Jpn. J. Cancer Res. 85, 601–609, June 1994

Subcutaneous Sarcomas of Probable Neuronal Origin in a Transgenic Mouse Strain Containing an Albumin Promoter-fused Simian Virus 40 Large T Antigen Gene

Beniyo Kawabuchi,[1,4,5] Kimie Nomura,[1] Keiko Ohtake,[1] Okio Hino,[2] Shinichi Aizawa,[3] Rikuo Machinami[4] and Tomoyuki Kitagawa[1]

Departments of [1]Pathology and [2]Experimental Pathology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, [3]Laboratory of Molecular Oncology, Tsukuba Life Science Center, Institute of Physical and Chemical Research, 3-1-1 Koyadai, Tsukuba, Ibaraki 305 and [4]Department of Pathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

Frequent development of subcutaneous neurogenic sarcomas was observed in a hepatocellular carcinoma-producing transgenic mouse strain harboring an albumin-promoted simian virus 40 (SV40) large T antigen gene. Found unexpectedly in 19 out of 306 mice (6.2%) by 6 months of age, all the sarcomas were similar and were characterized as neurogenic on the basis of histological features including Homer-Wright type rosette formation, the presence of dense core granules of 100–200 nm diameter under the electron microscope, expression of neuron specific enolase, S-100 protein, and catecholamines, and nerve cell-like differentiation in culture in response to But2cAMP. Immunohistochemical study revealed tiny clusters of SV40 T antigen-expressing cells with neurogenic character in normal-appearing adult mouse subcutis as candidate progenitors of the sarcomas. The tumor cells strongly expressed large T antigen but did not express albumin or albumin mRNA at the detection sensitivity used. Transient transfection assay (CAT assay), however, revealed the presence of transcriptional factor(s) acting on the albumin promoter in tumor cells. Thus, the present investigation suggested the presence of specifically differentiated neurogenic cells in the mouse subcutis with aberrant expression of the transgene.

Key words: Neurogenic sarcoma — Transgenic mouse — Albumin — Albumin promoter — Simian virus 40 large T antigen

Transgenic animal systems have proven advantageous for detecting promoter-specific unknown gene expression in cells or tissues.[1-5] A transgenic mouse strain harboring the SV40 large tumor antigen (T antigen) gene conjugated with an albumin promoter sequence was generated by Aizawa *et al.*[6,7] The T antigen is strongly expressed in hepatocytes from the late embryonic stage onwards and multiple hepatocellular carcinomas (HCCs) develop by 6 months after birth.[6,7] In the course of studying the process of hepatocarcinogenesis, a relatively frequent occurrence of sarcomas expressing T antigen was observed in the subcutis of the transgenic mice. This was quite unexpected, because albumin and consequently T antigen expression were thought to take place mainly in the liver[8-12] and, in addition, the kidney, the gastrointestinal tract[12-14] and possibly the brain,[11,15,16] but not in the subcutis of the animals. These observations prompted us to study the nature and the histogenesis of the sarcomatous tumors. We now report the results of our investigations.

[5] To whom requests for reprints should be addressed, at the Department of Pathology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170.

MATERIALS AND METHODS

Transgenic mouse line The generation of the transgenic mouse used in this study was reported previously.[6,7] In brief, the expression construct illustrated in Fig. 1 was microinjected into male pronuclei of F2 zygotes between B6 and SJL mice. The generated T-mouse has a single integration site of the transgene which is expressed in hepatocytes. Descendants of the T-mouse have been produced by crossing with normal C3H female mice. Transgene-positive mice were identified by Southern blot hybridization of tail DNA.

Histological and immunohistochemical examinations Tumor tissues obtained after the mice were killed or grown in nude mouse subcutis were processed for routine light microscopic examination. Four-μm thick sections from paraffin-embedded tissue were stained with hematoxylin and eosin (HE).

For immunostaining of T antigen, the whole bodies of mice were perfused *in situ*, by cannulating the right ventricle of the heart, with 10% phosphate-buffered formalin (5 ml/min) at room temperature for 1 h. Various tissues including liver, skin, brain, spinal cord, heart, lungs, stomach, intestine, pancreas, kidneys, subcutaneous tissue, and muscle were then sectioned, processed

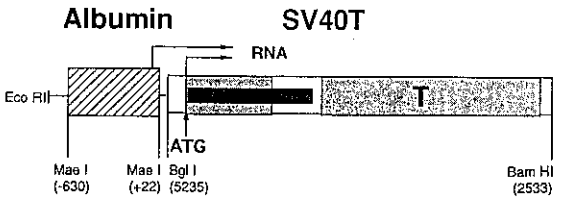

Fig. 1. Structure of the hybrid albumin-SV40 T antigen gene. The hatched box denotes the mouse albumin promoter gene and the open box the SV40 early gene, in which the coding sequences of large T and small t antigen genes are indicated by the stippled and filled boxes, respectively. The restriction sites are given as the sites in the original DNA clones for each unit with base numbers in parentheses. Transcription and translation start sites are also indicated.

