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Expression and amplification in transgenic mice of a polyoma virus mutant regulatory region

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ABSTRACT

Two hybrid gene constructs consisting of wild-type and mutant polyoma regulatory regions fused to a bacterial reporter gene were inserted in the mouse germline. Both transgenes were expressed in a large number of different organs. However, marker gene expression controlled by the polyoma wild-type regulatory region was not detectable in the early embryo and remained low throughout the life of the animal while expression controlled by the polyoma F9-1 mutation was detectable in blastocysts and was significantly higher at later stages of development. The F9-1 hybrid gene was also amplifiable when large T-antigen was supplied *in trans* to mice or to kidney cells derived from these transgenic mice. Amplification resulted in the appearance of several hundred copies of episomal transgenes and a marked increase of marker gene RNA and protein. Our results suggest that the F9-1 mutation does not alter the target spectrum of gene expression *in vivo* but does create a more efficient enhancer element in the polyoma early control region. Transgene amplification based upon use of the polyoma regulatory elements may be a means of increasing expression of genes in transgenic mice.

INTRODUCTION

The complex regulatory region of the murine polyoma virus (Py) lies between the early and late transcriptional units on the circular genome. Individual elements located in this regulatory region include the early and late promoter, transcriptional enhancers, and the origin (ORI) of replication. ORI is a short region of dyad symmetry (1, 2) regulated by an early gene product of Py, the large T (LT) antigen (3). Py LT specifically binds to DNA sites containing repeats of pentanucleotide recognition sequences located at ORI and in the early promoter region (4-6). Py LT also mediates transcription. While Py LT binding to the early promoter results in repression of early transcription (7), the protein can stimulate late gene expression in the presence of a functional enhancer (8). The 244 base pair (bp) Py enhancer consists of at least 4 separate domains designated A, B, C, and D (9, 10). The individual parts of the enhancer functionally depend on each other and show homology to other viral and cellular "core" enhancer sequences. These *cis*-acting elements not only stimulate transcription from the viral promoters, but are also essential for viral DNA replication (11-14).

Mutations in the enhancer can alter the viral host spectrum. Wild-type Py grows in differentiated mouse cells, but not in embryonal carcinoma (EC) cells which are similar to cells in the early mouse embryo. Several host range mutants of Py have been selected which grow in EC cells and carry DNA sequence reorganizations in the enhancer (15-17). One of these, the F9-1 mutant, has been derived from the wild-type strain A2. However, its enhancer is more closely related to that of strain A3. A single A:T to G:C transition at position 5230 distinguishes the F9-1 mutant enhancer from that of the A3 strain (16) and generates the E element of the enhancer (10).

The F9-1 mutant replicates in both undifferentiated and differentiated F9 embryonal carcinoma cells (14, 15).

Py enhancer function is thought to be mediated by proteins binding specifically to enhancer elements (10, 18-20). Recently, a protein factor has been detected in undifferentiated EC cells that binds preferentially to the F9-1 mutant sequence (21). The similarity between EC and normal embryonic cells suggests that the F9-1 regulatory sequence may be activated in the mouse embryo. We tested this by inserting into the germline of the mouse two chimeric genes, PywtCAT and PyF9-1CAT, which contain regulatory regions of wild-type and F9-1 mutant Py, respectively, fused to the chloramphenicol acetyl transferase (CAT) marker gene (22). The transgenic mice thus generated allowed us to compare the activities of the two Py control regions in the living organism in various tissues and at different stages of development.

The PyCAT transgenes contain the Py ORI, enhancers and large T-antigen binding sites, elements which are required in *cis* for viral DNA replication (23). Pelligrini and Basilico (24) have determined that in tissue culture cells containing these elements, ORI and sequences linked *in cis* are amplifiable and excise from the chromosome if large T-antigen is also supplied. Our aim was to test whether PyCAT sequences in the transgenic mouse would also amplify and excise if large T-antigen was supplied *in trans*. Since attempts to modulate transgene expression with the help of inducible promoter elements have met with limited success (25), we wanted to explore the possibilities of inducible gene amplification *in vivo* as a means of increasing transgene expression.

MATERIALS AND METHODS

Gene construction and embryo microinjection

The constructs PywtCAT and PyF9-1CAT were obtained by inserting the enhancer/promoter fragment (*Bam*HI/*Hph*I) (position 4631 to 163) from either Py wild-type strain A3 or the polyoma F9-1 mutant into the *Bgl*II site of the vector CAT3N (22). The Py insert 5′ of the CAT gene contains all known viral transcriptional elements and ORI. Abutting the CAT gene at the 3′ side are an intron and the polyadenylation signal of SV40. Plasmid DNA was digested with *Hin*dIII and was electrophoresed through a 1% agarose gel. The linear 2.8 kb *Hin*dIII fragments containing the PyCAT sequences were electroeluted. DNA was extracted with phenol, precipitated with ethanol, dissolved at 2μg/ml in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and microinjected into one-cell embryos of the inbred FVB/N strain (26). Transgene sequences were identified and copy number determined by Southern hybridization analysis of DNA prepared from mouse tail tissue (27).

