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A Truncated Herpes Simplex Virus Thymidine Kinase Phosphorylates Thymidine and Nucleoside Analogs and Does Not Cause Sterility in Transgenic Mice

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Dividing eukaryotic cells expressing the herpes simplex virus type 1 thymidine kinase (TK) gene are sensitive to the cytotoxic effect of nucleoside analogs such as acyclovir or ganciclovir (GCV). Transgenic mice with cell-targeted expression of this conditional toxin have been used to create animals with temporally controlled cell-specific ablation. In these animal models, which allow the study of the physiological importance of a cell type, males are sterile. In this study, we showed that this phenomenon is due to testis-specific high-level expression of short TK transcripts initiated mainly upstream of the second internal ATG of the TK gene. This expression is DNA methylation independent. To obtain a suicide gene that does not cause male infertility, we generated and analyzed the properties of a truncated TK (ΔTK) lacking the sequences upstream of the second ATG. We showed that when expressed at sufficient levels, the functional properties of ΔTK are similar to those of TK in terms of thymidine or GCV phosphorylation. This translated into a similar GCV-dependent toxicity for ΔTK- or TK-expressing cells, both in vitro and in transgenic mice. However, ΔTK behaved differently from TK in two ways. First, it did not cause sterility in ΔTK transgenic males. Second, low-level ΔTK RNA expression did not confer sensitivity to GCV. The uses of ΔTK in cell-specific ablation in transgenic mice and in gene therapy are discussed.

The herpes simplex virus type 1 thymidine kinase (TK) gene has gained considerable importance in clinical medicine and as a research tool. TK can substitute for cellular thymidine kinase in the metabolic pathway of thymidine, and eukaryotic cells lacking functional TK can be rescued from culture in hypoxanthine-aminopterin-thymidine (HAT) selection medium by TK expression (44). Furthermore, TK can phosphorylate certain nucleoside analogs that are not metabolized by cellular enzymes (21). This property has allowed the discovery of nucleoside analogs such as acyclovir and ganciclovir (GCV) that possess strong activity against herpesvirus infections (19, 21). This same property turned out to be useful for negative selection of TK-expressing cells. Phosphorylated-nucleoside analogs such as acyclovir triphosphate or GCV triphosphate are potent toxic metabolites for dividing cells. They are incorporated into elongating DNA by cellular DNA polymerase and induce chain termination and eventually cell death (20, 36, 42).

This conditional toxicity of TK can be utilized in vivo. TK-mediated destruction of undesirable cells is being developed for gene therapy of cancer or human immunodeficiency virus (HIV) infection (10, 11, 15, 36). Effectiveness has been demonstrated in animal models, and clinical trials for cancer gene therapy have already been initiated (14). In addition, Heyman et al. developed the concept of TK obliteration, the ablation of a specific cell type in transgenic mice (26). This conditional cell knockout is obtained by expressing TK with a cell-specific promoter. This system offers the valuable advantages of allowing cell ablation at times and for durations that are fully controlled by the investigator by administration of nucleoside analogs. Such a system is a powerful tool for appreciating both the physiological importance and the lineage of a given cell type and has been used for depletion of lymphoid cells, growth hormone-secreting cells, interleukin-2- and interleukin-4-expressing cells, or dendritic cells (DC) (6, 18, 26, 30, 34, 40).

One complication observed in transgenic mice harboring the TK gene is that males were found to be sterile (1, 2, 9, 18, 26, 29, 37). Breeding of these transgenic mice is tedious, and therefore it is difficult to generate enough mice for experimental studies. This sterility was correlated with high-level expression of TK in the testes (2, 9). Testis transcripts are shorter than full-length TK transcripts and are initiated within the TK coding sequence under the control of a "cryptic promoter" (2). We made similar observations when we generated transgenic mice harboring the TK gene under the transcriptional control of the HIV long terminal repeat (LTR) sequences with the aim of directing expression to DC (40). In the transgenic lines in which full-length transcripts were expressed in DC, allowing DC ablation, the males were sterile and short TK transcripts were abundant in the testes. In the others, there was no significant transgene expression in either DC or the testes and the males were fertile. We further investigated this phenomenon and sought to generate fertile transgenic mice with a conditional toxin capable of performing cell ablation.

In this study, we first analyzed the methylation pattern and the precise transcription initiations of the TK gene in the testes and somatic tissues. The results led us to conclude that sterility should be due to the presence of a cryptic testis-specific enhancer which induces strong expression of short transcripts initiated mainly upstream of the second ATG of the TK gene. We thus generated a truncated TK (ΔTK) gene by deletion of

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sequences upstream of the second ATG, since some evidence suggested that the product of such a gene might retain enzymatic activity (13, 28, 41, 45). We showed that this protein possesses thymidine kinase activity and can phosphorylate nucleoside analogs such as GCV. Furthermore, we constructed transgenic mice expressing ΔTK and showed that GCV-induced DC ablation could be achieved in these mice in a fashion similar to that seen with TK, demonstrating its functionality in vivo. Finally, these ΔTK-expressing transgenic males were fertile.

MATERIALS AND METHODS

Plasmids. The construction of the pLTR-TK plasmid has been reported elsewhere (10). For the pLTR-ΔTK plasmid, a ΔTK gene sequence was amplified by PCR with the TK gene as the template. The 5′ oligonucleotide 5′CCGAATTC AAGCTT**ATG**CCCACGCTACTGCGG3′ contains *Eco*RI and *Hin*dIII sites (underlined) and hybridizes to the sequence downstream of the second ATG (in boldface). The 3′ oligonucleotide 5′CCGGATCCACCCGTGCG**TTTTATT**CT GTCT3′ contains a *Bam*HI site (underlined) and hybridizes to the sequence surrounding the second polyadenylation site (in boldface) (33). The *Hin*dIII-*Bam*HI TK gene fragment from pLTR-TK was replaced by the amplified 1.05-kb *Hin*dIII-*Bam*HI ΔTK gene fragment, generating pLTR-ΔTK.

pLTR-ΔTKhygro was generated by inserting the 1.25-kb *Xho*I-*Bam*HI LTR-ΔTK fragment of the pLTR-ΔTK plasmid into the 4.4-kb *Bam*HI-*Sal*I fragment of pHygro (43). The pNCTat retroviral vector was obtained by cloning the 0.4-kb Tat cDNA fragment of pCV-1 (4) downstream of the cytomegalovirus promoter of pNC. This plasmid contains a neomycin resistance gene under the transcriptional control of the 5′ LTR of Moloney murine leukemia virus and an internal cytomegalovirus immediate-early promoter.

