Transgene Expression and Repression in Transgenic Rats Bearing the Phosphoenolpyruvate Carboxykinase-Simian Virus 40 T Antigen or the Phosphoenolpyruvate Carboxykinase-Transforming Growth Factor- α Constructs

Michael J. Haas, Yvonne P. Dragan, Hiroshi Hikita, Randee Shimel, Koichi Takimoto, Susan Heath, Jennifer Vaughan, and Henry C. Pitot

From the McArdle Laboratory for Cancer Research, Departments of Oncology and Pathology, The Medical School, University of Wisconsin, Madison, Wisconsin

Transgenic Sprague-Dawley rats expressing either human transforming growth factor- α (TGF α) or simian virus 40 large and small T antigen (Tag), each under the control of the phosphoenolpyruvate carboxykinase (PEPCK) promoter, were developed as an approach to the study of the promotion of hepatocarcinogenesis in the presence of a transgene regulatable by diet and/or hormones. Five lines of PEPCK-TGF α transgenic rats were established, each genetic line containing from one to several copies of the transgene per haploid genome. Two PEPCK-Tag transgenic founder rats were obtained, each with multiple copies of the transgene. Expression of the transgene was undetectable in the TGF α transgenic rats and could not be induced when the animals were placed on a high-protein, low-carbohydrate diet. The transgene was found to be highly methylated in all of these lines. No pathological alterations in the liver and intestine were observed at any time (up to 2 years) during the lives of these rats. One line of transgenic rats expressing the PEPCK-Tag transgene developed pancreatic islet cell hyperplasias and carcinomas, with few normal islets evident in the pancreas. This transgene is integrated as a hypomethylated tandem array of 10 to 12 copies on chromosome 8q11. Expression of large T antigen is highest in pancreatic neoplasms, but is also detectable in the normal brain, kidney, and liver. Mortality is most rapid in males, starting at 5 months of age and reaching 100% by 8 months. Morphologically, islet cell differentiation in the tumors ranges from poor to well differentiated, with regions of necrosis and fibrosis. Spontaneous metastasis of Tag-positive tumor cells to regional

lymph nodes was observed. These studies indicate the importance of DNA methylation in the repression of specific transgenes in the rat. However, the expression of the PEPCK-Tag induces neoplastic transformation in islet cells, probably late in neuroendocrine cell differentiation. T antigen expression during neoplastic development may result in a pervasive change in the islet cell growth properties with selection of a transformed phenotype as a possible requirement for cell viability. (Am J Pathol 1999, 155:183–192)

Numerous examples of transgenic carcinogenesis in mice have now been reported.^{1–3} However, transgenic carcinogenesis in other rodent species has remained relatively rare. Our laboratory reported in this journal several years ago the development of a transgenic strain of rats expressing the simian virus (SV)40 T antigen regulated by the albumin enhancer/promoter region of the mouse genome. These animals all developed hepatic neoplasms, and approximately one-third developed non-functioning islet cell neoplasms of the pancreas.⁴ As an extension of these studies, we have been able to develop several lines of transgenic rats, some with the phosphoenolpyruvate carboxykinase (PEPCK) promoter coupled to the transforming growth factor- α (TGF α) cDNA and others with the PEPCK promoter coupled to the SV40 T antigen gene. These lines of transgenic rats were developed in hopes that the expression of the transgene could be regulated by dietary and hormonal means as has been done with this promoter in several mouse transgenic lines.^{5,6} Transgenic mouse lines have been established that express TGF α under the control of various tissue-specific gene promoters.^{7–9} Hyperplasia and eventual carcinoma development was observed in or-

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Address reprint requests to Dr. Henry C. Pitot, Departments of Oncology and Pathology, McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, WI 53706. E-mail: pitot@oncology.wisc.edu.

gans that expressed the transgene. The SV40 T antigen (Tag) gene has been used extensively in the construction of transgenic animal models of carcinoma development.¹⁰⁻¹²

We developed constructs with either the human TGF α cDNA or the SV40 small and large TAG coding regions under the control of the rat phosphoenolpyruvate carboxykinase (PEPCK) 5'-flanking region. *cis* elements that mediate induction by gluconeogenesis and liver-specific expression are located in the proximal promoter of the PEPCK gene and have been used in transgenic mice.^{5,6} Although we were unable to discern a phenotype in transgenic rats that expressed the TGF α transgene, 100% of animals of one PEPCK-TAG transgenic rat line developed rapidly growing islet cell tumors. A morphological analysis and a characterization of the molecular changes inherent to the neoplasms of this line of transgenic rats are reported here.