through an alcohol-chloroform series and embedded in soft paraffin at 54°C. The standard avidin-biotin-horseradish peroxidase method was employed (Vectastain ABC kit, Vector Laboratories, USA). The 4 μm sections were incubated with a monoclonal antibody against T antigen (PAb101), a generous gift of Dr. N. Yamaguchi (University of Tokyo, Tokyo) for 2 h at 37°C. Other antibodies used in this study were polyclonal antibody for neuron-specific enolase (NSE) (Dakopatts, Denmark) at a dilution of 1:500, S-100 protein (Dakopatts) at a dilution of 1:1000, vimentin (Dakopatts) at a dilution of 1:100, desmin (Bio-Science, Switzerland) at a dilution of 1:100, keratin (Dako Corporation, USA), α-fetoprotein (AFP) (Dakopatts) at a dilution of 1:200, and albumin (Seikagaku Co., Tokyo) at a dilution of 1:1000.

Immunostaining of the cell lines was carried out on cells grown on culture dishes and fixed in acetone/methanol (1:1) at −20°C for 20 min after rinsing 3 times with phosphate-buffered saline (Dulbecco's PBS, Nissui, Tokyo). The fixed cells were air-dried and rehydrated in PBS.

For electron microscopic examination, small fragments of fresh subcutaneous tumor tissues and cultured cells after rinsing with PBS were fixed with 3% glutaraldehyde in 0.1 *M* cacodylate buffer at 4°C. They were then post-fixed in 1% osmium tetroxide and embedded in Epon 812. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL JEM-1200 EX electron microscope.

Establishment of subcutaneous tumor cell lines Tumor tissues obtained after the animals had been killed were cut up aseptically and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and Dispase (Godo Shusei Co., Ltd., Tokyo) for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Thereafter they were seeded in 60-mm^2 dishes (Corning 60 mm tissue culture dishes, Iwaki Glass, Tokyo) containing DMEM supplemented with 10% FCS and maintained.

Determination of the growth rate of subcutaneous tumor cell lines The cells were trypsinized and seeded on 35-mm tissue culture dishes (Falcon 1008, Becton Dickinson, USA) at a concentration of 2×10^5 cells/dish. Numbers of cells were counted at 24, 48, 72, and 96 h after trypan blue staining with a hemocytometer.

Heterotransplantation Subcutaneous tumors were harvested by treatment with 0.05% trypsin, washed three times with PBS, and suspended in DMEM. Suspensions of approximately 10^7 cells were injected into the back subcutis of nude mice (CD-1(ICR) nu/nu, male, Charles River Japan Inc., Kanagawa). They were kept under specific pathogen-free conditions, under laminar air flow, and provided with sterilized food, water, cages, and bedding.

Catecholamine analysis The amounts of adrenalin, noradrenalin, and dopamine in perchloric acid extracts of cultured cells were analyzed with an automated catecholamine analyzer (HLC-725CA, Tosoh, Tokyo) by SRL Inc. (Tokyo).[17]

But2cAMP treatment N^6,$O^{2'}$-Dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (But2cAMP, Sigma, Grade II) treatment was performed according to the method described in the literature.[18,19] The dishes was adjusted to contain 2.0×10^5 cells/60 mm^2 in 5 ml of DMEM with 10% FCS. The medium in one half of the dishes was replaced with medium containing 500 μg/ml of But2cAMP while the other half were cultured with the usual medium as controls. Living cell lines were observed continually by phase contrast microscopy (Olympus CK2-TRC-2, Tokyo).

Northern blot analysis Total cellular RNA was extracted by the method of Chomczynski and Sacchi.[20] Aliquots of total RNA (20 μg) were electrophoresed onto 1.2% agarose gel, blotted onto nylon membrane (Hybond-N$^+$, Amersham Japan, Tokyo), and then probed with rat albumin cDNA[21] or the *Hpa*I fragment of SV40 genome ^{32}P-labeled by the multi-primer method as described previously.[22] After hybridization at 42°C for 16 h, sheets were washed and exposed to X-ray film (Kodak X-OMAT AR) at −70°C overnight.

