autopsy specimens was not sufficiently intact for Northern blot analysis but would not necessarily have been a suitable control for basal expression anyway (see below). Interestingly, hybridization to total RNA extracted from surgically removed seizure foci, from the brains of patients with epilepsy, showed that the expression levels of pTB16 RNA transcripts in these nontumor samples were similar to those found in gliomas (Fig. 2*A*). Homologous transcripts detected in rat whole brain served as molecular size references (Fig. 2*A*). The presence of RNA in lanes showing low signals with probe pTB16 subfragment was demonstrated by hybridization of the same blot with probes for actin and histone (Fig. 2 *B* and *C*, respectively). Taken together, the results from Fig. 2 *A* and *C* demonstrated that highly proliferating tissues or cells do not invariably express high levels of pTB16 transcripts.

***In Situ* Hybridization.** Surgical specimens were also examined by *in situ* hybridization with ^{35}S-labeled complementary RNA antisense probe to assess expression of pTB16-related transcripts. Very high levels of expression were detected in

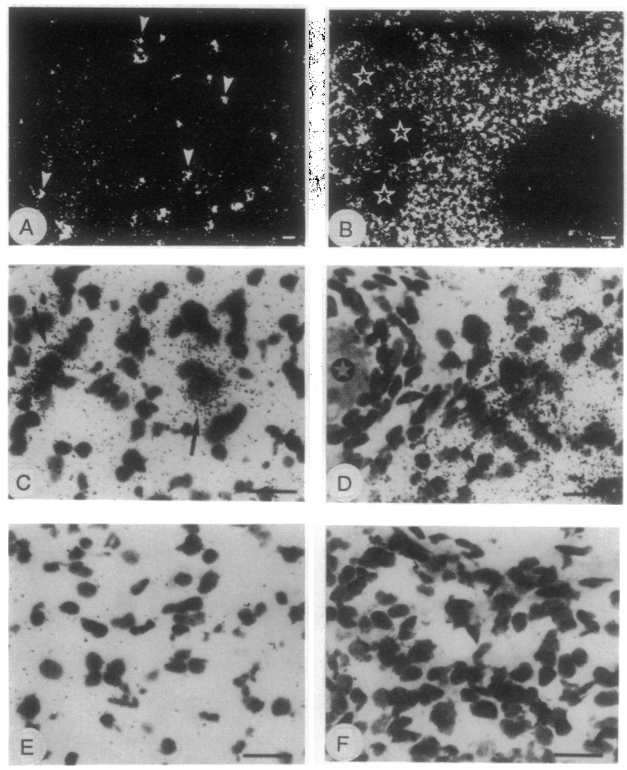

FIG. 4. Lower-power darkfield and high-power brightfield photomicrographs demonstrating the results of *in situ* hybridization with pTB16 antisense RNA probe in benign (*A* and *C*) and malignant (*B* and *D*) gliomas. Intensively labeled pTB16 mRNA-positive cells (arrows) were detected in benign tumor tissue (*C*). In malignant glioma, numerous labeled cells were seen (*D*). Angiogenic foci (blood vessels) are identified by stars (*B* and *D*). (*E* and *F*) Control hybridization using sense probe in benign and malignant gliomas, respectively. All sections were counterstained with cresyl violet. (Bars = 20 μm.)

all three gliomas as compared to the PNET, which demonstrated hardly any specific labeling (Fig. 3), thereby corroborating the results obtained by Northern analysis. In these gliomas, we observed intense overall labeling around microcysts with clusters of silver grains over many nuclei of variable shape (Fig. 3). In parallel, we performed *in situ* hybridization to stereotaxic biopsies of nine other gliomas (astrocytomas and mixed gliomas) from untreated patients. Again in each section, tumor cells were strongly positive, with clusters of grains being larger but mostly more numerous in tumors of higher grade, with no apparent relation with tumor cell density (examples are shown in Fig. 4). Noteworthy was the fact that capillaries and areas of endothelial proliferation did not show any hybridization (Fig. 4). In addition, in the only metastasis to brain examined (adenocarcinoma of digestive origin) a few clusters of grains were observed over invaded parenchymal cells but not over the metastatic cells (Fig. 5). In all cases, the sense probe gave little or no hybridization.