Transgenic mice. LTR-TK and LTR-ΔTK transgenic mice were generated as previously described (40). LTR-TK transgenic mice contain 10, 10, 150, and 1 copies of the transgene per cell for lines 14, 8, 12, and 17, respectively. The 13 transgenic lines with the LTR-ΔTK sequence contain 1 to 200 copies of the transgene per cell. Transgenic mice expressing the Tat protein under the control of the promoter of the hydroxymethylglutaryl-coenzyme A reductase-encoding mouse housekeeping gene were provided by Majid Mehtali (Transgene, Strasbourg, France).

Southern analysis and RNase protection assay. *Msp*I- or *Hpa*II-digested genomic DNA was electrophoresed in a 1% agarose gel and transferred to a nylon membrane. Southern blots were hybridized to the ^{32}P-labeled 490-bp *Bgl*II-*Sac*I TK gene fragment surrounding the first four ATG codons (see Fig. 1).

Testis RNA extraction and the RNase protection assay were performed as previously described (40), with two RNA probes. The antisense S probe extended from the 5′ *Eco*RV site in the TK sequence to the *Sca*I site in the HIV LTR sequence (40). The P probe was generated from the 490-bp *Bgl*II-*Sac*I TK gene fragment cloned into the multiple cloning site sequence of the pGEM3Z vector (Promega, Madison, Wis.). This plasmid was linearized with *Pvu*II and used as the template for transcription of a 720-base [^{32}P]UTP-labeled antisense probe synthesized with T7 RNA polymerase.

Cell culture, DNA transfection, and GCV sensitivity. Cellular TK-deficient L cells were maintained in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and antibiotics. Cells were transfected by the calcium phosphate procedure either with 1 μg of plasmid pLTR-TK and growth in HAT selection medium or with 20 μg of plasmid pLTR-ΔTK and 1 μg of plasmid pHygro (43), containing the hygromycin resistance gene, and growth in hygromycin selection medium (300 μg/ml). After 2 to 3 weeks in the selection media, HAT- or hygromycin resistance-selected colonies were picked up with sterile cylinders and individual clones were further expanded. The hygromycin-selected LTR-ΔTK(C1) clone, harboring plasmid pLTR-ΔTK, was then infected with the supernatant of Ψ-CRIP cells (16) stably transfected with pNCTat. Subconfluent cells were cultured with the viral supernatant for 4 h with Polybrene (8 μg/ml). At 48 h later, the cells were selected in HAT medium until clones appeared and were expanded. Tat-expressing HeLa cells (17), cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics, were transfected by the calcium phosphate procedure with 10 μg of pLTR-ΔTKhygro and were selected with 300 μg of hygromycin per ml. Individual clones were picked up and further expanded.

The GCV sensitivity of TK- and ΔTK-expressing L cells or Tat-expressing HeLa cells was determined by using a cell growth assay. L cells were cultured in medium (Dulbecco's modified Eagle's medium-serum-antibiotics) supplemented with hypoxanthine and thymidine for 2 days and then cultured in medium alone before addition of GCV. L cells or Tat-expressing HeLa cells were seeded in multiwell (6- or 12-well) plates at 1/150 confluence and cultured with various GCV concentrations. L cells were detached after 4 days by trypsinization, and viable cells, excluding trypan blue, were counted. Tat-expressing HeLa cells were fixed and stained with Giemsa after 9 days, and we visually determined the GCV dose that reduced the cell density by 50%.

Nucleotide analysis by fast protein liquid chromatography. Cell extracts were prepared as follows. Cells (2×10^6) were incubated for 12 h with 0.18 μM [8-^{3}H]GCV (22 C/mmol) from Moravek Biochemicals (Brea, Calif.) in a final volume of 5 ml. The medium was then removed, and the cells were washed twice with phosphate-buffered saline. Metabolites were extracted overnight with 0.5 ml of 60% methanol at −20°C. After centrifugation to remove cell debris, the supernatant was removed under reduced pressure and the dried extracts were resuspended in 500 μl of deionized water and filtered through a 0.22-μm-pore-size filter. The samples were analyzed by fast protein liquid chromatography (Pharmacia-LKB Instruments, Uppsala, Sweden) on a polymer-based monoQ HR 5/5 strong anion-exchange column and eluted with ammonium phosphate buffer (pH 7.0) by using an improved gradient program in the concentration range of 0.02 to 0.5 M (31). Radioactivity was counted at the column exit with a Radiomatic Flo-one Beta A-500 apparatus (Packard), and peaks were analyzed with Flo-one/Data software.

TABLE 1. TK RNA expression in skin and testes of LTR-TK transgenic lines[a]

Tissue	Mean expression ± SD in transgenic line(s):		
	8, 14	12	17
Skin	1.3 ± 0.9	0.02 ± 0.01	0.00
Testis	10.8 ± 7.7	0.25 ± 0.19	0.00

[a] Amounts of full-length TK transcripts in skin samples and those of short TK transcripts in testis samples were estimated by RNase protection assay. Values are presented as picograms of TK RNA per 10 μg of total RNA. Three to five transgenic mice each from lines 8, 12, and 14 contributed to the calculation of the mean and the standard deviation. The values for lines 8 and 14 were comparable and were pooled.

RESULTS

Methylation patterns of the transgene in LTR-TK transgenic mice. The sterility of transgenic mice harboring TK correlated with high-level expression of short TK transcripts in the male germinal line. Among our four LTR-TK transgenic lines, two had a high-level expression of short TK transcripts in the testes and sterile males, whereas in the two others, little or no TK expression was detected in the testes and the males were fertile. In addition, the mice in which the cryptic promoter was active in the testes (lines 8 and 14) had a functional HIV LTR promoter in somatic tissues. In the others (lines 12 and 17), the cryptic promoter in the testes and the HIV LTR promoter in the skin were both weakly expressed or completely silent (Table 1). Therefore, the two promoters were simultaneously activated or silent.