Analysis of transgene expression

Mouse zygotes were isolated as described (28) and cultured to the blastocyst stage in M16 medium (29). Blastocysts and embryonic and adult tissues were homogenized and assayed for CAT activity under conditions at which the conversion of chloramphenicol to an acetylated form progressed linearly (30), unless indicated otherwise. All values given are significantly higher than background levels obtained with wild-type tissue.

Cells and viruses

Kidneys from an adult transgenic mouse carrying the PyF9-1CAT fusion gene were isolated, minced and treated with 0.25% trypsin for 1 hr. The cell suspension was filtered through one layer of Nitrex membrane, centrifuged at 200 x g for 10 min and resuspended in DME medium containing 10% fetal calf serum. Cells were seeded at a concentration of 2×10^7 cells per 100 mm culture dish. They were infected with wild-type Py strain A2 at multiplicities of 50-100 plaque forming units (pfu) per cell. Plaque purified virus was kindly provided by Dr. A.M. Lewis, Jr. and Dr. J.B. Bolen. To inhibit DNA synthesis, 30 μg of cytosine arabinoside per ml were present during and after infection (31). Newborn offspring of the transgenic strain PyF9-1CAT were inoculated subcutaneously with 10^8 pfu of Py at 14 hrs after birth. The kidneys were isolated 5, 7, or 8 days after viral infection.

Preparation of DNA and RNA

DNA was prepared by the Hirt procedure (32). High molecular weight chromosomal DNA was recovered by resuspension of Hirt pellets in 10 mM Tris-HCl, pH 8.0 1 mM EDTA. The material was digested with 100 μg per ml RNaseA, extracted with phenol and precipitated with ethanol. Total cellular RNA was prepared by extraction with 4 M guanidine isothiocyanate and centrifugation through a 5.7 M CsCl solution as described by Chirgwin et al (33).

Southern hybridizations and dot blot analysis

Hirt supernatant or pellet DNAs, restricted as described in the text, were electrophoresed on 1% agarose gels and transferred to nitrocellulose membranes (34). A CAT fragment from the plasmid pαCAT3M (22) was used as hybridization probe. Hybridization to a ^{32}P nick-translated probe (35) was carried out at 42°C in 50% formamide (36). DNA was quantitated by scanning autoradiographs. Hirt supernatant DNA isolated from homogenized kidneys was analyzed by dot blot hybridization (27) and probed with the purified CAT fragment or with the *Eco*RI/*Bam*HI fragment of the Py genome. This fragment contains the COOH terminal of LT antigen and the coding sequence of VP1. The plasmid was kindly provided by Dr. Robert Freund.

Primer extension analysis

Quantitative primer extension analysis was performed on 20 μg of total RNA as described by Phelps and Howley (37). The oligodeoxyribonucleotide, 5'-TCCATTTTAGCTTCCTTAGC-3' was obtained from Dr. P. Lambert and Dr. P. Howley and is complementary to CAT mRNA at a position 75 bases downstream from the junction of CAT and Py sequences (37). Quantitation of RNA was achieved by scanning densitometry (see above).

Other techniques

PyF9-1CAT kidney cells were microinjected via glass capillaries into the nucleus as described previously (38).

RESULTS

Two gene constructs, PywtCAT and PyF9-1CAT, were introduced in the mouse germline by embryo microinjection. The construction of the hybrid genes has been described (22). Fig. 1 shows the composition of the 2.8 kb *Hin*dIII fragment which was injected in the zygote. It consists of the regulatory region of either wild-type or PyF9-1 mutant linked to the CAT reporter gene and to SV40 splice and polyadenylation signals. The positions of the enhancer elements A-E, the F9-1 point mutation, the ORI element, the TATA box, and the start site of early polyoma transcription are indicated in the figure. Four PywtCAT and three PyF9-1CAT transgenic mice were obtained. From these founder mice, three PywtCAT and two PyF9-1CAT lines were generated. Southern blot and segregation analysis (not shown) established that each had one site of integration. As indicated in Table 1, the number of transgene copies per haploid mouse genome varies from one line to the other. Transgene expression was tested in