Materials and Methods

Construction of the Transgenic Rats

The PEPCK-TGF α construct was generated by inserting the 617 bp *Bam*HI/*Bgl*II fragment of the rat PEPCK gene promoter⁶ into the *Bgl*II site of the plasmid pCMVTGF-Neo. This plasmid contains a cDNA of human TGF α flanked by the cytomegalovirus (CMV) immediate-early enhancer-promoter region and the SV40 late polyadenylation signal, and 5' to the former is the Neo cassette, with the whole construct inserted into the plasmid pBR322. The PEPCK-pCMVTGF sequence was removed from the plasmid by digestion with *Sal*I and *Pvu*II. The PEPCK-TAG transgene was constructed by ligating the same PEPCK promoter fragment into the *Stu*I/*Bam*HI site of the plasmid pSVA-1. This construct was prepared for microinjection by digestion with *Cla*I, followed by electrophoresis and purification of the linearized DNA. Diagrams of the two transgene constructs are seen in Figure 1. Microinjection of the transgene constructs into the pronucleus of Sprague-Dawley transgenic rats was done in a manner similar to that used to generate transgenic mice¹³ at the Wisconsin Biotechnology Center at the University of Wisconsin-Madison (Madison, WI). Animals with the PEPCK-TAG transgene were outbred as heterozygotes to minimize the effects of peripheral neuropathy.⁴

Southern Blotting, Northern Blotting, and RT-PCR Analysis

DNA and RNA were isolated from multiple tissues as described.¹⁴ Southern blot analysis using DNA digested with *Bam*HI was performed to examine the copy number and orientation of transgene arrays, whereas transgene methylation was assessed using the methylation-sensitive isoschizomers *Msp*I and *Hpa*II. Screening of PEPCK-TAG animals to determine carriers of the transgene was performed by polymerase chain reaction (PCR) as described,⁴ whereas Southern blotting was used to screen the PEPCK-TGF α animals. Northern blot analyses were

pPEPCK-TGF α

pPCKSVT

* Altered during construction.

■ 617 bp Rat PEPCK promoter.

□ Human TGF α cDNA.

■ Large and small TAG coding region.

Figure 1. Transgenes used in the construction of transgenic rats. The rat PEPCK promoter was ligated into either pCMVTGF-Neo (to construct pPEPCK-TGF α) or into pSVA-1 (to construct pPCKSVT) (see Materials and Methods). Restriction enzyme cleavage sites used to linearize the plasmid DNA before microinjection are shown at the 3' and 5' termini of the constructs. The TGF α cDNA was linked 3' to the SV40 polyadenylation signal.

carried out with probes labeled to high specific activity with ³²P by oligo-labeling, as has been described.¹⁵

Reverse transcriptase (RT)-PCR was performed for the assessment of minute levels of transgene expression. One microgram of total RNA was digested with RQ1 DNase for 30 minutes at 37°C in 1X PCR buffer (Perkin Elmer-Roche, Branchburg, NJ). The DNase was then inactivated and RNA denatured by heating the samples at 95°C for 5 minutes. At this time, random hexamers, dNTPs (200 μ mol/L), and 20 U of AMV or MULV RT were added, followed by an incubation at room temperature for 20 minutes and then at 37°C for 2 hours. The samples were again heated to 95°C for 10 minutes, at which time the remaining components (primers and *Taq* polymerase) and a small amount of [α -³²P]dCTP were added. The primers used to amplify a segment of the TAG mRNA were those used to screen the animals for the presence of the transgene as described above.¹⁶ Those used to amplify β -actin mRNA¹⁷ are as follows: 5'-GGTCT-CACGTCA GTG TACAGG-3' and 5'-CCGCAAATGCT-TCTAGGC-3'. Amplification was performed as follows: 95°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 2 minutes for 40 cycles. Twenty-five microliters of each sample was then fractionated on a 5% polyacrylamide gel in 1X Tris-buffered ethanolamine, dried, and exposed to Kodak XAR-5 film.