Previous transfection experiments showed that CpG methylation in the 5′ end of the TK coding sequence could completely block expression of the gene (23). We thus analyzed the methylation pattern of the TK gene in the four LTR-TK transgenic lines. Tail and testis DNAs were digested with restriction enzyme *Msp*I or *Hpa*II and analyzed by Southern blotting. The two enzymes have the same restriction site (CCGG), but only *Hpa*II is sensitive to methylation at the CpG dinucleotide. Thus, the comparison of the digestion patterns of the two enzymes reveals CpG methylation. After complete *Msp*I digestion of the LTR-TK transgene, three DNA fragments of 405, 245, and 122 bp were expected to hybridize with a probe which overlaps the second and third *Msp*I-*Hpa*II sites (Fig. 1). These three fragments were detected in the four transgenic lines. Two other bands in line 12 and one in line 14 probably corresponded to junction fragments at the transgene integration sites (Fig. 2). After complete *Hpa*II digestion, the same pattern was observed in transgenic lines 8 and 14. The band corresponding to the 122-bp fragment (not visible in the Southern blot of Fig. 2) became visible with longer DNA migration (data not shown). In these two transgenic lines, an additional band corresponding to a 650-bp fragment was detected in tail but not testis DNA, indicating that the second *Msp*I-*Hpa*II site of

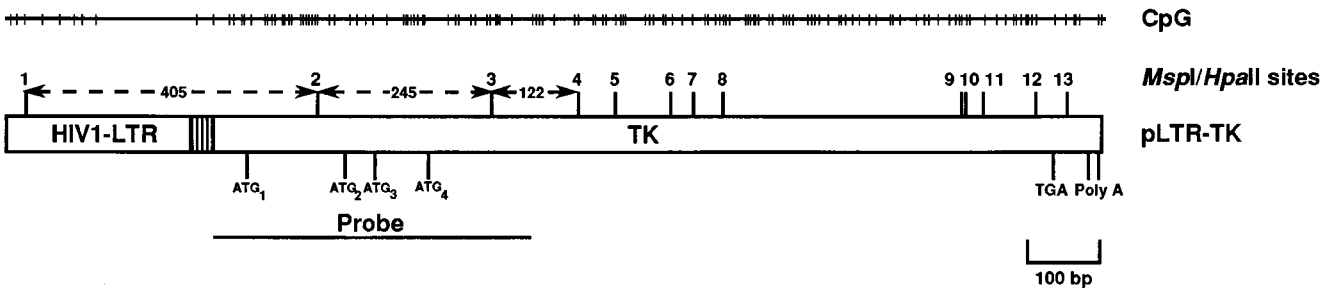

FIG. 1. Structure of the LTR-TK transgene. The top line shows the CpG dinucleotide distribution. The 13 *Msp*I-*Hpa*II restriction sites are numbered, and distances between the first four sites are indicated. The barred rectangle between the HIV LTR and TK sequences represents part of the multiple cloning site sequence of the PGEM3Z vector (Promega). The first four start codons (ATG), the stop codon (TGA), and the two polyadenylation sites (Poly A) of the TK gene are indicated. The construct is drawn to scale. The probe used in the Southern blot analysis is indicated.

the transgene was weakly methylated in tail DNA and not at all in testis DNA. In contrast in line 12, most of *Hpa*II-digested tail and testis DNA fragments were longer than 2.85 kb, the size of the transgene. This indicated that all of the *Msp*I-*Hpa*II sites of the TK gene were methylated. Finally, in line 17, which contains only one LTR-TK copy per cell, the transgene seemed to be partially methylated in somatic tissues and less methylated in the testes (Fig. 2). Altogether, these results indicate (i) that TK methylation is associated with inactivation of both the HIV and the cryptic TK promoters and (ii) that testis-specific expression of TK is not correlated with testis-specific undermethylation of this gene.

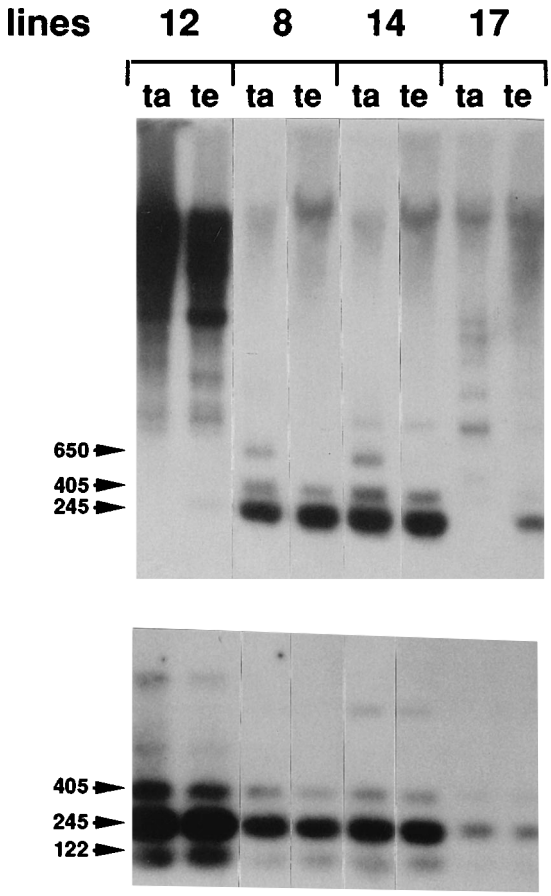

FIG. 2. Transgene methylation pattern of the four LTR-TK transgenic lines. Samples (3, 10, 10, and 30 μg) of tail (ta) or testis (te) DNA of transgenic lines 12, 8, 14, and 17, respectively, were digested with *Hpa*II (top) or *Msp*I (bottom) and analyzed by Southern blotting with the probe indicated in Fig. 1. The migration positions of some *Msp*I-*Hpa*II restriction fragments are indicated on the left (sizes are in base pairs).

