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# Hepatocarcinogenesis in Transgenic Mice Carrying Albumin-promoted SV40 T Antigen Gene

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**We have developed transgenic mice that inherit albumin promoter-regulated simian virus 40 (SV40) large T antigen gene, expressed specifically in hepatocytes. These mice all develop multifocal hepatocellular carcinomas at around 5 months and die of liver insufficiency by 7 months. Sequential morphological observation of hepatocarcinogenesis revealed 5 distinct stages: (I) newborn to 2 weeks of age, neither recognizable histological changes nor cellular replication in spite of T antigen expression; (II) between 3 and 7 weeks, diffuse cytomegalic change of hepatocytes with numerous abnormal mitoses, usually resulting in cell death; (III) from 7 weeks onwards, quasi-regenerative small hepatocyte foci with a decreased tendency for cytomegaly in spite of T antigen expression, rapidly replacing the hepatic tissue; (IV) 11 weeks of age and thereafter, neoplastic foci and nodules with enzymatic alteration; (V) 20 weeks of age and thereafter, gross hepatocellular carcinomas with occasional pulmonary metastases. Considerable variation existed both in morphological and enzymatic features and T antigen expression among neoplastic lesions, including carcinomas. Thus, these transgenic mice clearly show a multistep process in hepatocarcinogenesis with remarkable synchrony and provide a promising model for analyzing the essential events of carcinogenesis at different stages.**

Key words: Multistep carcinogenesis — Hepatocarcinogenesis — Transgenic mouse — SV40 T antigen — Albumin

SV40 T antigen is well-known as a very potent viral transforming protein which immortalizes and transforms cells in culture[1] and causes malignant tumors in transgenic mice.[2] T antigen alone, however, appears insufficient for causing true malignant change, because in culture, it requires a long period of time for the immortalized cells to acquire true malignant potential and in transgenic mice multistep carcinogenesis was recognized.[1,2]

We produced a transgenic mouse line carrying an albumin promoter-regulated SV40 T antigen gene expressed specifically in hepatocytes. As described previously in a meeting abstract,[3] this transgenic mouse (T-mouse) revealed a distinct multistep progression of hepatocarcinogenesis, resulting in multiple hepatocellular carcinomas at around 6 months with amazing synchrony. In this T-mouse system observations have also been made concerning H-*ras* activation and chromosomal changes during carcinogenesis.[4–6]

In the present paper we describe detailed histological and histochemical observations in this useful system for analyzing the underlying mechanisms of multistep carcinogenesis.

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## MATERIALS AND METHODS

**Construction of the albumin-T antigen hybrid gene** A PUC 19-derived plasmid containing an *Mae*I fragment (−630 to +22) of the mouse albumin promoter gene at the *Sma*I site was kindly provided by Dr. Ueli Schibler.[7] The plasmid was digested with *Eco*RI and *Bam*HI, and the albumin promoter gene was inserted into the *Eco*RI-*Bgl*II site of the plasmid pMT-T,[8] yielding the plasmid pAlbT. The SV40 sequence contains the transcription start site and ATG codon, but lacks TATA box, promoter and enhancer domains. Hybrid DNA (Alb-T) is composed of the 5′ upstream promoter region of the mouse albumin gene, and the coding and 3′ downstream termination regions of SV40 early gene (Fig. 1).

**Microinjection** The recombinant plasmid was digested with *Eco*RI and *Bam*HI to remove the vector sequence (Fig. 1). Then the DNA solution [1 μg/ml in 10 m*M* Tris HCl (pH 7.4), 0.25 m*M* EDTA] was microinjected into male pronuclei of F2 zygotes between B6 and SJL mice as previously described.[9] In total, 250 fertilized zygotes obtained from mating of F1 (B6×SJL)×F1 (B6×SJL) mice were microinjected and transferred into oviducts of pseudopregnant CDI females.