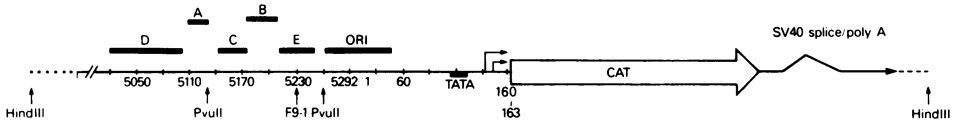

Figure 1. Structure of the microinjected gene constructs. Shown is the 2.8 kb fragment containing Py regulatory sequences fused to the bacterial CAT gene and to SV40 splice and polyadenylation sequences. The F9-1 mutation at position 5230 is indicated. Also shown are the enhancer elements A-E (10), ORI, the TATA box and the start sites of RNA transcription from this gene. Numbering is according to the Py A2 system (47). The Py sequences are selectively enlarged.

TABLE 1. Transgenic mouse lines generated for this study.

line		copy number	CAT activity[a]
PyF9-1CAT	1	5	15.4
	2	20	17.8
PywtCAT	1	1-2	0.4
	2	10	0.3

[a]An average of all adult organs tested, expressed as percent conversion of chloramphenicol to acetylated forms per 50 μg total protein.

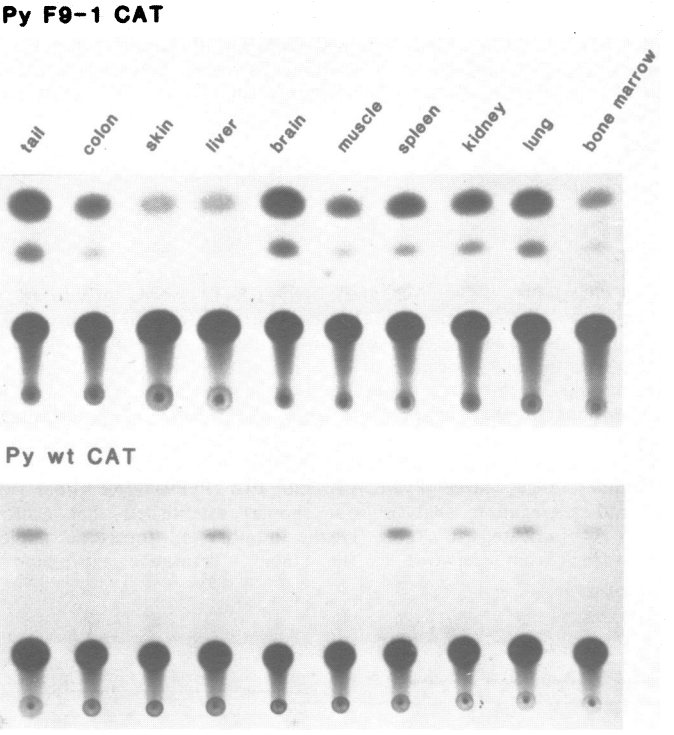

Figure 2. CAT activity in tissues derived from 3½ month old PyF9-1CAT (line 2) and PywtCAT (line 2) animals, respectively. Assays contained 10 μg of total protein of PyF9-1CAT and 50 μg of PywtCAT tissue extracts, respectively. Assays were incubated for 1 hr.

TABLE 2. CAT activity[a] in selected organs of transgenic mice.

Organ	PyF9-1CAT		PywtCAT	
	line 1	line 2	line 1	line 2
Tail	30.5	27.0	0.6	0.4
Colon	9.5	12.0	0.3	0.2
Skin	3.0	7.0	0.3	0.2
Liver	8.0	4.0	0.4	0.3
Brain	25.0	16.5	0.25	0.3
Muscle	15.0	25.0	0.2	0.2
Spleen	17.0	19.0	0.75	0.2
Kidney	13.0	20.5	0.4	0.2
Lung	28.5	39.5	0.5	1.6
Bone marrow	4.0	7.5	0.3	0.2

[a]See legend of Table 1.

progeny of four of the lines by examining CAT activity in many different organs and tissues (see below). Table 1 summarizes the CAT activity expressed as an average of all tissues tested. As can be seen, the average CAT activity was about 50-fold higher in the mice carrying the F9-1 mutant regulatory region. Of the founders, one PywtCAT and one PyF9-1CAT mouse expressed CAT activity in all organs tested at levels comparable to those found in the respective lines listed, but failed to pass their transgenes on to progeny. These two mice may have been mosaic and were therefore not included in Table 1. One PywtCAT line did not contain any detectable CAT activity. The reason for the apparent failure of transgene expression in these mice was not explored.