Immunohistochemistry

Sections of pancreas, liver, kidney, cerebrum, cerebellum, adrenal, testis, ovary, and lung were fixed in 10% neutral buffered formalin for 8 to 10 hours and used for routine hematoxylin and eosin (H&E) staining and immunostaining for TGF α and TAG. Sections were stained

using a modification¹⁸ of the method of Hsu et al.¹⁹ Briefly, endogenous peroxidase activity was blocked with 0.23% periodic acid in a PBS solution for 2 minutes. Endogenous biotin was blocked by incubation with a solution containing avidin (50 µg/ml) for 30 minutes. Non-specific protein binding was minimized by use of Blotto for 30 minutes (Zymed, South San Francisco, CA). The antibody was diluted appropriately in 1% bovine serum albumin and incubated with streptavidin bound to β-galactosidase (Sigma Chemical Co., St. Louis, MO). Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in dH₂O for 20 minutes. Antigen retrieval was performed as follows: for TGFα, 0.1 mol/L Tris/HCl, pH 9, with 5% urea; for SV40 TAG, 0.1 mol/L sodium citrate/HCl, pH 6, was used. TGFα primary antibody was applied at 1/50 in Blotto and SV40 TAG primary antibody at 1/25 in Blotto. The primary antibodies were incubated at 4°C overnight on the tissue sections. Biotinylated secondary goat anti-mouse IgG was applied at 1/200 in PBS for 30 minutes. The following conjugates were used: for TAG, X-avidin at 1/200 for 1 hour; for TGFα, streptavidin/horseradish peroxidase at 1/250 in PBS for 30 minutes. Slides were then stained with aminoethylcarbazole and subsequently counterstained with Mayer's hematoxylin. Slides stained for TAG were incubated with biotinylated tyramine at 1/50 in 0.05 mol/L Tris-buffered saline at pH 8 for 30 minutes, followed by a second incubation of streptavidin/horseradish peroxidase at 1/100 in PBS for 30 minutes. The chromogen aminoethylcarbazole was applied and then counterstained with Mayer's hematoxylin. A PBS wash of three changes for a total of 5 minutes was used between each of the steps. Liver sections from Alb-SV40 TAG transgenic rats⁴ were used as a positive control for TAG expression.

Fluorescence in Situ Hybridization (FISH)

The transgene-containing plasmid was nick translated in the presence of biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) and used as a probe. Lymphocytes obtained from transgenic rats were cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 2 mmol/L L-glutamine (Sigma), and penicillin plus streptomycin (Sigma) in a 37°C incubator and 5% CO₂. Lymphocytes were stimulated with a mixture of phytohemagglutinin (10 µg/ml; Life Technologies, Gaithersburg, MD), concanavalin A (3 µg/ml, type IV; Sigma), and lipopolysaccharide (3.3 µg/ml, *Escherichia coli* 0111-B4; Sigma) for 68 hours, at which time they were treated with colcemid (20 ng/ml; Life Technologies) for 1 hour. Metaphase spreads were prepared as described previously²⁰ and chromosomes stained with Giemsa as described elsewhere²¹ and photographed before *in situ* hybridization. The chromosomes were destained by washing with xylene/ethanol (1:1, v/v) and methanol/acetic acid (3:1, v/v) and post-fixed in 0.4% paraformaldehyde in PBS for 10 minutes. After washing in 2X standard saline citrate (SSC), FISH was performed as described^{22,23} with the following modifications. The DNA was denatured for 2 minutes in 70% formamide, 2X SSC