**DNA and RNA analysis** For Southern blot and Northern blot analysis, total DNA and RNA were extracted

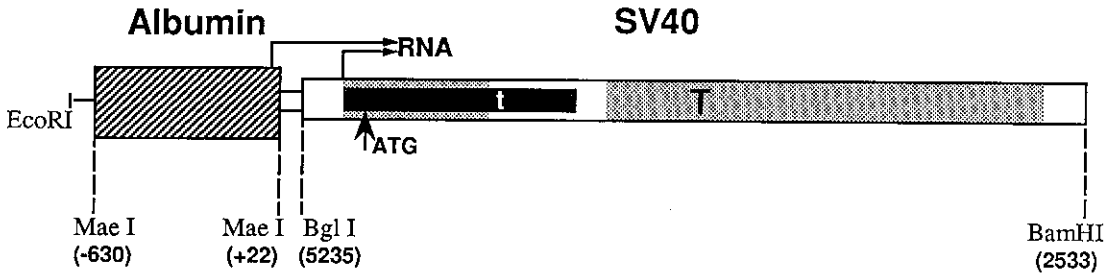


Fig. 1. Structure of the hybrid albumin-SV40 T antigen gene. The hatched box denotes mouse albumin promoter gene and the open box SV40 early gene, in which the coding sequences of large T and small t antigen genes are indicated by the striped and the filled boxes, respectively. The restriction sites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

from tail and/or liver, lung, pancreas, spleen and kidney as previously reported.[10, 11] The $^{32}$P-labeled probe used was the multiprimered *Hpa*I fragment (2666–3733:1067 bp) of SV40 T antigen gene.

**Histology** For sequential morphological observation in the liver of the AT-1 lineage (T mice), tissue samples were taken at 0, 2, 3, 4, 5, 7, 10, 12, 15, 20 and 24 weeks of age, from at least 4 animals at each time point, including both sexes. Transgene-negative littermates of mice were analyzed in parallel as controls for the different stages of tumorigenesis. Tissue were fixed in 10% formalin and routine histological examination was carried out on hematoxylin and eosin-stained paraffin sections.

**Enzyme histochemical and immunohistochemical analysis** Frozen sections, 12 $\mu$m thick, were made in a cryostat and glucose-6-phosphatase (G-6-P)[4] and $\gamma$-glutamyltranspeptidase (GGT) activities were demonstrated by the methods of Wachstein and Meisel[12] and Rutenburg *et al.*,[13] respectively. For immunostaining of T antigen, the liver was perfused *in situ* with 10% phosphate-buffered formalin, 5 ml/min, at room temperature for 1 h. The tissue was then sectioned, prossed in an alcohol-chloroform series and embedded in soft paraffin at 52–54°C. The standard avidin-biotin-horseradish peroxidase method was employed (Vectastain ABC KIT, Vector Laboratories Inc., Burlingame, CA). A monoclonal antibody against T antigen (PAb101) was a generous gift of Dr. N. Yamaguchi (The Institute of Medical Science, The University of Tokyo) and the biotin-conjugated mouse anti-rabbit IgG was purchased from Vector Laboratories Inc. The 4 $\mu$m sections were incubated with the first antibody for 2 h at room temperature.

[4] Abbreviations used: GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; G-6-P, glucose-6-phosphatase; GGT, $\gamma$-glutamyltranspeptidase.

## RESULTS

**Establishment of the transgenic mouse lines** Forty-seven pups were born and six animals harbored the intact transgenes. Those transgenic mice were designated AT1 through AT6. One of the founders (AT5) was sterile, and one (AT6) did not transmit the transgene to its offspring. In the others (AT1–4), the albumin promoter-T antigen gene was passed to the offspring. Unexpected pancreatic tumors of islet cell origin developed in one line (AT2) of mice. In founders AT1, 3 and 4 and their offspring, however, the tumors developed in the liver exclusively. In this study, we analyzed only the AT1 line (designated T-mouse in this paper) in detail, because the other 2 lines (AT3 and 4) showed basically the same processes with AT4 surviving slightly longer (up to 10 months) than AT1 and AT3. Descendants of the T-mouse were produced by crossing to normal B6 (+/+). Approximately 50% of the offspring were affected, as expected for dominant Mendelian inheritance.

In the T-mouse, one band (14 kb) or two bands (4.0 kb and 7.6 kb) were observed on Southern blotting of the DNA after digestion with *Bam*HI or double digestion with *Bam*HI and *Eco*RI (data not shown), respectively. These two bands were stable and not segregated during transmission, indicating a single integration site of the transgene. The T antigen gene was expressed specifically in hepatocytes from birth (Fig. 2a, b), although expression at the embryonic stage was not examined.