Tissue specific expression of the Py-CAT transgenes

We determined the spectrum of tissues expressing the two different Py regulatory regions in the adult mouse by measuring CAT activity in a variety of organs. The result is shown in Fig. 2. Heterozygous animals of the PyF9-1CAT and the PywtCAT lines contained CAT activity in every tissue tested. A quantitative assessment is shown in Table 2. Data derived from the two PyF9-1CAT and the two PywtCAT lines demonstrate that expression levels from the mutant regulatory region of Py are clearly higher than those of the wild-type regulatory region in all tissues tested. CAT activities based on protein content in tissue extracts vary slightly from tissue to tissue within a given line. However, the profiles of individual lines derived from the same construct show a remarkable degree of conformity. No correlation between gene copy number (Table 1) and the level of expression (Table 2) was apparent.

Expression of the transgenes during development

Transgene expression during fetal stages of development generally reflected the findings obtained with adult animals. Table 3 compares CAT activity in total embryo

TABLE 3. CAT activity[a] during development.

Transgene	12 day old embryo	17 day old embryo	adult tissues[b]
PyF9-1CAT (line 2)	2.9	6.6	17.8
PywtCAT (line 2)	0.1	0.6	0.3

[a]See legend of Table 1.

[b]Average value for all tissues analyzed.

extracts with the average CAT activity of adult tissues. While expression from the Py wild-type regulatory region is marginal at all stages, there is considerable expression of the PyF9-1CAT transgene in the 12-day old embryo and this expression increases during subsequent stages of development. Extraembryonic tissues also expressed CAT activity (data not shown).

At earlier stages of development, CAT activity was clearly present in the day 7 PyF9-1CAT embryo. In contrast, no activity could be detected in the PywtCAT embryo (data not shown). In an effort to demonstrate CAT activity at an even earlier developmental stage in the PyF9-1CAT transgenic mice, we prepared extracts from 280 homozygous PyF9-1CAT (line 2) blastocysts, from an equal number of non-transgenic

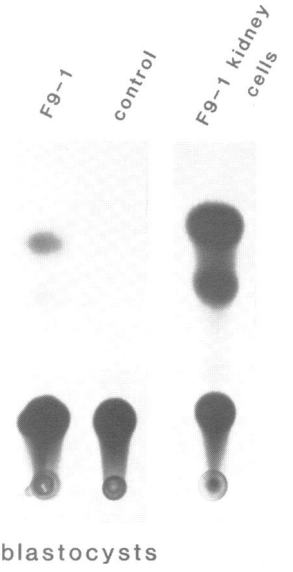

Figure 3. CAT activity in PyF9-1CAT (line 2) blastocysts. Lanes labeled *F9-1* and *control* represent extracts of 280 blastocysts derived from homozygous PyF9-1CAT mice and from non-transgenic mice, respectively. The lane labeled *F9-1 kidney cells* contains an extract of heterozygous PyF9-1CAT (line 2) kidney cells established in culture. The number of kidney cells used roughly reflects the number of cells present in the blastocyst extracts. We used a minimum estimate of 32 cells per blastocyst (48). Assays were incubated overnight.

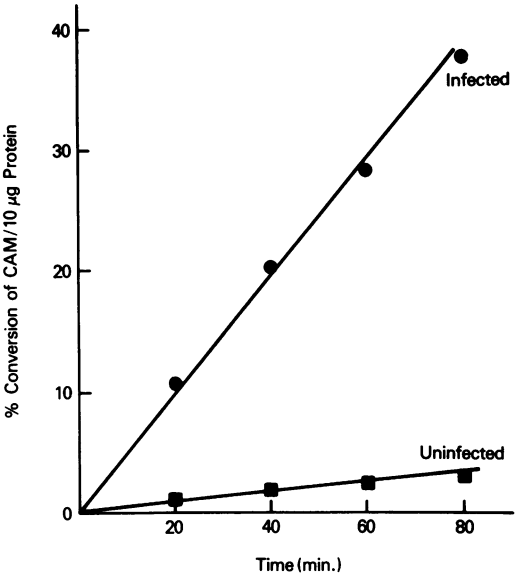

Figure 4. CAT activity in mock infected and in Py infected PyF9-1CAT cells. Ten µg of protein extract from uninfected cells or from cells harvested 36 hrs after infection with Py strain A2 were assayed for CAT activity at the times indicated. Filled dots indicate extracts from infected cells, filled squares extracts from uninfected cells.

FVB/N blastocysts, and from cultured kidney cells of a heterozygous PyF9-1CAT (line 2) transgenic mouse. The result of the CAT assay performed on these extracts is shown in Fig. 3. Assays were incubated overnight in order to optimize detection of CAT activity in the limited number of PyF9-1CAT blastocysts available. The experiment gives a clear indication that CAT activity is present in the homozygous PyF9-1CAT blastocyst, and that this activity, on a per cell basis, is much lower than in the heterozygous somatic cells derived from the adult animal.