at 72°C, dehydrated in an ethanol series, and air dried. Hybridization of the probe was performed at 37°C in a moist chamber for 14 to 20 hours and then washed in 50% formamide, 2X SSC for 15 minutes at 37°C, in 2X SSC for 10 minutes at 47°C, and in 1X SSC for 10 minutes at 47°C. The slides were then incubated in 2% bovine serum albumin (Sigma), 4X SSC for 20 minutes at 25°C, and fluorescein isothiocyanate (FITC)-conjugated avidin (Oncor, Gaithersburg, MD) for 45 minutes at 37°C and then washed with 4X SSC for 8 minutes and then in 4X SSC, 0.05% Triton X-100 for 8 minutes at 25°C. Biotin-conjugated goat anti-avidin (Oncor) and FITC-avidin were used next to amplify the signal. Each incubation was followed by a wash in 4X SSC for 8 minutes and 0.05% Triton X-100 for 8 minutes, and a final wash in 2X SSC for 5 minutes. The chromosomes were counterstained and mounted in 90% glycerol, 8% PBS, 1 µg/ml propidium iodide (Sigma), and 2% 1,4-diazabicyclo-(2,2,2)-octane (Sigma) and visualized by epifluorescence microscopy on an Olympus microscope and filter set (470-nm excitation, 520-nm emission).

Protein Extract Preparation and Western Blotting

Tissue samples (100 mg), frozen in liquid nitrogen at the time of sacrifice, were pulverized and added to 10 ml of PBS containing aprotinin (10 µg/ml), leupeptin (10 µg/ml), and phenylmethylsulfonyl fluoride (1 mmol/L). Cells were homogenized and collected by centrifugation at 1000 × *g* for 5 minutes. The pellet was washed three times with PBS, and after the last wash, 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris/HCl, pH 8.0, 100 mmol/L NaCl, 1% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride) was added. Samples were rocked for 30 minutes at 4°C and transferred to a new tube, and particulate material was removed after a brief spin (12,000 × *g* for 10 minutes). Protein concentrations were determined by the method of Bradford²⁴ using bovine serum albumin as a standard. TAG and p53 proteins were immunoprecipitated from 50 µg of extract protein as previously described using the antibodies pAB419 and pAB421 (Oncogene Science, Uniondale, NY) to precipitate TAG and p53, respectively.²⁵

Results

Generation of Transgenic Rats

A 617-bp DNA fragment containing the PEPCK 5'-flanking region⁶ was fused 5'-proximal to the coding regions for both large and small TAG and for human TGFα cDNA and, after being linearized (Figure 1), was used to construct transgenic rats. DNA isolated from rat tails was screened by Southern blotting, and five pPEPCK-TGFα and two pPCKSVT (Figure 1) F0 founder animals were identified. All founder animals transmitted the transgene to progeny in a Mendelian fashion, but in the case of the TGFα and one TAG line, no transgene expression was

Table 1. Transgene Characterization

Line	Gene dosage*	DNA methylation*	Transgene expression	Chromosome localization
pPEPCK-TGF α				
FA	1	ND	—	ND
FB	1	ND	—	ND
FC	3–4	+++	—	13q22
FD	3–4	+++	—	17p12
FE	>10	+++	—	ND
pPCKSVT				
1	>10	ND	—	ND
2	10–12	+	++	8q11

The state of transgene integration was analyzed by Southern blotting and Phosphorimager analysis to determine gene dosage and methylation status. Gene dosage is indicated for the heterozygous animal. Northern blots were used to detect expression of either the TGF α or TAg transgenes. Chromosome localization was determined by FISH, subsequent to staining with Giemsa.

*Copies of transgene/diploid genome. +++, complete methylation of restriction sites; +, partial methylation; ++, significant transgene expression; ND, not determined.

observed in any tissue, nor could it be induced when animals were placed on a high-protein diet (Table 1). No discernible phenotype was observed in any of the five pPEPCK-TGF α lines and the one pPCKSVT line even after careful analysis of several generations and up to 24 months of age. Analysis of the transgene by Southern blot and FISH was performed to determine the transgene dosage and methylation status and how it was integrated into the genome (Table 1). From 1 to 10 or more copies of the transgene were integrated into the genome of the pPEPCK-TGF α transgenic rats (Table 1). The transgene arrays were heavily methylated, as demonstrated in Figure 2A. Digestion of rat liver DNA to completion with the restriction enzyme *Hpa*II, which is sensitive to methylation and will not cleave when the internal CpG is methylated, liberates only a high molecular weight band of the transgene, indicating that the array is extensively methylated. Digestion with *Msp*I, which recognizes the same restriction site as *Hpa*II but is not sensitive to the methylation status of the internal CpG, liberates only small fragments of the transgene. No hybridization is observed in the DNA samples from the nontransgenic littermate due to the stringent washing conditions used. These lines were not analyzed further.