Northern blot analysis has demonstrated significant expression of pTB16 RNA transcripts in foci of seizure from epileptics (see above). *In situ* hybridization with the pTB16 antisense probe on these same brain specimens revealed that expression was mainly restricted to a few scattered cells showing marked positivity (Fig. 3*H*). Grain clusters were found mostly over round nuclei but sometimes over larger triangle-shaped nuclei reminiscent of neurons. Similar findings were observed in postmortem human brain cortex where clusters of silver grains were distributed throughout the gray matter (Fig. 6).

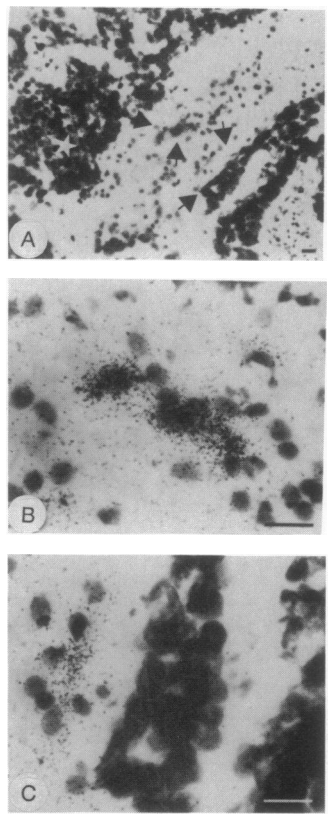

FIG. 5. Brightfield photomicrographs demonstrating the results of *in situ* hybridization with pTB16 antisense RNA probe in brain metastasis (adenocarcinoma of digestive origin). (*A*) Low-power photomicrograph showing tumor tissue (e.g., white star) adjacent to normal brain tissue (center). (*B* and *C*) Enlargements of the areas indicated by arrows in *A*. All *in situ* hybridization sections were counterstained with cresyl violet. (Bars = 20 μm.)

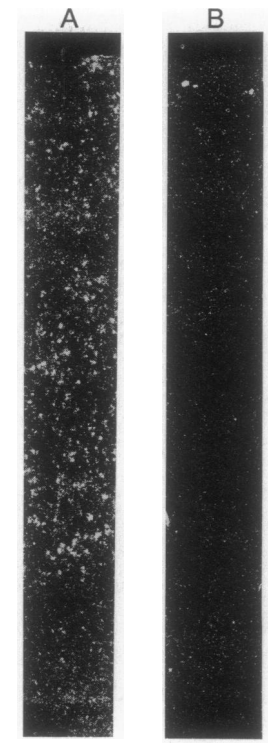

FIG. 6. Darkfield montage photomicrographs of emulsion-coated sections from postmortem human brain temporal cortex demonstrating the results of *in situ* hybridization with pTB16 antisense RNA probe. (*A*) Numerous clusters of silver grains (white dots) representing pTB16 mRNA-positive cells were detected throughout the gray matter. (*B*) No specific hybridization signal was found after hybridization of consecutive sections of cerebral cortex with the sense probe.

DISCUSSION

In this communication, we report the isolation of clone pTB16 by differential screening of a cDNA library we have constructed from a human malignant astrocytoma. Northern blot analysis as well as *in situ* hybridization showed that overexpression of the corresponding gene was not restricted to this single case but was also found in every glioma we examined. Furthermore, a strikingly more intense labeling was seen in glioblastomas than in benign or mixed cell gliomas. Tumors diagnosed as glioblastoma multiforme or anaplastic astrocytoma had on average more grains over more nuclei than those diagnosed as benign gliomas. This observation was most obvious in sections of glioma G2 where two neighboring populations of different stages of the tumor could be easily compared (M.D. and J.-G.C., unpublished observations). In contrast, *in situ* hybridization demonstrated that pTB16 expression was virtually absent in PNET and metastatic adenocarcinoma cells. Our results suggest that, in tumors, the expression level of pTB16 mRNA could be related to type and stage. Other types of brain tumors and tumors of other origins must be screened before we can draw any definite conclusion concerning the exclusivity of pTB16 expression in tumors to those of glial origin.