Construction of CAT plasmids A PUC 19-derived plasmid containing an *Mae*I fragment (−0.63 to +0.022) of the mouse albumin promoter gene was digested with *Eco*RI and *Hin*dIII and separated on a 1.2% low-melting-point agarose gel. Then the fragment (about 0.65 kbp) containing the albumin promoter gene was isolated. After phenol extraction, this fragment was blunt-ended by the Klenow fragment of DNA polymerase I (Takara, Kyoto) and inserted by blunt-end ligation (DNA ligation kit, Takara) into the *Hin*dIII site of pSV00CAT (Wako, Tokyo) after filling in with the Klenow enzyme. The

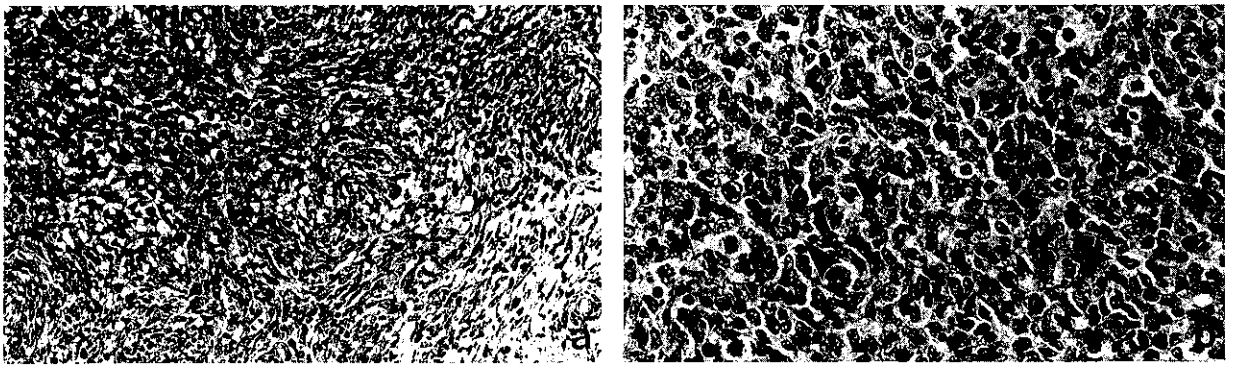

Fig. 2. (a) Histological appearance of a subcutaneous tumor showing dense proliferation of short-spindle cells in fascicles. (b) Another lesion demonstrating small round cells forming abortive Homer-Wright rosettes (arrows). (HE, ×20)

plasmid containing the translationally oriented albumin promoter gene insert was selected by restriction enzyme analysis and designated AL0.6-CAT (Fig. 12).

Cell transfection and CAT assay Transfections were performed by the lipofection method.[23] Subcutaneous tumor and HCC cells plated subconfluently in 35-mm dishes were washed three times with DMEM and immersed in 3 ml of DMEM, then cultured for 12 h at 37°C after addition of 100 μl of plasmid-lipofectin reagent (Gibco BRL, Life Technologies, Inc., USA) mixture containing 2 μg of a β-galactosidase expression vector RSVgal (gift of Dr. J. Inoue, University of Tokyo) and 20 μg of a reporter plasmid (AL0.6-CAT), and then a further 3 ml of DMEM supplemented with 20% FCS was added. After 48 h of cultivation in this medium, cell extracts were prepared by freezing and thawing followed by centrifugation. β-Galactosidase activity[24] was used to standardize the transfection efficiency. CAT assays were performed for 3 h at 37°C as previously described.[25]

RESULTS

Development of subcutaneous tumors Palpable subcutaneous tumors began to develop around 4 months of age. Of 306 transgenic mice, 19 (19/306, 6.2%) developed such subcutaneous tumors concomitantly with HCCs by around 6 months of age, when all the mice with/without subcutaneous tumor died, principally from hepatic insufficiency. The affected sites and the numbers of tumors were five on the back, four on the neck, three each on the face, chest, and leg, and one on the abdomen. All the tumors were single whitish firm nodules 0.5 cm to 3 cm in size, located in the subcutis covered by intact epidermis, showing occasional infiltration to the underlying connective tissue. Distant metastasis was rarely seen.

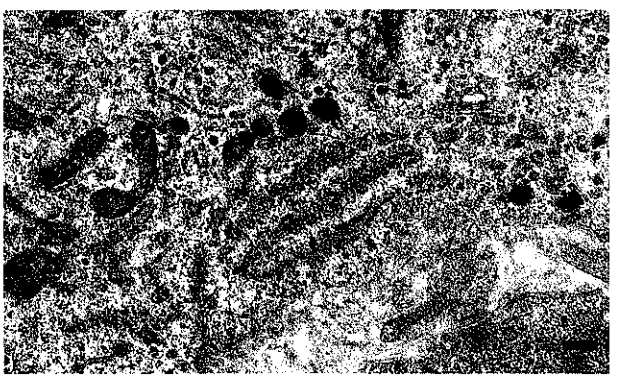

Fig. 3. Electron micrograph of a sarcoma cell illustrating electron dense granules measuring 100–200 nm in the cytoplasm. (×20,000) (Bar, 200 nm)