Because a phenotype was observed in the pPCKSVT animals, they were examined in detail. Rat liver DNA was digested with *Bam*HI, producing a unit-length transgene fragment and a larger band when the blot was hybridized with a TAg probe (Figure 2B, lanes 3 and 4), indicating that multiple copies of the transgene are integrated in a tandem array. Lane 1 in Figure 2B contains genomic DNA from a nontransgenic littermate, whereas lane 2 contains the same DNA as lane 1 spiked with a haploid amount of transgene-containing plasmid. Experiments that compare the hybridization signal in tail DNA to known amounts of transgene (from haploid on up) indicate that 10 to 12 copies are present in other heterozygous animals (data not shown). Digestion of liver DNA from two transgenic animals with *Msp*I and *Hpa*II followed by blotting with a TAg probe demonstrates that the transgene is not highly methylated in this tissue, suggesting that it may be expressed (Figure 2C, compare lanes 1 and 2 with 3 and 4).

The integration site of the pPCKSVT transgene in the genome was determined by performing FISH subsequent to Giemsa staining of chromosomes. Metaphase spreads prepared from stimulated lymphocytes were stained with Giemsa and hybridized to a biotin-labeled probe prepared with the transgene-containing plasmid. A compar-

Figure 2. Transgene analysis. A: Liver DNA from a pPEPCK-TGF α transgenic rat (+) or a nontransgenic littermate (—) was digested to completion with either *Hpa*II (H) or *Msp*I (M), blotted, and hybridized to a probe specific to human TGF α . B: Tail DNA from either a nontransgenic animal (FIT26) or its transgenic littermates (FIT25 and FIT32) was digested with *Bam*HI, blotted, and hybridized to a probe specific to TAg. The hybridization signal from a haploid amount of transgene was generated by spiking the DNA from FIT26 with the transgene-containing plasmid before digestion. C: Liver DNA from transgenic animals FIT25 and FIT32 was digested with either *Msp*I (M) or *Hpa*II (H), blotted, and hybridized to a probe specific to TAg.

Figure 3. Chromosome localization. The identification of the transgene integration site was determined by FISH with the pPCKSVT plasmid, subsequent to staining with Giemsa as described in Materials and Methods. **A:** Giemsa. **B:** FISH.

ison of the location of the FISH signal (Figure 3B) with the corresponding Giemsa banding pattern (Figure 3A) demonstrated that the transgene array is located on chromosome 8q11.

Animal Survival

Similar to the pAlbSV40 transgenic rats,⁴ the pPCKSVT rats have a shortened life span, which is different in males and females (Figure 4). In males, survival starts to decline gradually by 125 days of age, whereas in females this decline begins at approximately day 225. Both sexes reach a maximal life span of approximately 260 days. It is in this final stage when a peripheral neuropathy similar to that observed in pAlb-SV40 animals⁴ was seen. This condition is likely attributable to demyelination, which is evident in sections of the spinal cord.⁴

Islet Cell Hyperplasia and Carcinoma Development

Islet cell neoplasms were examined by H&E staining of lesions and normal-appearing pancreatic tissue from transgenic animals and their normal nontransgenic control littermates. One hundred percent of the pPCKSVT rats in each generation tested developed multiple hyperplastic islets, adenomas, and islet cell carcinomas. Nor-

Figure 4. pPCKSVT transgenic rat survival curve. The percent survival of male and female transgenic rats as a function of days surviving demonstrates that the males die sooner than females, suggesting a faster tumor growth in males than females. Animals were routinely sacrificed at the onset of peripheral neuropathy as suggested by the animal care personnel, as death normally ensued 48 to 72 hours later.