The fact that astrocytomas and glioma cell lines express pTB16 may indicate that the astrocytes are accountable for the basal expression seen throughout the cortex of normal brain. Furthermore, rat hippocampal fissure, which is strongly positive for the glial fibrillary acidic protein, showed marked differential labeling with pTB16 antisense probe (M.D. and J.-G.C., unpublished observations). In contrast, capillaries in glioma did not show any hybridization (nor was any observed in capillaries of rat brain and of other organs examined; M.D. and J.-G.C., unpublished observations). On the other hand, neurons may also have the potential of expressing pTB16 transcripts (10, 23). We have reported here that neuronal nuclei were labeled in epileptic foci as well as in the cortex of postmortem brain. Although seizure is a common feature of patients with cerebral tumors, no histological (or clinical) evidence of an underlying tumor could be found in the three cases of epilepsy we have studied.

Nucleotide sequence analysis has revealed that pTB16 is homologous to several cDNA sequences cloned from various tissues in different species (4–14). Some *in vitro* biological properties have been demonstrated for the encoded proteins, such as the induction of Sertoli cell or erythrocyte aggregation (24) and the inhibition or enhancement of hemolysis by the complement terminal complexes (13, 25). Still, the precise physiological function for these proteins remains to be determined. It is also possible that the protein plays a different role in the vascular compartment from that in the reproductive system and that posttranslational processing would be responsible for this multifunction (26, 27). The fate and functional significance of the brain-derived counterpart is currently unknown. However, a close association of SGP-2 expression (also known as TRPM-2) with cell death has been demonstrated in rodent models, including prostate involution after orchiectomy (7, 9), renal tissue damage by acute ureteral obstruction (7), interdigital tissue regression during embryogenesis (7), and chemotherapy-induced tumor regression (7, 28). Furthermore, it is well known that in primary tumors there is a variable rate of cell death believed to be caused mainly by the limited diffusion of nutrient metabolites from blood vessels, accumulation of toxic catabolites, and immunologic host defense mechanisms. Accordingly, one

could expect the induction of a TRPM-2/SGP-2 homologous transcript (pTB16) in human gliomas. Moreover, we have demonstrated that the levels of this induction do not correlate to the rate of tissue proliferation. Thus, pTB16 could be associated with cell death rather than cell proliferation. Also supportive of the cell death hypothesis is the remarkable absence of pTB16 expression in the vicinity of blood vessels. Thus, the labeling of presumably normal parenchymal cells on the metastasis tissue section might be explained by their isolation from blood supply. Alternatively, as might also be expected from nontumor tissue, like in epileptic foci, pTB16 mRNA induction could be a consequence of reactive gliosis. However, it was reported that gliosis is neither an antecedant nor a consequence in certain types of seizures (29). Also arguing against the gliosis hypothesis is the fact that some neurons were positive for pTB16 expression. Furthermore, it is likely that degenerating cells, associated with the process of normal aging and postmortem anoxia, are responsible for the observed clustering and "patchy" distribution of the antisense RNA probe in the brain cortex of the 81-year-old woman who died of a myocardial infarction.

Our studies support and extend two recent reports on the increase of SGP-2 expression in CNS degenerative diseases. Duguid *et al.* (6) have found a 10-fold increase of SGP-2 transcripts in scrapie-infected hamster brain while pADHC-9/SGP-2 was found to be elevated 2-fold in the hippocampus of Alzheimer patients (23). In this latter report, complementary observations made with experimental lesions in rats lead the authors to suggest a role for the SGP-2 homologue in ongoing degenerative and/or regenerative processes. Regenerative activity together with tissue remodeling are also consistent with epilepsy where sprouting of fibers, synaptogenesis, and reorganization of neuronal circuitry are salient features.

Thus, in brain, pTB16/SGP-2 gene induction is not solely restricted to neurodegenerative diseases, such as Alzheimer disease, but is also seen in other conditions including gliomas, epilepsy, and probably normal aging. This suggests that pTB16/SGP-2 represents a transcript whose level of expression is associated with the extent of cellular damage. Whether this induction is characteristic of cellular suicide or of an attempt to recover from cell injury is yet to be determined, although our results tend to support the former possibility. Southern analysis ruled out amplification or rearrangement of the gene encoding pTB16 as major causes for its upregulation in glioma G1 (M.D., C.C., A.-L.B., and M.S., unpublished results). Factors and circumstances governing the regulation of this gene remain to be investigated. A better understanding of the mechanisms controlling pTB16 gene expression together with a determination of the precise function(s) of its protein product(s) in the CNS should provide more information concerning the mechanisms involved in apoptosis and/or plasticity in aging and brain tumors.

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