Infection of PyF9-1CAT cells with Py leads to a marked increase in CAT activity

Since the PyF9-1CAT mice contain Py control elements for DNA replication as well as for gene expression, the inserted DNA may be a target for Py LT dependent gene amplification through an "onion skin" amplification and excision mechanism (39, 40). Increases in gene expression may result due to the increased number of templates for transcription. Mice carrying multiple tandem copies of the PyF9-1CAT construct (line 2) were used in the studies described below.

Our first attempt to directly demonstrate this sequence of events involved kidney cells derived from PyF9-1CAT (line 2) animals. These cells were carried through over 14 passages. The PyF9-1CAT gene copy number in this cell line was the same as that observed in the mice from which it was derived, and the CAT activity did not noticeably change during passaging (data not shown). We asked whether transgene sequences (unit length 2.8 kb) could be induced to amplify and to be excised from the PyF9-1CAT cell genome in the presence of Py LT, and whether this would result in an increase in CAT gene expression. Py LT was introduced into the kidney cells by infection with the wild-type Py strain A2 at a multiplicity of infection of 50 pfu per cell. About 70% of the infected cells showed bright T antigen specific fluorescence in immunostaining experiments (data not shown). The cells were harvested 36 hrs after infection and assayed for CAT activity. From the rate analysis shown in Fig. 4, we

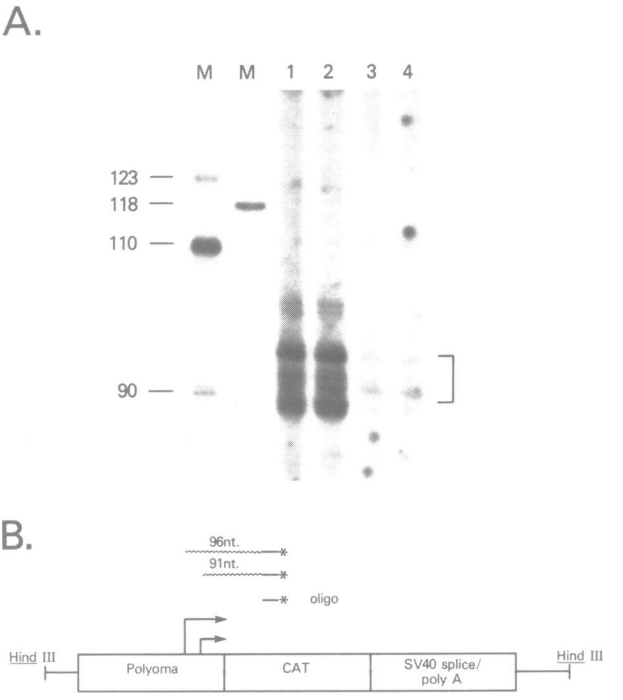

Figure 5. Primer extension analysis of CAT transcripts in mock infected and in Py infected PyF9-1CAT cells. Panel A. Primer extension analysis was performed on 20 μg of total RNA. Lanes 1 and 2 contain RNA from infected cells, lanes 3 and 4 from mock infected cells; M lanes contain ^{32}P øx174 DNA digested with *Hae*III, and ^{32}P pBR322 DNA digested with *Msp*I, respectively. The 91 and 96 nucleotide products represent mRNA start sites at the Py early promoter. Panel B. Predicted sizes of primer extended CAT RNAs initiated at the early Py promoter start sites. Shown is the 2.8 kb *Hin*dIII fragment of PyF9-1CAT, the transgene in these cells.

conclude that CAT activity is stimulated 13-fold in Py infected PyF9-1CAT cells relative to uninfected cells.

Next, we examined whether the increase in CAT activity coincided with an increase in the steady state levels of CAT messenger RNA, and whether this RNA was correctly initiated within the early Py promoter. Results of a primer extension analysis of RNA isolated from uninfected cells or from cells harvested 36 hrs after infection with Py virus are shown in Fig. 5. The expected sizes of the primer extension products, 91 and 96 nucleotides, respectively, (Fig. 5B) are based on the PyF9-1CAT gene construction (22), the positions of the Py early start sites for mRNA synthesis (41) and the position of annealing of the CAT oligonucleotide primer (37) to the RNA. Fig. 5A shows that CAT transcripts originating from the Py early promoter are detectable in uninfected cells. Upon infection of the cells with Py, the amount of CAT transcripts initiated at the early promoter start sites increased by 15-fold as measured by scanning densitometry. A minority of transcripts corresponded to sizes expected from products initiating elsewhere in the promoter region (42). We conclude that Py infection of PyF9-1CAT kidney cells results in a markedly increased expression of transgene sequences regulated by the Py early promoter. The increase in the