**Sequential morphological and biochemical observation of hepatocarcinogenesis** Up to 2 weeks of age, no recognizable histological change was observed in the T-mouse liver in comparison with the normal counterpart (non transgenic litter mate), although T-antigen was clearly expressed in all of the hepatocyte nuclei (Fig. 3). Mitotic indices of T-mouse and normal mouse hepatocytes at the

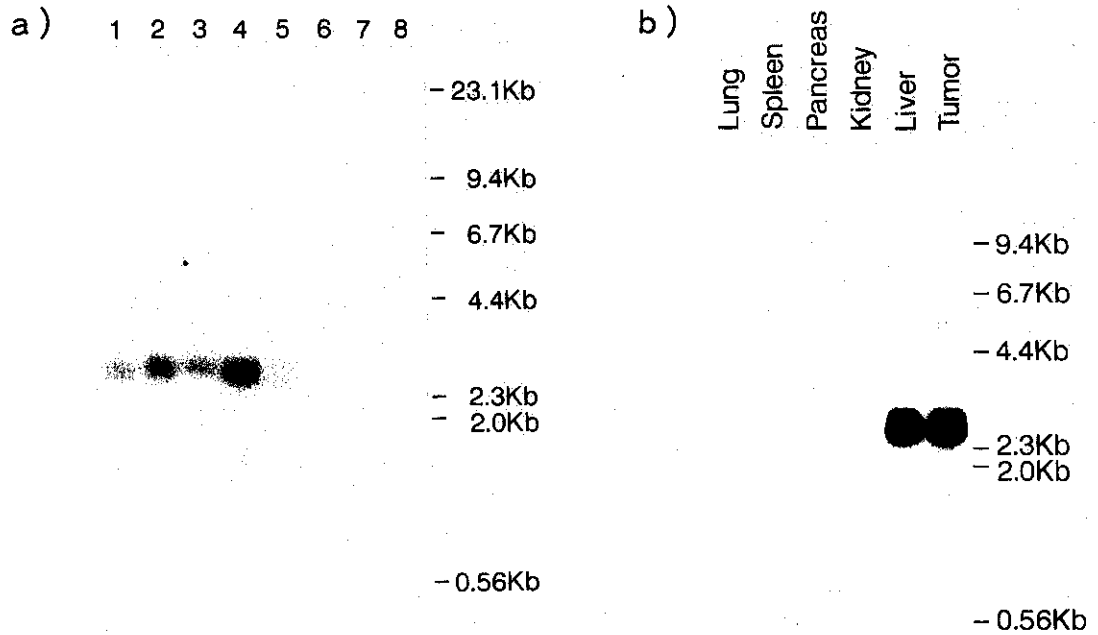


Fig. 2. Northern blot analysis on expression of T antigen in T-mice. (a) Day of birth (liver). Lanes 1–5 are T-mice. Lanes 6–8 are transgene-negative littermates. (b) T antigen is expressed specifically in liver. Tumor and liver indicate HCC and surrounding liver tissue at 5 months of age, respectively.

Table I. Mitotic Indices of T-Mouse and Normal Mouse Hepatocytes at the 2nd Week

| Animals | Tag | No. nuclei counted | No. of mitoses[a)] | % | Mean±SD (%) |
|---|---|---|---|---|---|
| 1 | + | 2901 | 11 | 0.38 | |
| 2 | + | 3122 | 8 | 0.26 | 0.330±0.063 |
| 3 | + | 1714 | 6 | 0.35 | |
| 4 | − | 1687 | 6 | 0.36 | |
| 5 | − | 1562 | 7 | 0.45 | 0.395±0.087 |
| 6 | − | 2433 | 7 | 0.29 | |
| 7 | − | 1451 | 7 | 0.48 | |

*a*) Metaphase and anaphase.

end of the 2nd week about the same, 0.330±0.063 and 0.395±0.087%, respectively (Table I).

During the 3rd week, hepatocytes with enlarged nuclei began to appear randomly in the lobules. This cytomegalic change became prominent in most hepatocytes in subsequent weeks (Fig. 4). Many abnormal mitoses and necrotic single cells were scattered, reflected in high serum transaminase level (700–800 IU of GOT and GPT). Nuclei of the cytomegalic cells were huge and often bizarre in shape with coarse granular heterochromatin and multiple small nucleoli. Although the atypia of these cells was striking, they did not behave as malignant cells, and the normal architecture of the liver tissue was maintained. T-Antigen was demonstrated weakly in the nuclei.