Morphological characterization of subcutaneous sarcomas The histological features of the nineteen subcutaneous tumors were essentially similar. Representative pictures are presented in Fig. 2. The tumors were composed of short-spindle cells arranged in sweeping fascicles (Fig. 2a) or round-oval cells occasionally forming abortive Homer-Wright type rosettes (Fig. 2b). High cellularity, marked cellular atypia, and numerous mitotic figures were indicative of a highly malignant nature. Ultrastructural examination revealed abundant rough endoplasmic reticulum, dense core granules measuring from 100–200 nm, compatible with neurosecretory granules, in the cytoplasm (Fig. 3) and occasional cytoplasmic processes forming junctional complexes (Fig. 4).

Sarcoma cell lines Four cell lines were established from different subcutaneous sarcomas. They grew in monolayer with much higher proliferation rates than HCC cell lines (Fig. 5) and comprised either small spiny cells

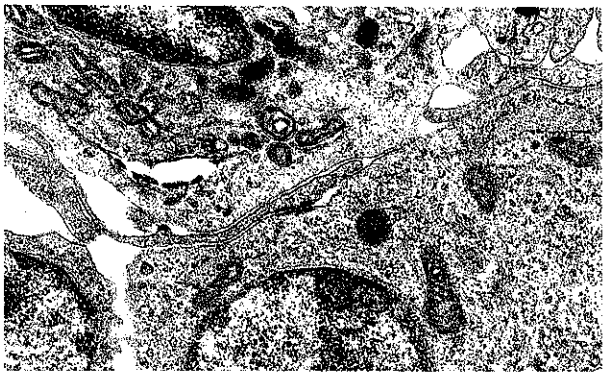

Fig. 4. Electron micrograph of sarcoma cells revealing cytoplasmic processes forming a junctional complex. (×20,000)

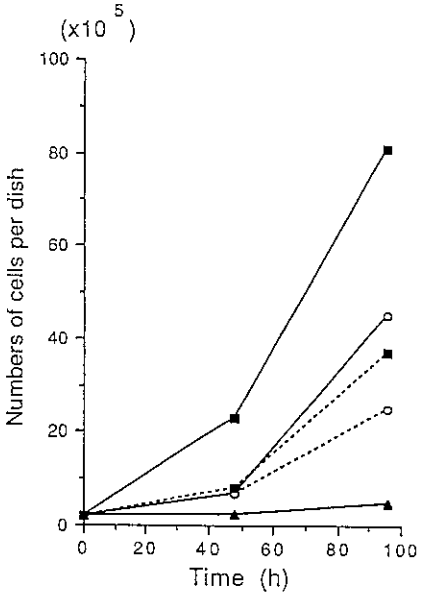

Fig. 5. Growth rates of sarcomas and HCC cells in culture with or without But2cAMP. Sarcomas 1 (Sa.1) and 2 (Sa.2) were established from different tumors. HCC is a cell line established from an HCC of the same transgenic mouse line. Note much higher growth rates of sarcoma cell lines than HCC. On But2cAMP treatment of sarcoma cells, growth retardation is evident. —○— Sa.1, ····○···· Sa.1 treated with But2cAMP. —■— Sa.2, ····■···· Sa.2 treated with But2cAMP, —▲— HCC.

(Type A) (Fig. 6a) or large polygonal cells (Type B) (Fig. 6b). On But2cAMP treatment, a shift from Type B to Type A cells was observed (Fig. 6c) in association with marked growth inhibition (Fig. 5). When inoculated into nude mouse subcutis (1.0×10^7 cells), all the sarcoma cell lines formed palpable tumors within two weeks.