mal islets were only rarely seen in animals 3 months of age or older even before the beginning of spontaneous deaths (Figure 4). On average, only one or two islets were noted in each section (1 to 2 cm² examined). Hyperplastic islets, which occurred in younger animals even in the absence of adenomas, consisting of increased numbers of slightly basophilic islet cells with prominent nuclei (Figure 5A), were somewhat more frequently seen, up to four or five per section. Very low levels of TAg expression could be detected in some of the hyperplastic lesions by immunohistochemistry, but no TAg was ever detectable in normal-appearing islets. Islet cell adenomas were present in the pancreas of every animal sacrificed or which died. The exact number per pancreas was not determined, although frequently one or two adenomas could be noted in one or two sections examined from each animal. Most of the adenomas showed varying degrees of anaplasia.

Individual islet cell carcinomas displayed a variation of differentiation states. Rosette formation was commonly observed in some areas (Figure 5B), whereas other areas consist of sheets of small basophilic cells with a small nucleus/cytoplasm ratio (Figure 5C). Regions of necrosis were evident in many neoplasms, along with many mitoses. A large amount of extracellular matrix deposition also occurred in some tumors. The malignant nature of the neoplasms is further exemplified by the occurrence of metastases of small TAg-expressing basophilic carcinoma cells in peri-pancreatic lymph nodes (Figure 5D). Metastases to other organs have not been observed, possibly due to the relatively short life span of the animals (Figure 4). More than 30 animals of both sexes were necropsied for this study, and each animal exhibited at least one but usually three or four islet cell carcinomas.

TAg Expression

TAg protein expression was assessed using immunohistochemistry and Western blotting. Formalin-fixed sections

Figure 5. Pancreatic lesions in pPEPCK-TGF α transgenic rat. **A:** Hyperplastic islet. Magnification, $\times 100$. **B:** Well differentiated. Magnification, $\times 50$. **C:** Poorly differentiated. Magnification, $\times 50$. **D:** Peri-pancreatic metastasis. Magnification, $\times 50$.

from pancreas, liver, cerebrum, cerebellum, adrenal glands, testis/ovary, uterus, kidney, intestine, and lung were examined for TAg expression by immunohistochemistry using the mouse monoclonal antibody pAb419. All pancreatic neoplasms stained intensely for nuclear TAg (Figure 6A). In the other sites examined, nuclear TAg and/or cytoplasmic staining was observed primarily in the proximal tubule cells of the kidney and in most regions of the cerebellum and cerebrum (Figure 6, B and C). Occasional staining was observed in a few hepatocytes (data not shown). No staining was observed in lung, testis/ovary, intestine, uterus, or adrenal glands.

When TAg mRNA expression was examined by Northern blotting with 10 μ g of total RNA and a TAg probe, only the pancreatic tumors expressed detectable amounts of TAg (Figure 7A, lanes 4 and 8). Equivalent amounts of total RNA per sample were analyzed as indicated in the ethidium bromide-stained gel (Figure 7B). As immunoreactive material could be detected in other tissues, RT-PCR was performed to detect minor levels of transgene expression. Total RNA isolated from the pancreas, liver, lung, kidney, and brain was used to construct cDNAs from which the TAg mRNA was amplified as described above. A 313-bp DNA fragment, indicative of the presence of the TAg mRNA, was observed in the pancreas, the major site of TAg expression, and in the brain (Figure 8, lanes 3 and 7). A smaller amount was detected in the kidney (lane 6), whereas the liver and lung showed no

expression. No amplification product was observed in the control lanes, where either no RNA or no RT was added (lanes 1 and 2, respectively). Expression of β -actin could be detected in all of the RNA samples, indicating that they were all competent for this analysis (data not shown).

IP/Western blotting confirmed many of the results observed with both RT-PCR and immunohistochemistry. TAg was immunoprecipitated with pAB419 from protein extracts prepared from pancreas, kidney, liver, and brain (Figure 9A, lanes 2, 4, 6, and 8). p53 was immunoprecipitated from the same extracts with pAB421 antibody. In all tissues examined, TAg was found to be complexed to p53. Western blotting performed with an antibody to TAg (pAB419) detected the presence of TAg in proteins immunoprecipitated with an anti-p53 antibody (pAB421) (Figure 9A, lanes 3, 5, 7, and 9). Reciprocal experiments in which p53 was detected by Western blot (Figure 9B) confirmed these results.