From 7 weeks onwards, foci of quasi-regenerative hepatocytes appeared randomly in the hepatic lobules (Fig. 4 arrowhead), rapidly replacing the cytomegalic hepatocytes so that most of the liver tissue was replaced by the new cells by 12 weeks (Fig. 5a). T-Antigen was expressed in these cells to the same degree as seen in the original hepatocytes in the initial weeks, whereas it was very weak or not demonstrated in the original hepatocyte-derived cytomegalic cells at this phase (Fig. 5b). Neither ductal nor periportal "oval cell" proliferation was observed.

From 11 weeks of age, foci showing a distinctly nodular growth pattern appeared. They were comprised of eosinophilic, basophilic, or clear cells with some structural atypia (Fig. 6a, b). While regenerative hepatocytes showed normal levels of G-6-p activity, the cells of foci were characterized by reduced levels of this enzyme (Fig. 7a), or occasionally by the appearance of GGT activity (Fig. 7b), reminiscent of altered cell foci seen in rodent chemical carcinogenesis.[14] In these foci, T antigen was expressed generally at a moderate level but occasionally at higher or reduced levels. The lesions consisting of hepatocytes with eosinophilic hepatocytes tend to show

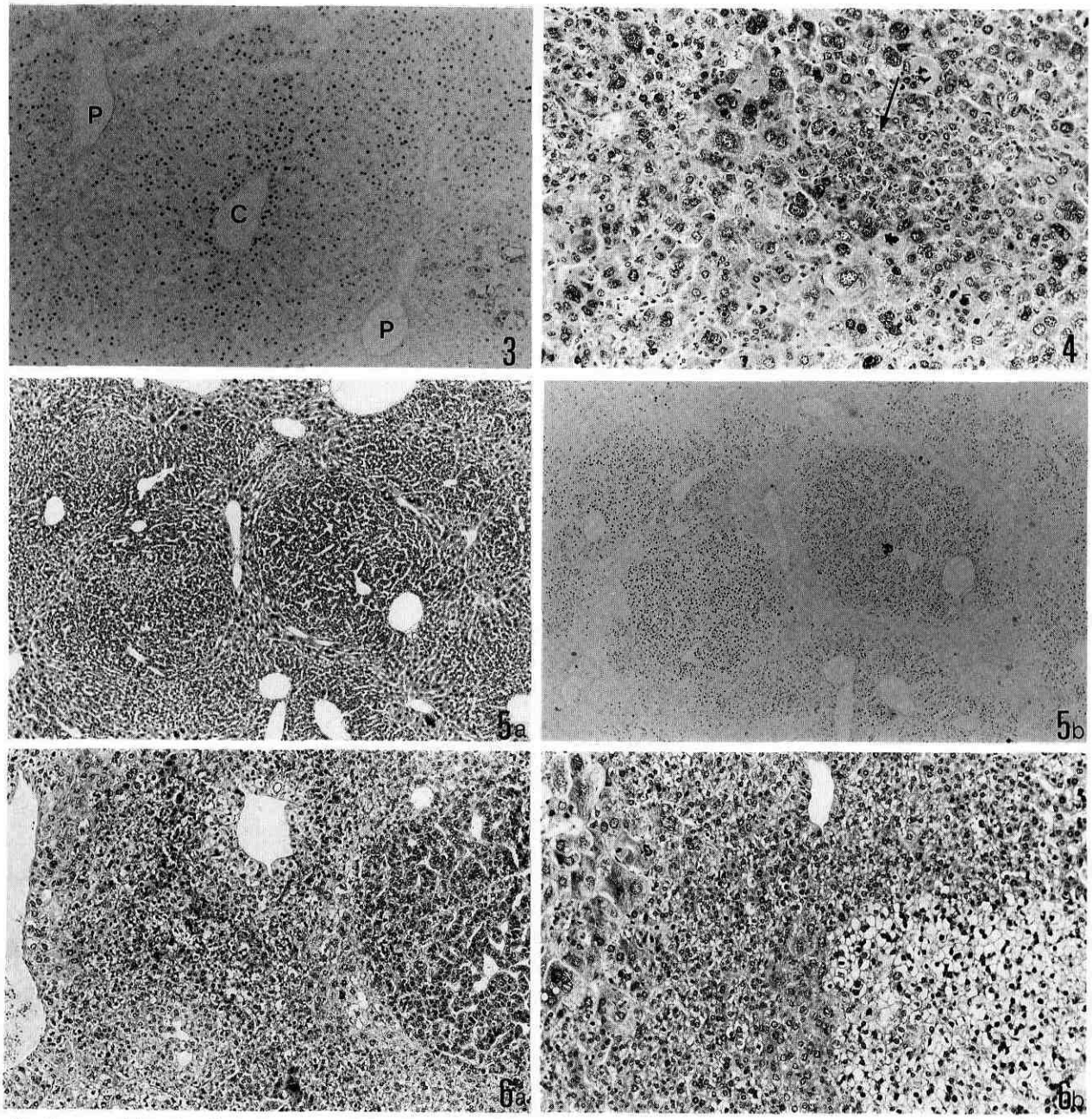


Fig. 3. Immunohistochemical demonstration of SV40 T antigen in the liver of T-mouse at 3 weeks of age. Nuclei of all the hepatocytes are stained positive. Note the normality of the liver tissue with uniform size of hepatocyte nuclei. The nuclei located around the central vein (C) present stronger reaction. P indicates the portal vein. ×120.

Fig. 4. Liver of T-mouse at 7 weeks of age showing striking cytomegalic change of hepatocytes. Note scattered atypical mitotic figures and a tiny focus of quasi-regenerative hepatocytes with small nuclei (arrowhead). HE, ×150.

Fig. 5. (a) Liver of T-mouse at 12 weeks of age. Foci of quasi-regenerative hepatocytes are replacing the cytomegalic hepatocytes. HE. ×50. (b) A serial section showing distinct expression of T antigen in quasi-regenerative hepatocytes and very weak reaction in cytomegalic original cells.

Fig. 6. Liver of T-mouse at 12 weeks of age. Picture showing foci comprised of basophilic cells (a) or clear cells (b), with background of regenerative and cytomegalic hepatocytes. HE, ×100 and ×150, respectively.