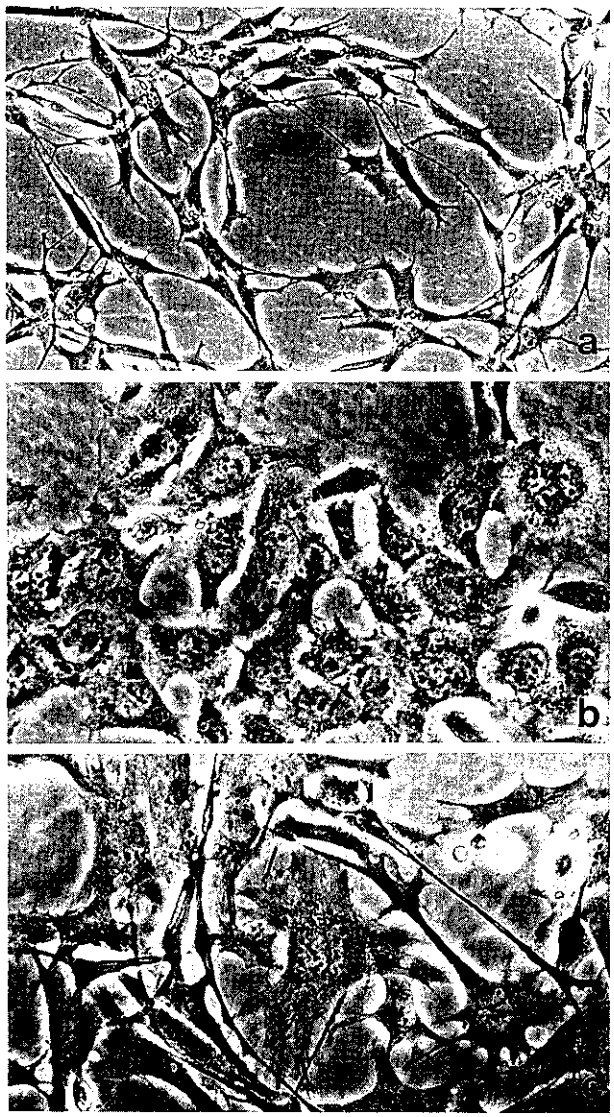

Fig. 6. Morphology of sarcoma cell lines. (a) Small spiny cells (Type A) (×20) or (b) large polygonal cells (Type B) (×40). (c) On But2cAMP treatment, Sarcoma-2 cells shifted to Sarcoma-1 cells. (×40)

Catecholamine contents in cell lines The results are summarized in Table I. Adrenalin was detected in sarcoma cell lines 1 and 2 at the concentrations of 29 and 73 pg/g protein, respectively, whereas noradrenalin was detected in sarcoma cell lines 1, 2, 3, and 4 at the concentrations of 86, 200, 114, and 86 pg/g protein, respectively. In contrast, all these catecholamines were below the detection limit in HCC and fibroblast cell lines.

Immunohistochemical examination The tumor cells, both *in vivo* and *in vitro*, were positively stained for S-100

Table I. Results of Catecholamine Analysis

	Adrenalin	Noradrenalin	Dopamine
Sarcoma 1	29	86	—
Sarcoma 2	73	200	—
Sarcoma 3	—	114	—
Sarcoma 4	—	86	—
HCC	—	—	—
Fibroblast	—	—	—

Values are in pg/g protein. — means below the detection limit.

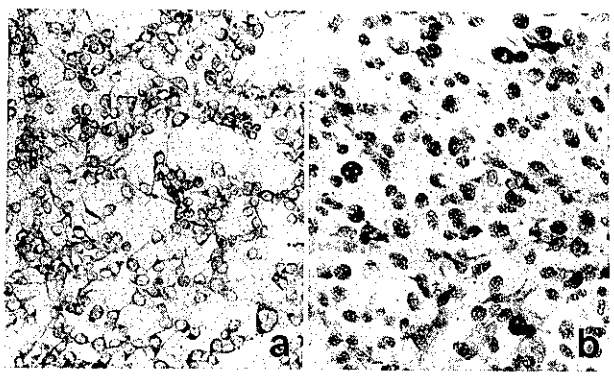

Fig. 7. Immunostaining of sarcoma cells showing NSE in the cytoplasm (a) and T antigen in nuclei (b). (×10)

protein and NSE (Fig. 7a) but were negative for keratin, vimentin, desmin, and AFP. These results strongly indicated neural and/or schwannian differentiation of the tumor cells and virtually ruled out the possibility of metastatic hepatocellular carcinomas. The NSE concentrations in the medium with these cells were 2- to 20-fold higher that those in the medium with HCC or fibroblast cell lines (data not shown).

Expression of T antigen and albumin On immunohistochemical staining, T antigen was always distinctly positive in the nuclei of subcutaneous sarcoma cells (Fig. 7b), HCC cells, and hepatocytes, both *in vivo* and *in vitro*. Among the various organs, T antigen expression was noted in nuclei of occasional distal renal tubules (Fig. 8) and juxta-forestomach mucus glands (Fig. 9). Detailed microscopical studies also revealed the very occasional existence of small clusters of both SV40 T antigen and S-100 protein-positive cells in the subcutis of adult mice free from sarcomas (Fig. 10). Albumin was positively stained only in hepatocytes.