Testis-specific short TK transcripts are initiated within 145 bp of the TK coding sequence. The presence of a cryptic promoter in the coding sequences of TK was first suggested by Northern (RNA) blot analysis. In transfection experiments with cellular TK-deficient cells with a promoterless TK gene, followed by HAT selection, Roberts and Axel detected two short TK transcripts of 1.1 and 0.9 kb (38). Similarly, Al-Shawi et al. found TK transcripts of 1.2 and 0.9 kb in the testes of transgenic mice harboring TK. A more precise analysis of these transcripts by RNase protection assay and primer extension revealed the presence of five transcriptional initiation sites in an 80-bp sequence between the first and the second ATGs (2). However, under their experimental conditions, they could not detect transcripts initiated downstream of the second ATG. To find these eventual additional transcriptional initiation sites, we performed an RNase protection assay with two RNA probes, P and S, that overlap the 5′ end of the TK gene by 500 and 290 bases, respectively (Fig. 3). We detected at least eight groups of transcriptional initiation sites that span a 145-bp sequence (Fig. 3 and 4). The transcriptional initiation sites of the longest TK RNA were identical when mapped with probes P and S (Fig. 4). Therefore, the whole transcriptional initiation window lies between the first and fourth ATGs and the more abundant transcripts are initiated upstream of the second ATG.

In vitro functional properties of ΔTK. We desired a TK mutant retaining its enzymatic activity without causing sterility in transgenic males. The localization of the short TK transcripts in the testes led us to construct a truncated TK gene by deleting the 5′ fragment upstream of the second ATG. To compare the functional properties of ΔTK and TK, ΔTK was placed under the same transcriptional control of the HIV LTR promoter used in our previous experiments with TK (40). In the absence of the viral transactivator Tat, the HIV LTR is a weak promoter. This allowed us to analyze the functional properties of ΔTK at basal and transactivated levels.

pLTR-ΔTK and pLTR-TK expression vectors were transfected into L cells deficient in cellular TK, and the cells were then selected in HAT medium. LTR-TK-transfected clones appeared and grew normally, whereas parental cells died by 5 days. LTR-ΔTK-transfected cells appeared but died during week 2 of culture in the selection medium (data not shown). This suggested that low-level expression of ΔTK could produce reduced TK activity compared with expression of the full-length TK gene. Thus, we generated LTR-ΔTK transfectants

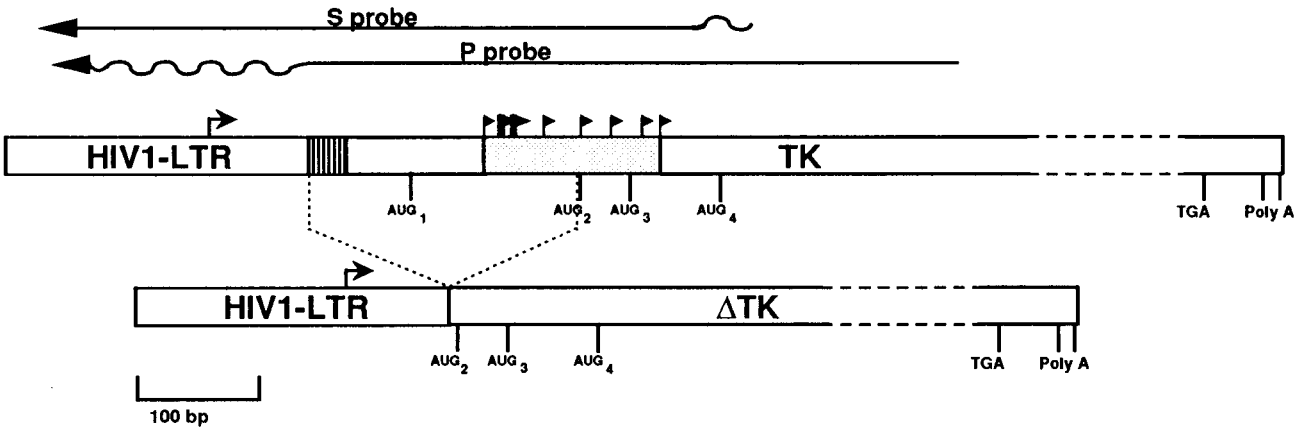

FIG. 3. Schematic representation of the testis-specific TK transcript 5′ ends and of the LTR-ΔTK transgene. The locations of the S and P probes used in the RNase protection assay relative to the LTR-TK transgene are shown. The wavy lines represent sequences of the PGEM3Z vector. The bent arrow indicates the transcription initiation site of the HIV type 1 LTR. The flags represent the eight groups of transcription initiation sites of the testis promoter, and the grey region of the TK gene delimits the transcription initiation window. The region of the TK gene deleted to form the ΔTK gene is indicated by dotted lines. Other details are described in the legend to Fig. 1. Constructs are drawn to scale except in the dashed-line regions.

by cotransfection of L cells deficient in cellular TK with pLTR-ΔTK and pHygro and then subjected them to selection with hygromycin. We subsequently transactivated ΔTK RNA expression in these transfectants by expression of Tat. This was achieved by infection with the supernatant of a packaging cell line transfected with pNCTat, followed by HAT selection. Several clones and the bulk of the stable LTR-ΔTK+Tat transfectants were selected and expanded in HAT medium without mortality. We analyzed TK transcript amounts in these different cells. In three LTR-TK clones, five LTR-ΔTK clones, and three LTR-ΔTK+Tat clones, RNA expression was estimated to be 5, 1 to 3, and 50 RNA copies per cell, respectively (data not shown). These cells were then tested for GCV sensitivity. The 50% inhibitory doses (ID_{50}) were 20 μM for parental L cells and 0.02 μM for most of the LTR-TK clones. The ID_{50} of the six LTR-ΔTK clones tested was 20 μM, whereas the ID_{50} of the two clones and the bulk of the LTR-ΔTK+Tat cells ranged between 0.03 and 0.08 μM (Fig. 5 and data not shown).