Discussion

Microinjection of DNA constructs into fertilized eggs at the one-cell stage is one of the principal methods for producing transgenic animals of a variety of species with mice predominating. If more than one copy of the DNA integrates into the genome, multiple copies are typically

Figure 6. Immunoperoxidase staining for TAg. Five-micron formalin-fixed, paraffin-embedded tissues were stained for TAg and localized with an immunoperoxidase label. **A:** Pancreatic islet cell neoplasm. **B:** Kidney. **C:** Cerebellum. Magnification, $\times 50$.

arranged in tandem arrays containing up to several hundred copies.²⁶ In the rat lines described in this paper, up to 10 to 12 copies of the transgene were incorporated in some lines. Most genes that had been microinjected into mice are expressed,²⁶ but the level of expression is unpredictable and usually does not correlate with gene copy number. In the studies in this paper, lack of expression of the transgenes correlated with extensive methylation of the transgene constructs incorporated into the rat genome. Although some evidence indicates that reduction in the copy number of silenced transgenes leads to an increase in their expression, this did not appear to be so in animals bearing the PEPCK-TGF α transgene where

Figure 7. TAG mRNA expression. **A:** Total RNA from the liver, kidney, brain, and pancreatic tumor was blotted and hybridized to a probe specific to TAg. **B:** Gel from **A** stained with ethidium bromide to demonstrate equal loading of the 28 S and 18 S rRNAs.

from 1 to 10 copies of the transgene were found in different lines, all with no transgene expression.²⁷ Chromosomal insertion of foreign DNA has been associated with enhanced methylation of cellular DNA,²⁸ and it has been proposed that DNA methylation to inactivate foreign DNA may be a host defense mechanism.²⁹ The exact mechanism, however, of the extensive methylation of the

Figure 8. Detection of low-level TAg expression by RT-PCR. One microgram of total RNA was reverse transcribed and used in a PCR reaction to amplify a portion of the TAg message. No RNA, no RNA in the RT reaction; No RT, no RT was included in the RT reaction, which included 1 μ g of total RNA from a pancreatic tumor.

Figure 9. Complex formation between TAg and p53 by IP/Western blotting. Protein extracts from a pancreatic neoplasm (Panc), brain (Br), kidney (Kid), and liver (Li) were either immunoprecipitated with pAB419 (TA α) or pAB421 (p53), separated by electrophoresis through a 10% SDS-polyacrylamide gel, and subjected to Western blotting with either an antibody to TAg (pAB419, A) or p53 (pAB421, B).

PEPCK-TGF α construct as a transgene *in vivo* is not clear. The size of the construct does not appear to be a factor as the PEPCK-SV40TA α construct, at least in one founder, was not suppressed. The SV40 TAg gene has been a component of many constructs for transgenic carcinogenesis in mice^{10–12} and rats.⁴ The ability of the SV40 TAg to inactivate the retinoblastoma tumor suppressor gene by forming a complex with the hypophosphorylated form of the molecule³⁰ allows cell cycle progression from G1 to S. This stimulus, under most circumstances, would induce apoptosis, but TAg's ability to complex with p53 inhibits this process, allowing the cell cycle to continue without consequences.³¹ This ability to stimulate cell cycle progression has the effect both of perturbing the differentiation process and allowing for the development of genomic instability, increasing the chances for a cell to become neoplastic.

The original aim in these studies was to use an enhancer/promoter component to the transgene that could be regulated by environmental factors. Previous studies have demonstrated several mechanisms of regulation of the phosphoenolpyruvate carboxykinase gene in several tissues of the rodent. In the mouse, high levels of PEPCK are found in the liver, kidney, and gut.^{32,33} The tissue-specific nature of this activity is due to transcriptional control of its expression.^{6,33} The 617-bp DNA fragment we used in our construct contains all of the information necessary to observe tissue-specific expression in these

tissues, as determined in transgenic mice. Low PEPCK levels are found in numerous tissues in the adult mouse, with the exception of the pancreas.³² The PEPCK gene is expressed in the pancreas from day 14 in embryonic development until 2 weeks postnatally. PEPCK expression in neural tissue occurs throughout embryonic development, continuing in the adult. However, no phenotype, neoplastic or non-neoplastic, was noted in tissues of rats bearing the pPEPCK-TGF α transgene when sacrificed at any age up to 24 months. The lack of a phenotype is presumably due to the extensive methylation in the regulatory region of the transgene. Attempts are now being made to demethylate the transgene by several different methods.