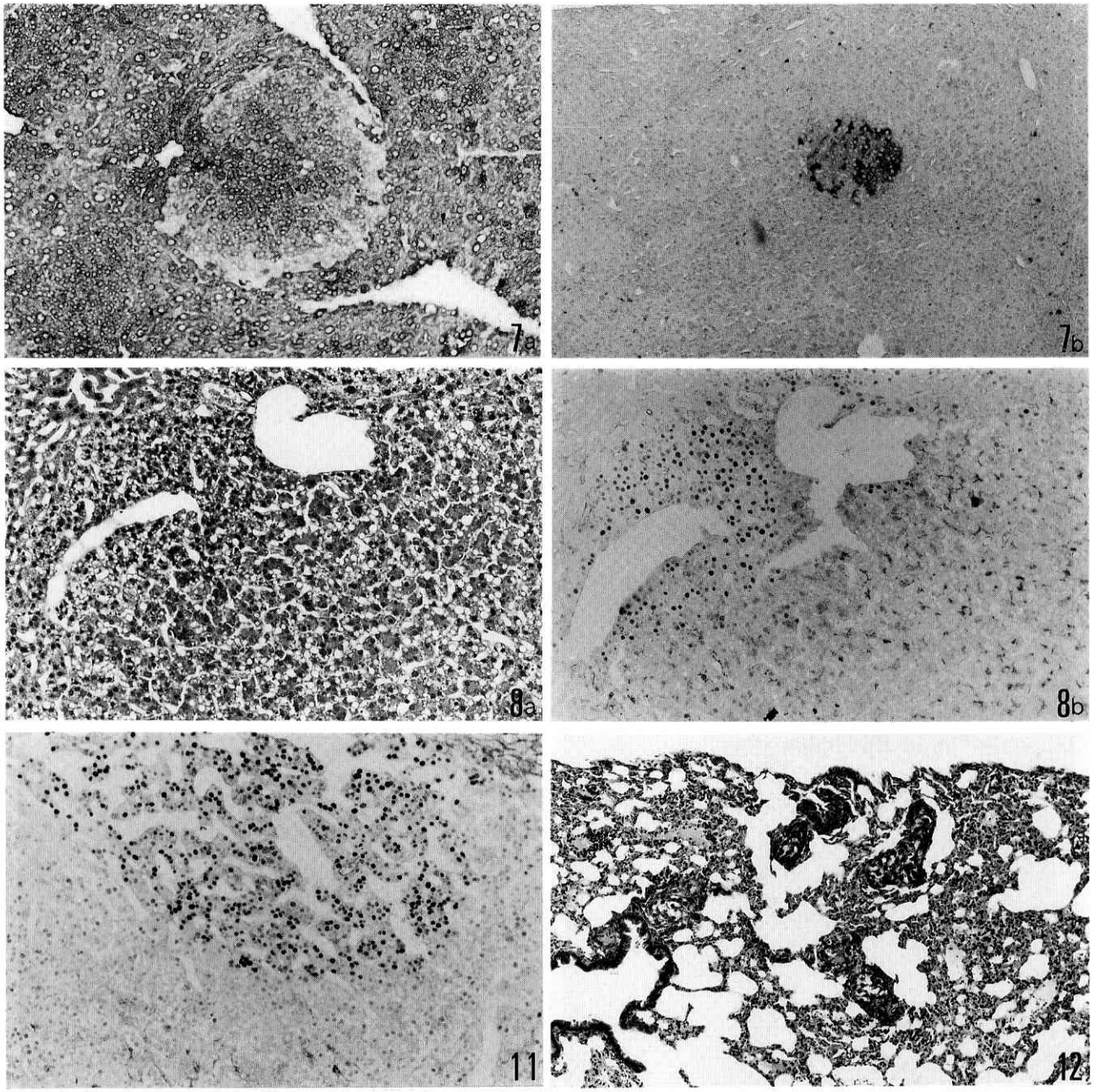


Fig. 7. Pictures showing a G-6-P-deficient focus (a) and a GGT-positive focus (b) developed by 12 weeks of age. ×100 and ×60, respectively.

Fig. 8. Serial sections demonstrating very weak T antigen expression in a focus comprised of eosinophilic cells. (a) HE staining, (b) immunohistochemistry of T antigen. ×120.

Fig. 11. Distinct difference in T antigen expression between two neoplastic lesions at 21 weeks of age. The one with trabecular structure and high T antigen expression was a microscopic focus found in a fingertip-sized clear cell node. ×150.

Fig. 12. Microscopic foci of pulmonary metastasis at 24 weeks of age. HE, ×100.

Fig. 9. Macroscopical liver tumor of a T-mouse at 6 months of age.

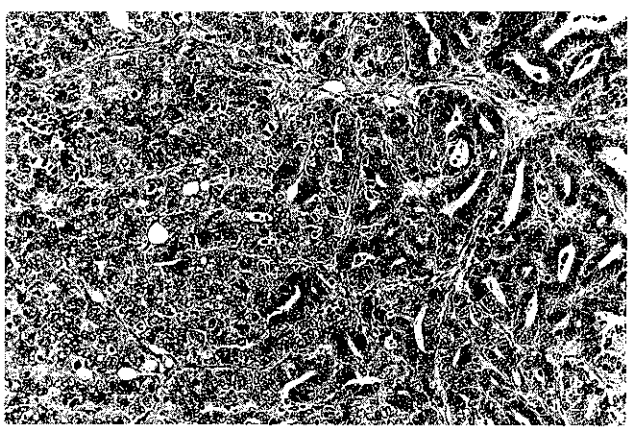

Fig. 10. Histology of a hepatocellular carcinoma showing solid, trabecular and psudoglandular structures. HE, ×150.

reduced levels, or absence, of T-antigen expression (Fig. 8a, b).

From around 20 weeks of age, gross hepatic tumors developed and the animals died by 7 months with bulky liver, more than 10 times normal size, filled with numerous tumors measuring up to 30 mm in diameter (Fig. 9). Many of the tumors were composed of cells similar to those of foci, but there were also overt hepatocellular carcinomas (HCCs) showing distinct cellular and structural atypia (Fig. 10). Occuasionally nodule-in-nodule and/or cancer-in-nodule patterns were observed (Fig. 11). Microscopic pulmonary metastases were detected, though infrequently (Fig. 12).

A tendency toward cytomegalic change was also seen in some of the quasi-regenerative and neoplastic cells, but it was generally much less conspicuous than that seen in the original hepatocytes. T-antigen, as measured previously by mRNA level,[4] was variable among gross nodules and HCCs (Fig. 11).

These sequential observations were consistent in subsequent generations and were not influenced by the sex of the host. Non-transgenic littermates did not show any significant histological abnormalities up to at least one year of age.