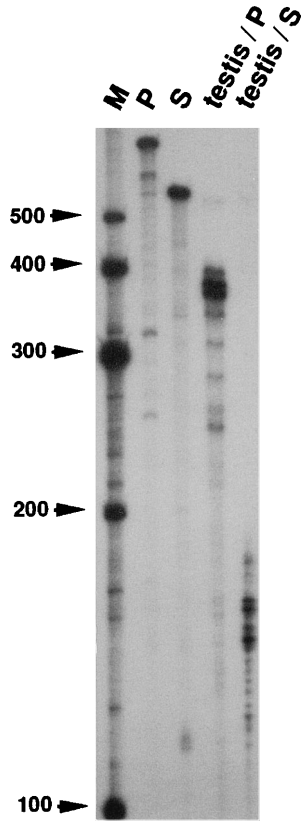

FIG. 4. Transcription initiation sites of the testis-specific cryptic promoter of the TK gene. Testis RNA (20 μg) from LTR-TK transgenic mouse line 14 was analyzed by an RNase protection assay with the P riboprobe (lane testis/P) and the S riboprobe (lane testis/S). The full-length, nondigested P probe (lane P) and S probe (lane S) migrated on the same 4% polyacrylamide gel. RNA molecular size markers (lane M) of 100, 200, 300, 400, and 500 bases were generated by in vitro transcription with an RNA marker template set from Ambion Inc.

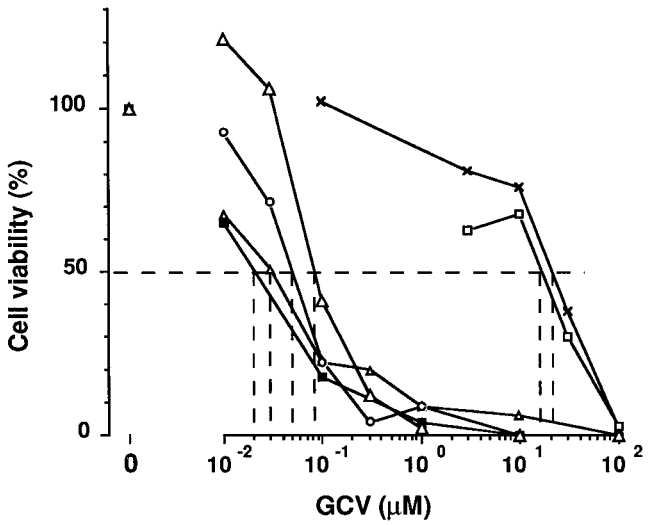

FIG. 5. GCV sensitivity of TK- and ΔTK-expressing L cells. Parental L cells, the representative C1 clones of LTR-TK and LTR-ΔTK transfectants, and the bulk and C1 and C2 clones of the LTR-ΔTK+Tat transfectants were cultured in the presence of increasing GCV concentrations for 4 days. The percentage of viable cells, relative to those in the culture without GCV, was determined. The dashed lines indicate the ID_{50}. Symbols: ×, L cells; ■, LTR-TK C1; □, LTR-ΔTK C1; ○, bulk of LTR-ΔTK+Tat; △, LTR-ΔTK+Tat C1; △, LTR-ΔTK+Tat C2.

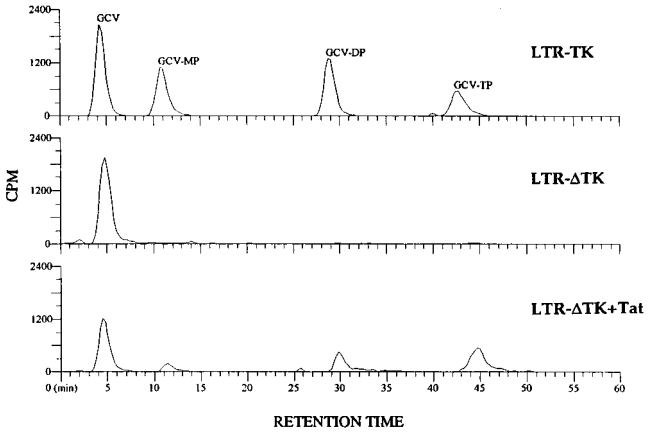

FIG. 6. Comparison of fast protein liquid chromatography profiles of the C1 clones of LTR-TK and LTR-ΔTK transfectants and those of the bulk of the LTR-ΔTK+Tat transfectants incubated with [^{3}H]GCV for 12 h. MP, monophosphate; DP, diphosphate; TP, triphosphate.

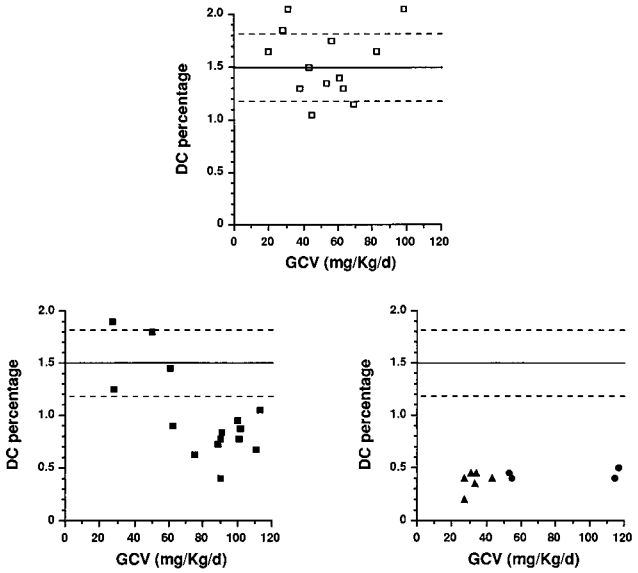

FIG. 7. GGV treatment of transgenic mice harboring ΔTK induces DC depletion. Control mice (□) and LTR-ΔTK (■), LTR-TK (▲), and LTR-ΔTK × Tat (●) transgenic mice were treated with various doses of GCV for 7 days. Splenic DC were then quantified by flow cytometry as previously described (40). The control mice were nontransgenic mice or Tat-expressing transgenic mice. The absolute numbers of splenocytes from control mice and LTR-ΔTK and LTR-ΔTK × Tat transgenic mice, treated or not treated with GCV, were similar. Continuous and dashed lines represent the mean ± 1 standard deviation, respectively, of DC percentages for control mice. At GCV doses higher than 70 mg/kg/day, the average percentages for LTR-ΔTK transgenic lines 3 and 72, from which are derived the LTR-ΔTK × Tat lines, were 0.7 and 0.6, respectively (three mice of each line were treated). The average percentages for the four other LTR-ΔTK transgenic lines were 0.5, 0.7, 0.7, and 0.8.