Current transgenic models of pancreatic cancer development involve primarily either islet or acinar cells and their neoplastic counterparts.^{34–37} In humans, the most prominent malignant neoplasm of the pancreas is ductular carcinoma, with acinar and islet cell neoplasms constituting a relative minority. Islet cell tumors are relatively common in transgenic mice expressing TAg regardless of the promoter used.^{16,34,37–39} TAg expression in our line of pPCKSVT transgenic rats was observed in most neoplastic islet cells, but was minimal in hyperplastic islets. Experiments under way to characterize the expression pattern in the fetal pancreas, neonates, and early lesions should clarify the role of the PEPCK promoter in driving expression of the transgene as we and others have observed that islet cells are particularly susceptible to transformation by TAg. Transgene expression may be due to the presence of an islet- or neural-cell-specific transcription factor binding site located within the TAg coding sequences. The peripheral neuropathy observed by many groups in transgenic mice⁴⁰ and in our transgenic rat line⁴ may have a similar explanation.

Although the original aim of the development of this line of transgenic rats was not realized, the spontaneous development of islet cell neoplasms in all animals of both sexes in this species is unique. In addition, whereas most transgenic mice that develop islet cell neoplasms also develop neoplasms of other tissues,^{37–39,41–43} the transgenic rat line described herein exclusively develops islet cell neoplasms. This model may thus afford a better situation in which to study the molecular pathogenesis of this neoplasm induced by a specific viral oncogene regardless of the regulatory elements presumably driving the expression of the oncogene in the transgenic animal. Furthermore, the unique nature of the islet cell tumors that develop in these animals is demonstrable by their ability to synthesize insulin, glucagon, and somatostatin.⁴⁴ Nests of endocrine hormone-secreting cells can be found scattered within each neoplasm, suggesting that a multipotent stem cell is the target for transformation by TAg. This tumor type is unique as most of the islet cell tumors found in transgenic mice synthesize only one endocrine product.^{11,16} This result is expected in situations where the oncogene is driven by an endocrine hormone gene promoter.¹⁶ The islet cell tumors that develop in the Alb-SV40 TAg transgenic rat⁴ do not synthesize an endocrine hormone gene product (unpublished observations), suggesting that the gene promoter selected for constructing

the PEPCK-SV40 TAG transgenic animals is important to the derivation of these hormone-producing neoplasms. These results also imply that the short life span of these animals is not due to impaired glucose homeostasis.

The appearance of immunoreactive TAG in the kidney is not due to uptake of the secreted protein by the proximal tubule cells as its mRNA is detectable by RT-PCR. The animals do not develop any renal pathology, but this may be due either to the short life span of the animals or a lack of susceptibility of this cell type to transformation by this oncogene. Whether or not the sequestration of TAG to the cytoplasm of these cells is relevant to the phenotype is unclear. TAG expression in the liver was sporadic. Secretion of insulin by neoplastic islet cells (M. J. Haas, C. A. Sattler, Y. P. Dragan, W. L. Gast, and H. C. Pitot, manuscript submitted) may repress transcription of the PEPCK promoter in hepatocytes. It is also possible that some hepatocytes may clear TAG from the blood; hence the protein is present in the cell, but the mRNA is not. In either event, TAG is localized in the hepatocyte nucleus and is complexed with p53. In fact, all of the tissues that express TAG by immunohistochemistry (pancreas, liver, cerebrum, and kidney) demonstrate complex formation between TAG and p53 (Figure 9). We are presently attempting to determine whether administration of chemical carcinogens may stimulate the expression of the TAG in these tissues with the development of subsequent neoplasia.

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