Northern blot analysis of T antigen and albumin expression HCCs as well as liver tissue expressed both the T antigen and albumin mRNAs, whereas subcutaneous sarcomas expressed only T antigen mRNA (Fig. 11). The

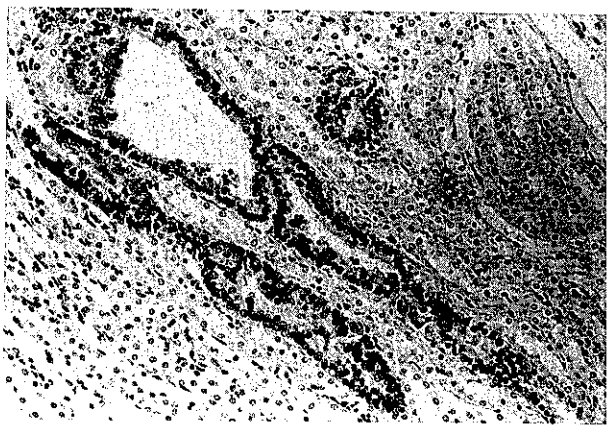

Fig. 8. Immunostaining showing T antigen in nuclei of several distal and/or collecting tubuli of the kidney. (×20)

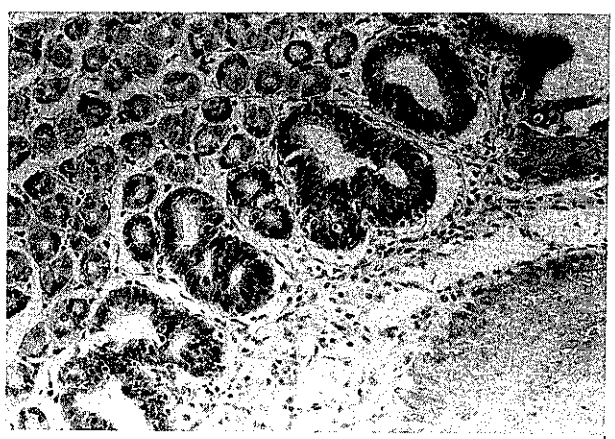

Fig. 9. Immunostaining showing T antigen expression in nuclei of the gastric mucus gland mucosa located near the forestomach. (×20)

T antigen mRNA was not detectable in other organs including lung, heart, spleen, brain, spinal cord, kidney, and subcutaneous tissue (data not shown).

CAT assay A representative result is shown in Fig. 12. Although variation was observed among individual sarcoma cell lines and CAT activities were demonstrated only at relatively low levels, all of them showed CAT activities, indicating the existence of transcriptional factor(s) acting on the albumin promoter.

DISCUSSION

The present results demonstrate a relatively frequent development of subcutaneous sarcomas in a transgenic mouse strain harboring albumin promoter-regulated