To rule out the possibility that the HAT selection of LTR-ΔTK+Tat clones biased the selection of ΔTK transfectants, we analyzed the GCV sensitivity of Tat-expressing HeLa cells transfected with pLTR-ΔTKhygro and selected with hygromycin. Of the six clones analyzed, four were sensitive to GCV. An RNase protection assay showed that they expressed 50 to 100 RNA copies per cell. The ID_{50} of GCV were below 0.01 μM for one clone, between 0.1 and 0.01 μM for the three others, and over 100 μM for parental cells (data not shown).

Altogether, these results demonstrate that ΔTK is a functional kinase which can phosphorylate thymidine and GCV. However, in contrast to those that express TK RNA, cells expressing a very low level of ΔTK RNA are not sensitive to GCV.

Comparison of GCV phosphorylation by TK and ΔTK. We directly investigated the enzymatic properties of ΔTK by looking at the GCV phosphorylation pattern in ΔTK-expressing cells. ^{3}H-labeled GCV was added to cultures of parental L cells and TK and ΔTK gene-transfected L cells. After 12 h, nucleoside pools were extracted from these cells and analyzed by fast protein liquid chromatography. Unphosphorylated GCV was the only form detected in parental cells (data not shown), while all three phosphorylated forms were found in TK-expressing cells. In LTR-ΔTK cells, phosphorylation appeared to be very inefficient, with only traces of phosphorylated GCV. In Tat-expressing LTR-ΔTK cells, the GCV phosphorylation pattern was similar to that of TK-expressing cells (Fig. 6). Therefore, ΔTK could phosphorylate GCV and there was a correlation between the GCV phosphorylation pattern and the GCV sensitivity of these cells.

ΔTK allows GCV-mediated cell ablation in transgenic mice and does not cause male sterility. Thirteen transgenic lines harboring the LTR-ΔTK sequence were generated. Six of the eight transgenic lines analyzed for transgene expression in skin had a significant level of ΔTK transcripts, comparable to the TK transcript level in LTR-TK transgenic lines 8 and 14 (data not shown). We then analyzed the abilities of different LTR-ΔTK transgenic lines to ablate DC. We previously demonstrated that proliferating DC precursors arising from granulocyte-macrophage colony-stimulating factor-treated bone marrow cultures derived from LTR-TK transgenic line 14 were sensitive to GCV. The ID_{50} of GCV for bone marrow-derived DC precursors from nontransgenic mice was 40 μM, and it was 0.04 μM for LTR-TK transgenic line 14 mice. We repeated this same experiment with two LTR-ΔTK transgenic lines. We found ID_{50} of 2 and 20 μM GCV for these two lines, indicating that the bone marrow cultures derived from LTR-ΔTK transgenic mice were sensitive to GCV, although to a lesser degree than those from LTR-TK transgenic mice (data not shown). We then tested GCV-dependent ablation of DC in LTR-ΔTK transgenic mice. The DC percentages were compared with those obtained with GCV-treated LTR-TK transgenic line 14 with the same genetic background. In LTR-ΔTK transgenic mice, GCV treatment led to a partial decrease of DC percentages in six different LTR-ΔTK transgenic lines and only at GCV doses higher than 70 mg/kg/day (Fig. 7). To increase the expression of ΔTK in these transgenic mice, and possibly improve DC depletion, we further crossed LTR-ΔTK transgenic lines 3 and 72 (Fig. 7, legend) with transgenic mice expressing the Tat protein under the control of a mouse housekeeping promoter. ΔTK RNA expression in the spleens of the LTR-ΔTK × Tat double-transgenic mice was increased three- to fivefold compared with that in the spleens of LTR-ΔTK transgenic mice. These two double-transgenic lines were both treated with GCV at 50 and 110 mg/kg/day. This resulted in DC depletion comparable to that obtained with LTR-TK transgenic mice (Fig. 7). These results are in agreement with those obtained in in vitro transfection experiments.

Finally, the LTR-ΔTK and LTR-ΔTK × Tat transgenic lines were tested for fertility. All transgenic males transmitted the transgene to their progeny in a Mendelian manner. We detected no short TK transcript in their testes with the RNase protection assay (data not shown).

DISCUSSION

The use of a conditional cell knockout in transgenic mice with TK is greatly complicated by a TK side effect that renders male transgenic mice sterile. This sterility is associated with high-level expression of short TK transcripts in postmeiotic germ line cells (9). There are few TK transgenic males that are fertile and have little or no TK transcript expression in the testes (Table 1) (2, 9). However, in these mice there is no significant TK RNA expression in somatic cells. We show here that this is correlated with transgene methylation (Fig. 2). This is in accordance with the observation that the methylation of one of the first *Msp*I-*Hpa*II sites of the TK coding region is sufficient to block TK expression, even from a heterologous promoter (23). Therefore, male fertility in a TK transgenic line is predictive of a lack of transgene expression in somatic tissues, precluding cell ablation with GCV.

We further investigated the cause of male sterility in transgenic mice harboring a TK transgene with the aim of generating a mutant functional enzyme that does not cause sterility. We first investigated whether the activity of the testis-specific cryptic promoter of TK is linked to testis-specific undermethylation for several reasons. (i) The TK gene is a GC-rich gene (65% GC and 15% CpG dinucleotide sites); thus, its expression may be methylation dependent (7). Indeed, this gene is considered to be a "methylation-sensitive" gene (23). (ii) CpG methylation has been involved in the transcriptional regulation of most tissue-specific genes (12). (iii) The methylation of germ line genomes is significantly different from the methylation of somatic cell genomes (35), and testis-specific expression of some endogenous genes or transgenes has been correlated with specific undermethylation of the locus in testis DNA (3, 39). We thus compared the TK methylation patterns in somatic and testis DNAs of LTR-TK transgenic lines. In lines 8 and 14, in which short TK transcripts are highly and specifically expressed in the testes, the transgene is undermethylated in both somatic and testis DNAs. Thus, testis-specific expression of TK is not due to a particular methylation pattern, strongly suggesting that this expression is rather due to the presence of an unidentified male germ line-specific enhancer(s) in the TK gene.

A truncated TK gene starting at the second ATG, with its major internal transcription initiation sites deleted, might therefore be silent in testes but still retain a functional activity. Indeed, in promoters that lack a TATA box or initiator element and have multiple transcriptional initiation sites, the boundaries of the initiation window might depend only on regulatory sequences around the 5′ end of this window (22). In addition, previous work has shown that truncated TK with these amino-terminal residues deleted can phosphorylate thymidine (25, 41, 45), in accordance with the localization of the two active sites of the enzyme (ATP and nucleoside-nucleotide-binding sites) 3′ to the second ATG (5, 32).