## DISCUSSION

We have described the multistep progression of hepatocarcinogenesis in our T-mouse system, i.e., initial cytomegalic degeneration followed by replacement by resistant cell populations, development of enzyme-altered foci and nodules reminiscent of those induced by chemical carcinogens[14] and finally carcinomas. In the literature, four groups have so far described hepatocarcinogenesis in SV40 T antigen-harboring mice.[15-18] In other reports, the SV40-T antigen gene was fused to the regulatory sequence of metallothionein,[15] major urinary protein,[16] $\alpha$-1-antitrypsin[17] and albumin,[18] respectively. Although the time span for development of tumors varied considerably, ranging from 7 weeks to over 1 year, even among founder mice of one and the same group, according to the difference in copy number,[15, 16, 18] there seems good similarity among observations in representative transgenic lines of individual groups describing the carcinogenic sequence starting from hyperplasia through dysplasia, nodule and carcinoma.[16-18]

In our T-mouse system, however, we were not impressed by initial "hyperplasia," since there was no difference in the mitotic index between T-mouse liver and its counterpart at the 2nd week, when there was no histological difference between them in spite of distinct T antigen expression in the former. What impressed us strongly was the rapid development of cytomegalic degeneration in the subsequent weeks, associated with considerable single cell necrosis and high serum transaminase level. We did observe numerous mitotic figures (almost all abnormal) of hepatocytes at this phase, a phenomenon that we were inclined to interpret as the response to the degeneration-provoked replication drive.

It is well known that toxicity-dependent degeneration-regeneration enhances both initiation and promotion processes during experimental and human hepatocarcinogenesis.[14, 19] Such a mechanism has also been shown recently in hepatocarcinogenesis of transgenic mice constructed with large S gene of human hepatitis B virus[20] and hereditary hepatitis of rats (LEC rat) with abnormal copper accumulation.[21] We consider that the initial event of hepatocarcinogenesis in SV40 T transgenic mouse systems would also be toxicity-dependent cell death and degeneration, apparently with SV40-T antigen acting as a cytotoxin.

Apart from the cytotoxicity, T antigen may also be acting as a mutagen. It has been reported recently that SV40 T antigen induces karyotypic instability and chromosomal aberrations.[5, 22, 23] As described in "Results," the quasi-regenerative cell populations appearing haphazardly among cytomegalic cells were less sensitive to the toxicity of T antigen, although the antigen was distinctly expressed. These cells might, therefore, be resistant mutants induced by the mutagenicity of T antigen and might have a selective growth advantage. Subsequent induction of enzyme-altered (neoplastic) foci and tumors might also be, to a certain extent, a function of the mutagenicity of T antigen.

Our sequential observations revealed that T antigen expression in the original hepatocytes becomes inconspicuous as the cytomegalic change proceeds (Fig. 5b). It would therefore be inadequate to describe the expression level of T antigen in various "dysplasias"[17] assuming the levels are fixed, or to describe the level in foci[16] in comparison with that of surrounding cytomegalic cells.

The ultimate role of T antigen in the neoplastic and/or malignant transformation in this system is not clear at present. Since T antigen expression was apt to be low or lacking in relatively mature neoplastic foci with eosinophilic cells, it may well be contributing to the enhancement of the malignancy of the lesions. On the other hand, T antigen expression was shown to be not essential in gross hepatic carcinomas.[4] There is now evidence that SV40 T antigen might exert its tumorigenic effect by complexing with products of anti-oncogenes, including the retinoblastoma and p53 genes.[24, 25] Although our preliminary studies using conventional Southern and Northern blotting revealed neither rearrangement nor deletion of these specific genes, search for loss of specific chromosomes in our T-mouse tumors is intriguing.[26]

Concerning the nature of events occurring during neoplastic progression, we have previously shown in our T-mouse system that c-H-*ras* point mutation at the second position of codon 61 was present in 10 out of 25 HCCs (40%) at 24 weeks of age and some tumors showed weak signals for mutated c-H-*ras*, implying that such tumors consisted of cells both with and without *ras* mutation, in other words, a mixed population of tumor cells.[4] Furthermore, we established 9 HCC lines from tumors at 24 weeks of age and found 3 out of 9 HCC lines to progress during culture over 6 months with respect to growth rate and growth in soft agar and in nude mice. This progression was associated with the appearance of activated c-H-*ras* oncogene.[5] These data indicate that c-H-*ras* activation may be an event occurring at a relatively late stage of hepatocarcinogenesis in our transgenic mouse system.

Thus, this transgenic mouse system provides a promising model for analyzing the essential events of hepatocarcinogenesis at different stages.

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