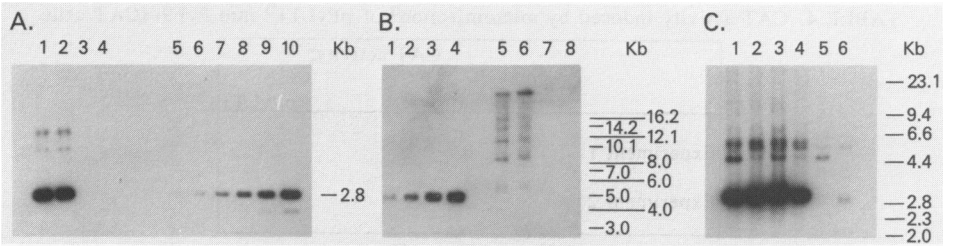

Figure 6. Analysis of Hirt supernatant and pellet DNAs from mock infected and infected PyF9-1CAT cells. DNA was prepared from mock infected cells or from cells harvested 36 hrs after infection with Py. Panel A. Hirt supernatant DNA representing approximately 7 x 10^5 cells was digested with *Bcl*I and subjected to Southern blot analysis using ^{32}P nick translated CAT fragment. DNA from infected cells is shown in lanes 1 and 2, DNA from mock infected cells in lanes 3 and 4. Lanes 5-10 show 5, 10, 25, 50, 100 and 250 μg, respectively, of the 2.8 kb PyF9-1CAT *Hin*dIII fragment. Panel B. Southern blot of undigested Hirt supernatant DNA. Lanes 1-4 contain 75, 150, 300 and 400 μg, respectively, of the 2.8 kb transgene fragment. DNA from infected cells is shown in lanes 5 and 6, DNA from mock infected cells in lanes 7 and 8. Panel C. Analysis of Hirt pellet DNA. Aliquots of 10 μg of DNA were digested with *Bcl*I or *Xba*I and analyzed as above. Lanes 1 and 3 contain *Xba*I digested DNA from infected cells, lanes 2 and 4 *Bcl*I digested DNA from infected cells, lanes 5 and 6 contain DNA from mock infected cells, digested with *Xba*I or *Bcl*I, respectively.

amount of steady state levels of CAT RNA corresponds to the observed increase in CAT activity.

The transgene DNA is amplified in Py infected PyF9-1CAT kidney cells

Py LT is known to promote the amplification of Py viral genomes integrated in chromosomal DNA of rat fibroblasts (23). Replication is mediated by the interaction of LT and the viral ORI element. This suggests that the increase in CAT expression observed in Py infected PyF9-1CAT kidney cells was caused by LT mediated amplification of the transgene. Increase in the number of transgene templates would explain the higher level of expression. According to the "onion skin" model, overreplicated DNA becomes excised from the chromosome through homologous recombination and is present in the form of episomes. In order to show this, we separated DNA by the Hirt procedure (32). Hirt supernatants contain episomal DNA; Hirt pellets contain mostly chromosomal DNA. DNA extracts were digested with *Bcl*I or *Xba*I, each of which cuts the transgene once. DNA digests were analyzed by Southern hybridization to a probe specific for CAT sequences. These results are shown in Fig. 6. CAT hybridizing DNA fragments corresponding in size to the 2.8 kb transgene were found in *Bcl*I or *Xba*I treated Hirt supernatants of Py infected PyF9-1CAT cells (Fig. 6A). This is best explained by assuming that the cells contained unit size transgene episomes excised from amplified DNA. From the number of cells analyzed in this experiment, and from the fact that at least 70% of them expressed LT (see above), we have calculated that each infected cell produced, on average, 200 - 800 episomal copies of the transgene. The experiment strongly suggests that Py LT activated replication and excision of transgene sequences occurred in the PyF9-1 kidney cells. Fig. 6B shows Southern blot analysis of undigested Hirt supernatant DNA from infected or uninfected cells hybridized to the CAT probe. DNA hybridizing to CAT probe corresponded in size to dimers, trimers, tetramers, etc. of the 2.8 kb unit length molecule presumed to be the primary excision product of amplified chromosomal transgene DNA. This suggests that, in the presence of Py LT, most of the excised monomers underwent further replication. Fig. 6C shows Southern blot analysis of Hirt pellet DNA analyzed similarly. The host cell chromosomal DNA, representing the bulk

TABLE 4. CAT activity induced by microinjection[a] of pPyLT1[b] into PyF9-1CAT cells.