We tested directly the enzymatic and functional properties of such a ΔTK as a conditional toxin for eukaryotic cells. We found that ΔTK can indeed phosphorylate both thymidine and GCV, but in contrast to those expressing TK RNA, cells expressing low levels of ΔTK RNA are not sensitive to GCV. Indeed, we have made numerous cell lines from different origins and species that express TK under the control of various promoters. The ID_{50} of GCV varies from one line to another, but for a given cell line, even very low-level TK RNA expression is sufficient to render cells fully sensitive to the toxic effect of GCV (data not shown). On the contrary, we show here that a low level of ΔTK RNA expression does not confer sensitivity to GCV. However, when RNA expression is increased, there is a correlated increase in sensitivity to GCV. The ID_{50} for L cells or Tat-expressing HeLa cells expressing a higher but still moderate level of ΔTK RNA reaches the ID_{50} for TK-expressing cells. These observations are in agreement with previous studies showing that some herpes simplex virus mutants synthesizing low levels of truncated TK are less sensitive to the antiviral effect of acyclovir or GCV than are wild-type viruses (13). This was correlated with the observation that the stability of truncated TK proteins could be influenced by their amounts (13, 24, 28). These in vitro observations translate in vivo. Indeed, we obtained good DC depletion in LTR-ΔTK × Tat double-transgenic mice, comparable to that achieved with LTR-TK transgenic mice. In contrast, only partial DC depletion was obtained with LTR-ΔTK transgenic mice which expressed low levels of ΔTK RNA. Finally, the males of these ΔTK transgenic mice were fertile and transmitted the transgene to their progeny, in agreement with the observation that no short TK transcripts could be detected in the testes. This suggests that the putative testis-specific enhancer is located between the first and second ATGs.

These results demonstrate that the ΔTK gene can be substituted for the intact TK gene to generate fertile male transgenic mice, allowing TK obliteration. Furthermore, the functional properties of ΔTK might offer several advantages over those of intact TK for in vivo use in transgenic mice and for gene therapy. The utilization of ΔTK should increase the specificity of cell-specific ablation in transgenic mice harboring a ΔTK gene controlled by a tissue-specific promoter. Indeed, tissue-specific promoters often demonstrate some leakiness in cell types other than those in which they should normally be expressed, the leaky expression usually being weak. Therefore, only cell-specific expression of ΔTK would be sufficient to confer sensitivity to GCV. Similarly, TK has been used to ablate cancer cells in experimental models, and in humans, after gene transfer with recombinant retroviruses and adenoviruses (14). It has been proposed to place the TK gene under the control of a cell-specific promoter to minimize possible deleterious effects resulting from the transduction of other cell types during in vivo transduction (27). However, internal heterologous promoters used in the context of viral vectors may lose part of their specificity. The use of ΔTK can offer additional safety in this setting, since leaky expression is often weaker than specific expression. This advantage is even more obvious when use of the TK gene for gene therapy of HIV infection is contemplated (10). In such a setting, the toxic gene must be present in the HIV target cell before infection but in a silent state that can be rapidly activated when HIV infection proceeds. This is best achieved by using the HIV regulatory sequences to control TK expression in which an increased level of TK will be induced, because of the ability of the Tat protein to transactivate the HIV LTR promoter in infected cells. However, it is difficult to avoid basal expression of TK in the absence of HIV infection (8). Therefore, by utilizing the ΔTK gene, instead of the intact TK gene, this therapy can result in the sole ablation of HIV-infected cells, which express a very high level of ΔTK.