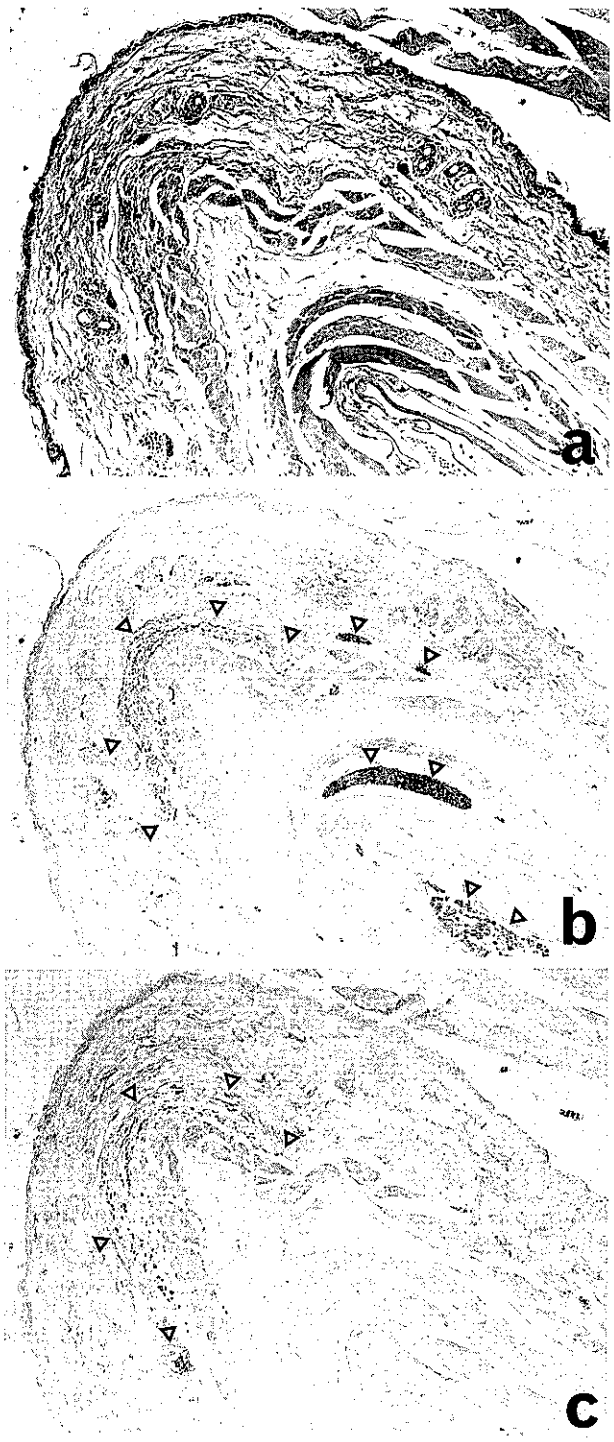

Fig. 10. T antigen-expressing cells in normal-appearing subcutaneous tissue. Serial sections stained with (a) HE or for (b) S-100 protein, or (c) T antigen. Note only a small part of the S-100 protein-positive nerve bundles shown in (b) (open arrows) contain T antigen-positive cells in (c) (open arrows).

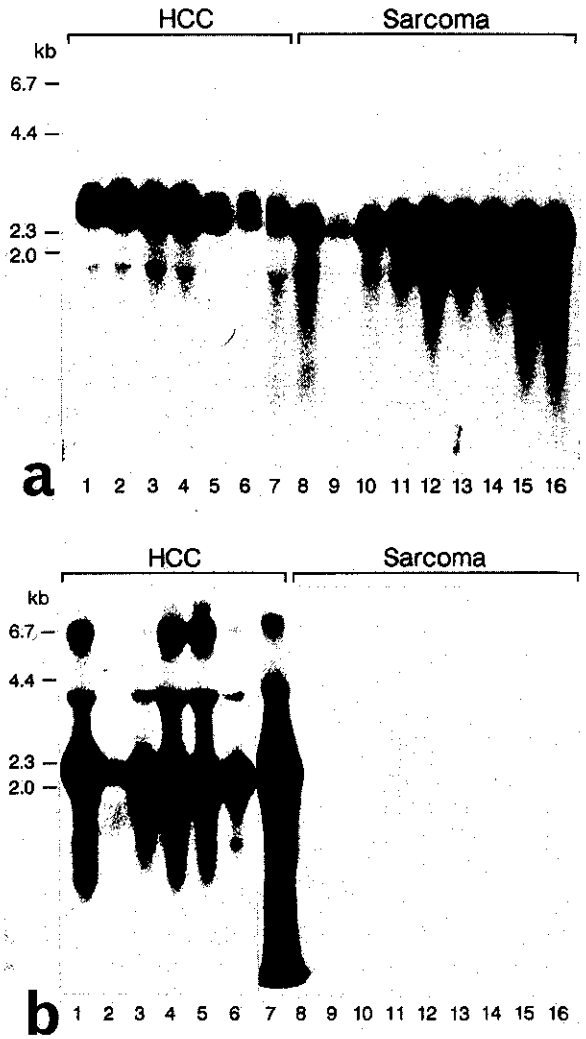

Fig. 11. Northern blot showing the expression of T antigen (a) and albumin (b) in SV40 T transgenic mouse HCCs (lanes 1–7) and subcutaneous sarcomas (lanes 8–16). Note remarkable T antigen but no albumin expression in all sarcomas.

SV40 T antigen gene. These tumors were characterized as neurogenic in nature on the basis of several lines of evidence, including Homer-Wright type rosette formation in histology, dense core granules in electron microscopy, expression of neuron specific enolase, S-100 protein and catecholamines, and nerve cell-like differentiation in culture in response to But2cAMP. Further, normal-appearing neurogenic cells with T antigen expression were identified in the transgenic mouse subcutis as possible progenitors of the sarcomas.

Sarcomas or non-epithelial tumors have been noted in transgenic mice harboring various transgenes[26]: fibrosar-