	CAT activity[c]	
	Control	pPyLT1
Experiment 1	0.7	2.4
Experiment 2	1.3	2.9

[a]Plasmid DNA (100 μg/ml) was injected into 100 cells (experiment 1) or 160 cells (experiment 2), respectively. Control cells received pBR322 vector DNA lacking the Py insert. Cells were maintained in culture for 20 hrs, and subsequently assayed for CAT activity. Assays were incubated overnight. [b]Kindly provided by Dr. F.G. Kern and Dr. C. Basilico. [c]Shown is the percentage of conversion of chloramphenicol to acetylated forms.

of the material shown here, was digested with enzymes that cut only once in the transgene. The large increase of CAT-specific DNA from infected cells over that of uninfected cells suggests that at least some of the transgene DNA had been amplified at the chromosomal site of transgene integration in the host cell. This amplified chromosomal DNA is thought to be the source for episomal transgene DNA found in the Hirt supernatants. Indeed, we were able to quench any increase of CAT activity in Py infected PyF9-1CAT cells by incubating them in the presence of cytosine arabinoside, an inhibitor of DNA synthesis (data not shown).

In order to show that Py LT alone can increase expression of the transgene in PyF9-1CAT cells, we microinjected into the nucleus of these cells the plasmid pPyLT1 (11). This plasmid is known to selectively express Py LT antigen. We observed a 3- to 4-fold increase in CAT activity in cells injected with pPyLT1 compared to control cells which had been microinjected with vector DNA lacking Py sequences (Table 4). This result supports the notion that LT is indeed responsible for the observed increase in transgene expression.

The transgene is amplified in Py infected newborn PyF9-1 CAT mice

Transgene amplification *in vivo* was examined in newborn PyF9-1CAT mice mock infected or infected with wild-type Py virus A2. At 5, 7, or 8 days after infection, the kidneys were removed and Hirt supernatant DNA was prepared and hybridized with a CAT specific probe. The result is shown in Fig. 7. We failed to detect significant

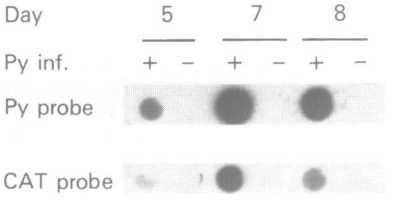

Figure 7. Dot blot analysis of kidney DNA from newborn PyF9-1CAT transgenic mice infected with Py. Animals were injected 14 hrs after birth with a lysate from mock infected cells (-) or of cells infected with Py (+). Low MW Hirt supernatant DNA was isolated from the kidneys and transferred to nitrocellulose filters. A ^{32}P labeled, primer extended *Eco*RI/*Bam*HI spanning the COOH terminus of Py LT antigen and the VP1 coding sequence fragment was used as a Py hybridization probe. The CAT probe was the primer extended CAT fragment. DNA was extracted from mouse kidneys at 5, 7 or 8 days after infection, as indicated.

TABLE 5. Infection[a] of newborn PyF9-1CAT mice with Py resulting in an increase of CAT activity in the kidney.

	CAT activity[b]	
	Mock	Py
Day 5	5.4	6.5
Day 7	6.1	11.2
Day 8	5.7	11.0

[a]Mice were mock infected or infected with Py as indicated in Materials and Methods. Extracts were prepared from kidneys 5, 7, and 8 days after infection. Aliquots containing 10 μg protein were assayed for CAT activity for 1 hr. [b]Shown is the percentage of conversion of chloramphenicol to acetylated forms.

levels of transgene sequences in the mock infected controls. In contrast, kidneys of Py infected animals contained significant amounts of episomal transgene DNA. The highest level was observed at day 7 after infection. Py infection of the kidneys was monitored with the help of a Py probe which encodes the virion protein VP1 and part of the C-terminus of LT, but not any sequences contained within the PyF9-1CAT transgene. The dot blot analysis of this experiment, included in Fig. 7, confirmed that productive Py infection had taken place in the kidneys of infected animals. Hence, we conclude that Py infection of the animals resulted in transgene amplification.

An increase in CAT activity comparing extracts from infected and uninfected kidneys was more difficult to demonstrate (Table 5). The observed increase appears small. However, the data given here clearly represent a minimal estimate because presumably only a small proportion of all cells present in the kidneys were infected with Py and therefore able to amplify and overexpress the transgene.

DISCUSSION

Our study on the PywtCAT and PyF9-1CAT lines of transgenic mice has enabled us to analyze expression of Py wild-type and mutant regulatory elements in the various tissues of the developing organism. In the adult animal, we detected CAT gene expression controlled by the wild-type or mutant regulatory region in each of a large number of different tissues tested. The results suggest that the point mutation does not alter the tissue distribution of gene expression. By contrast, the mutation causes a remarkable increase in gene activity. On average, CAT enzyme activity in adult tissues of PyF9-1CAT mice was about 50-fold higher than in the corresponding tissues of PywtCAT mice. High levels of CAT gene expression were measured in two independently generated F9-1CAT mouse lines, whereas low levels of expression were observed in all PywtCAT lines. Therefore, it appears unlikely that the chromosomal location of the integrated transgene is responsible for the enhanced expression in F9-1CAT mice.