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REFERENCES

1. **Al-Shawi, R., J. Burke, C. T. Jones, J. P. Simons, and J. O. Bishop.** 1988. A *Mup* promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. Mol. Cell. Biol. **8:**4821–4828.
2. **Al-Shawi, R., J. Burke, H. Wallace, C. Jones, S. Harrison, D. Buxton, S. Maley, A. Chandley, and J. O. Bishop.** 1991. The herpes simplex virus type 1 thymidine kinase is expressed in testes of transgenic mice under the control of a cryptic promoter. Mol. Cell. Biol. **11:**4207–4216.
3. **Ariel, M., J. McCarrey, and H. Cedar.** 1991. Methylation patterns of testis-specific genes. Proc. Natl. Acad. Sci. USA **88:**2317–2321.
4. **Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal.** 1985. Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229:**69–73.
5. **Black, M. E., and L. A. Loeb.** 1993. Identification of important residues within the putative nucleotide binding site of HSV-1 thymidine kinase by random sequence selection: analysis of selected mutants *in vitro*. Biochemistry **32:**11618–11626.
6. **Borrelli, E., R. A. Heyman, C. Arias, P. E. Sawchenko, and R. M. Evans.** 1989. Transgenic mice with inducible dwarfism. Nature (London) **339:**538–541.
7. **Boyes, J., and A. Bird.** 1992. Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J. **11:**327–333.
8. **Brady, H. J. M., C. G. Miles, D. J. Pennington, and E. A. Dzierzak.** 1994. Specific ablation of human immunodeficiency virus Tat-expressing cells by conditionally toxic retroviruses. Proc. Natl. Acad. Sci. USA **91:**365–369.
9. **Braun, R. E., D. Lo, C. A. Pinkert, G. Widera, R. A. Flavell, R. D. Palmiter, and R. L. Brinster.** 1990. Infertility in male transgenic mice: disruption of sperm development by HSV-tk expression in postmeiotic germ cells. Biol. Reprod. **43:**684–693.
10. **Caruso, M., and D. Klatzmann.** 1992. Selective killing of $CD4^+$ cells harboring a human immunodeficiency virus-inducible suicide gene prevents viral spread in an infected cell population. Proc. Natl. Acad. Sci. USA **89:**182–186.
11. **Caruso, M., Y. Panis, S. Gagandeep, D. Houssin, J.-L. Salzmann, and D. Klatzmann.** 1993. Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. Proc. Natl. Acad. Sci. USA **90:**7024–7028.
12. **Cedar, H.** 1988. DNA methylation and gene activity. Cell **53:**3–4.
13. **Coen, D. M., A. F. Irmiere, and J. G. Jacobson.** 1989. Low levels of herpes simplex virus thymidine-thymidylate kinase are not limiting for sensitivity to certain antiviral drugs or for latency in a mouse model. Virology **168:**221–231.
14. **Culver, K. W., and R. M. Blaese.** 1994. Gene therapy for cancer. Trends Genet. **10:**174–178.
15. **Culver, K. W., Z. Ram, S. Wallbridge, H. Ishii, E. H. Oldfield, and R. M. Blaese.** 1992. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science **256:**1550–1552.
16. **Danos, O., and R. C. Mulligan.** 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA **85:**6460–6464.
17. **Dragic, T., P. Charneau, F. Clavel, and M. Alizon.** 1992. Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. J. Virol. **66:**4794–4802.
18. **Dzierzak, E., B. Daly, P. Fraser, L. Larsson, and A. Müller.** 1993. Thy-1 tk transgenic mice with a conditional lymphocyte deficiency. Int. Immunol. **5:**975–984.
19. **Field, A. K., M. E. Davies, C. DeWitt, H. C. Perry, R. Liou, J. Germershausen, J. D. Karkas, W. T. Ashton, D. B. R. Johnston, and R. L. Tolman.** 1983. 9-{[2-Hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine: a selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. USA **80:**4139–4143.
20. **Furman, P. A., P. V. McGuirt, P. M. Keller, J. A. Fyfe, and G. B. Elion.** 1980. Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Virology **102:**420–430.
21. **Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion.** 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. **253:**8721–8727.
22. **Geng, Y., and L. F. Johnson.** 1993. Lack of an initiator element is responsible for multiple transcriptional initiation sites of the TATA-less mouse thymidylate synthase promoter. Mol. Cell. Biol. **13:**4894–4903.
23. **Graessmann, A., G. Sandberg, E. Guhl, and M. Graessmann.** 1994. Methylation of single sites within the herpes simplex virus *tk* coding region and the simian virus 40 T-antigen intron causes gene inactivation. Mol. Cell. Biol. **14:**2004–2010.
24. **Haarr, L., and T. Flatmark.** 1987. Evidence that deletion of coding sequences in the 5′ end of the thymidine kinase gene of herpes simplex virus type 1 affects the stability of the gene products. J. Gen. Virol. **68:**2817–2829.
25. **Halpern, M. E., and J. R. Smiley.** 1984. Effects of deletions on expression of the herpes simplex virus thymidine kinase gene from the intact viral genome: the amino terminus of the enzyme is dispensable for catalytic activity. J. Virol. **50:**733–738.
26. **Heyman, R. A., E. Borrelli, J. Lesley, A. Deborah, D. D. Richman, S. M. Baird, R. Hyman, and R. M. Evans.** 1989. Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. Proc. Natl. Acad. Sci. USA **86:**2698–2702.
27. **Huber, B. E., C. A. Richards, and T. A. Krenitsky.** 1991. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. Proc. Natl. Acad. Sci. USA **88:**8039–8043.
28. **Irmiere, A. F., M. M. Manos, J. G. Jacobson, J. S. Gibbs, and D. M. Coen.** 1989. Effect of an amber mutation in the herpes simplex virus thymidine kinase gene on polypeptide synthesis and stability. Virology **168:**210–220.
29. **Iwakura, Y., M. Asano, Y. Nishimune, and Y. Kawade.** 1988. Male sterility of transgenic mice carrying exogenous mouse interferon-beta gene under the control of metallothionein enhancer-promoter. EMBO J. **7:**3757–3762.
30. **Kamogawa, Y., L. E. Minasi, S. R. Carding, K. Bottomly, and R. A. Flavell.** 1993. The relationship of Il-4- and IFNγ-producing T cells studied by lineage ablation of Il-4-producing cells. Cell **75:**985–995.
31. **Kremmer, T., E. Paulik, M. Boldizsar, and I. Holenyi.** 1989. Application of the fast protein liquid chromatographic system and MonoQ HR 5/5 anion exchanger to the separation of nucleotides. J. Chromatogr. **493:**45–52.
32. **Liu, Q., and W. C. Summers.** 1988. Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. Virology **163:**638–642.
33. **McKnight, S. L.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. **8:**5949–5963.
34. **Minasi, L. E., Y. Kamogawa, S. R. Carding, K. Bottomly, and R. A. Flavell.** 1993. The selective ablation of interleukin-2-producing cells isolated from transgenic mice. J. Exp. Med. **177:**1451–1459.
35. **Monk, M., M. Boubelik, and S. Lehnert.** 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development **99:**371–382.
36. **Moolten, F. L.** 1986. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Res. **46:**5276–5281.
37. **Neznanov, N., I. S. Thorey, G. Ceceña, and R. G. Oshima.** 1993. Transcriptional insulation of the human keratin 18 gene in transgenic mice. Mol. Cell. Biol. **13:**2214–2223.
38. **Roberts, J. M., and R. Axel.** 1982. Gene amplification and gene correction in somatic cells. Cell **29:**109–119.
39. **Salehi-Ashtiani, K., R. J. Widrow, C. L. Markert, and E. Goldberg.** 1993. Testis-specific expression of a metallothionein I-driven transgene correlates with undermethylation of the locus in testicular DNA. Proc. Natl. Acad. Sci. USA **90:**8886–8890.
40. **Salomon, B., P. Lorès, C. Pioche, P. Racz, J. Jami, and D. Klatzmann.** 1994. Conditional ablation of dendritic cells in transgenic mice. J. Immunol. **152:**537–548.
41. **Smiley, J. R., H. Swan, M. M. Pater, A. Pater, and M. E. Halpern.** 1983. Positive control of the herpes simplex virus thymidine kinase gene requires upstream DNA sequences. J. Virol. **47:**301–310.
42. **St. Clair, M. H., C. U. Lambe, and P. A. Furman.** 1987. Inhibition by ganciclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Antimicrob. Agents Chemother. **31:**844–849.
43. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5:**410–413.
44. **Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11:**223–232.
45. **Zemskova, M. Y., and I. Fodor.** 1991. Transient expression of deletion mutants of the herpes simplex virus thymidine kinase-encoding gene in mouse fibroblast cells. Gene **106:**249–253.