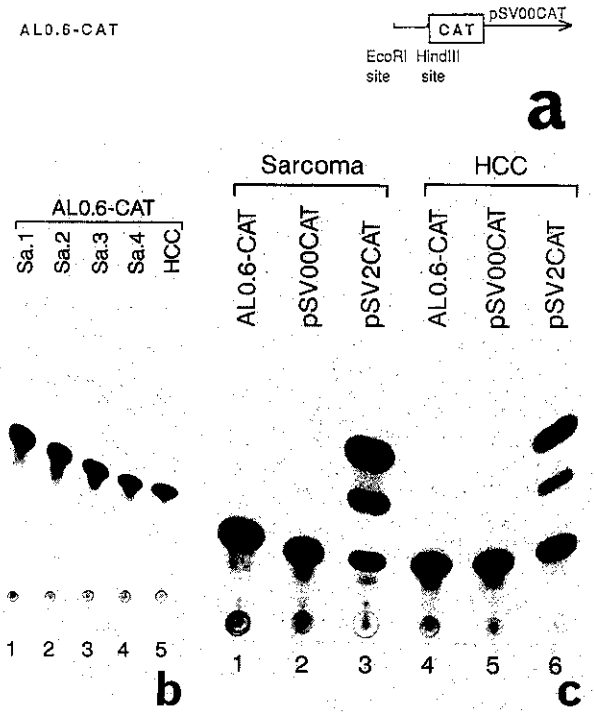

Fig. 12. CAT expression in cell lines. Transient expression analysis was carried out by transfecting the cells with AL0.6-CAT (a). CAT activity was evident in all the sarcoma lines (lanes 1–4) at similar levels to those in HCC cells (lane 5) (b). Another representative CAT assay autoradiograph is shown in (c). CAT activity was demonstrated only at relatively low levels with plasmid AL0.6-CAT, but did exist. pSV2CAT and pSV00CAT were used as positive and negative controls.

comas in BPV-mice,[27,28] Kaposi sarcomas or neurofibromas in tax-HTLV-1 LTR-mice,[29,30] endothelial cell tumors or angiosarcomas in PM-mT of Dy-mT-HSV-TK or human proto-ras-mice,[31–33] and osteosarcomas in c-fos-metallothionein promoter mice.[34,35] To our knowledge, however, this is the first report of such occurrence of neurogenic sarcomas.

Since all these neurogenic sarcomas strongly expressed T antigen both *in vivo* and *in vitro*, it may have been the principal causal agent. The stochastic mode of tumor development, however, indicated a multistep process of sarcomagenesis, as seen in hepatocarcinogenesis in the same mouse system.[6,7] In this transgenic mouse, T antigen expression was also seen in occasional distal tubules of the kidney and in the gastric mucosa, in addition to hepatocytes and the above-mentioned subcutaneous neurogenic cells. T antigen expression in the kidney and stomach is in agreement with previous studies detecting albumin expression in these organs.[12–14] Albumin expression in the subcutaneous tissue or in peripheral neurogenic cells, however, was never observed in those studies.[12] We also failed to detect T antigen mRNA expression in the subcutaneous tissue. These negative results, however, should be interpreted with caution because the T antigen-expressing cells comprise only a very small population of the tissue, as shown immunohistochemically in the present study.

Aberrant expression of hybrid genes containing the SV40 T antigen gene in association with tumors or hyperplasias has been reported previously.[1–5] Such unexpected transgene expression may be the result of 1) a previously unknown specificity of the promoter used,[1,4,5] or 2) a novel regulatory element(s) generated by the juxtaposition of the promoter next to the T antigen gene[36] or to the host flanking sequence, or 3) a positional effect of host regulatory gene(s) neighboring the transgene.[37–39] The albumin promoter used in the present transgenic mouse contains sufficient regions for tissue-specific expression of the mouse albumin gene[10,11,40–45] and, indeed, is strongly activated in hepatocytes, causing hepatocellular carcinomas.[6] Since we detected by CAT assay transcriptional factors acting on the albumin promoter in the tumor cells, the first possibility would appear most likely in the present case. Although albumin was negative in the tumor cells, our methodologies were not sensitive enough to rule out low-level albumin expression in these cells. Alternatively, it is possible that in these cells negative regulatory factors for albumin gene expression might be at work in the promoter construction upstream of the transgene, the binding site interacting with the negative factors having been deleted. Theoretically, the two other possibilities regarding aberrant gene expression can not be ruled out, assuming that cellular specificity to bring forth specific gene expression stems from juxtapositional or positional mechanisms.

Whatever the case, the present observations indicated the existence of specifically differentiated neurogenic cells in the transgenic mouse subcutis, in which aberrant expression of the trangene occurred. Whether the presence of such cells is limited to this particular transgenic mouse line or is more ubiquitous in the mouse or other animals requires further investigation. Studies in other transgenic mouse lines harboring the albumin promoter and oncogenes, including the SV40 T gene, are needed. In the four lines generated by Sandgren and his colleagues, development of subcutaneous sarcomas was not observed.[46] The founder mouse lines generated on the same occasion with the same transgene together with the present transgenic mouse line in Aizawa's laboratory were unfortunately not maintained.

(Received November 12, 1993/Accepted March 3, 1994)

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