The profile of CAT gene activity in a number of organs of two PyF9-1CAT mouse lines shows higher marker gene expression in lung, brain and tail. CAT activity was consistently low in bone marrow and skin. However, the very low levels of CAT of gene expression found in every organ of PywtCAT tissues do not allow us to investigate the tissue preference of the wild-type regulatory region.

No obvious differences of viral gene activity had been observed in established rodent cell lines infected with Py wild-type or F9-1 mutant viruses (43) or transfected with the PywtCAT or PyF9-1CAT gene constructs (22). In the mouse, the F9-1 mutation may generate a better recognition sequence for proteins interacting with Py

enhancer elements (44). Alternatively, or in addition, it may abrogate host mechanisms of Py repression. Consistent with this view, we detected CAT activity in PyF9-1CAT animals at prenatal stages of development, as early as the blastocyst stage. A parallel can be established between our result and the fact that F9-1 mutant virus replicates efficiently in undifferentiated cells of embryonic origin (43). It remains to be established whether, in the PyF9-1CAT blastocyst, the inner cell mass, the trophectoderm, or both, contribute to the observed CAT activity. The absence of CAT activity in early embryos of the PywtCAT lines may reflect the inability of wild type virus to replicate in undifferentiated cells of embryonic origin. However, given the large difference in the level of gene expression between PyF9-1CAT and PywtCAT lines, CAT activity may simply be below the level of detection in the PywtCAT early embryo.

The potentially most useful facets of our work concern the increased level and the apparent ubiquity of PyF9-1CAT expression in the adult animal. Measuring CAT activity in extracts obtained from the sum of all cells of a given organ or tissue does not allow us to conclude unequivocally that every cell in the organism expresses the Py regulatory region. We plan to corroborate this point by fusing to the PyF9-1 regulatory region a marker gene whose product can be examined by *in situ* staining techniques. If the PyF9-1 regulatory region indeed mediates gene expression in every cell in the adult animal, the interesting question arises of how the regulatory elements achieve expression in every tissue. From a practical standpoint, it should be possible to achieve ubiquitous expression, starting at an early stage of development, of any gene of choice by fusing it to the PyF9-1 regulatory region.

Our results also demonstrate that the chimeric PyF9-1CAT gene, composed of Py replication and transcription control elements and a reporter gene, can be amplified after infection with virus while resident in transgenic mice as well as in cells derived from them. Several hundred monomeric and multimeric episomal DNA copies were found in Hirt supernatants from Py infected PyF9-1CAT cells. In addition, transgene sequences were increased in Hirt pellets. These sequences may represent replication complexes of the transgene in the context of chromosomal DNA.

Replication of the transgene in Py infected cells resulted in a marked increase in reporter gene expression. Our data show that the increase in CAT activity is matched by an increase in steady state levels of CAT RNA, and that this RNA is correctly initiated at the early Py start sites present in the transgene. In our experiments the expression of the transgene appears to be less pronounced than the degree of DNA amplification. This may be explained by the fact that in the transgene CAT is directed by the early Py promoter which is known to be negatively controlled by Py LT (42, 45, 46). The late viral promoter is not a subject of this negative control (8) and may therefore be a better choice for future gene constructs intended for overexpression.

Increased expression of the transgene was also observed if a plasmid encoding only Py LT was introduced in these cells. This shows that Py LT is necessary and sufficient for the observed effect. Py small t antigen has been shown to enhance amplification and excision (45). This may serve to explain why we found the enhancement of gene expression in Py infected cells to be greater than in cells microinjected with a plasmid encoding Py LT alone.

The observed transgene amplification and increased expression in the intact animal suggests that a Py mediated amplification system can be experimentally manipulated to achieve high levels of transgene expression. Suitable transgenes would contain ORI, enhancer sequences and the LT binding sites, and at the same time incorporate elements which direct temporal and spatial patterns of expression. The Py LT, a key factor in the amplification scheme, can be added via viral infection as shown in this paper. Alternatively, T antigen can be supplied at the desired time and place in development by mating ORI containing transgenic mice to transgenic mice expressing Py LT from tissue specific regulatory elements. Thus it should be possible to selectively amplify and overexpress transgenes in specific tissues at specific times of development. In conclusion, there is good evidence that transgene amplification will

become a viable approach toward achieving high levels of specific gene transcripts and gene products in animals.

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