

# A Gene-Specific Promoter in Transgenic Mice Directs Testis-Specific Demethylation Prior to Transcriptional Activation In Vivo<sup>1</sup>

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## ABSTRACT

Transcription of the autosomal *phosphoglycerate kinase* gene, *Pgk-2*, is initiated at the onset of meiosis during spermatogenesis in mammals. However, in the mouse, the 5' portion of the endogenous *Pgk-2* coding sequence undergoes a specific demethylation event that precedes transcriptional activation by 10–12 days. Here we show that transgenes consisting of the *Pgk-2* core promoter ligated to the CAT reporter gene undergo a similar tissue-, stage-, and cell type-specific demethylation in the 5' portion of the CAT coding sequence, whereas transgenes consisting of the CAT reporter sequence alone, or of the CAT sequence ligated to the CpG island-containing *transferrin* gene promoter, demonstrate different patterns of demethylation. These results indicate that specific promoter sequences can influence the pattern of tissue-specific demethylation within different genes and that a signal for spermatogenic cell-specific demethylation resides within the core promoter of the mammalian *Pgk-2* gene.

## INTRODUCTION

In mammals, DNA methylation occurs at CpG dinucleotides at which methyl groups can become covalently bound to cytosine residues. CpG dinucleotides can be maintained in a fully demethylated state (unmethylated on both strands) or in a fully methylated state (methylated on both strands). The transient hemimethylated state that results from semi-conservative replication of fully methylated DNA is restored to a fully methylated state by the action of a maintenance DNA methyltransferase [1–3]. A fully unmethylated site can become fully methylated by the action of a de novo DNA methyltransferase [4]. A fully methylated site can be demethylated by the action of an RNA-mediated demethylating activity [5].

Changes in DNA methylation status have been correlated with many biological processes including gene regulation, X-chromosome inactivation, genomic imprinting, formation of heterochromatin, tumorigenesis, and aging [4, 6–8]. A knockout mutation of the gene encoding the maintenance DNA methyltransferase showed that this enzyme, and hence the ability to propagate DNA methylation patterns, is indispensably required for normal development in mice [2]. However, despite evidence implicating DNA methylation as a parameter of gene regulation, neither the mechanism by which changes in DNA methylation modu-

late gene expression, nor the mechanism by which these changes in methylation are themselves regulated, is well understood.

Many ubiquitously expressed housekeeping genes bear constitutively hypomethylated CpG islands, defined as sequences of > 100 base pairs (bp) with relatively high overall GC content (> 50%) that lack the typical underrepresentation of CpG dinucleotides seen in other regions of the mammalian genome [9]. For many tissue-specific genes that do not contain a CpG island, a tissue-specific correlation has been demonstrated between hypomethylation and transcription [10, 11]. In cases that have been examined in a developmental context, demethylation of tissue-specific genes typically occurs prior to the initiation of transcription [12, 13].

The autosomal *phosphoglycerate kinase-2* gene (*Pgk-2*) is a tissue-specific gene expressed according to developmental stage and cell type in the mammalian spermatogenic lineage [14]. *Pgk-2* transcription is initiated at the onset of meiosis in primary spermatocytes and continues in post-meiotic round spermatids. This requires both core promoter and enhancer sequences that interact with ubiquitous and testis-specific transcription factors, respectively [15, 16]. However, several days prior to the establishment of these protein-DNA interactions and the initiation of transcription, the 5' portion of the mouse *Pgk-2* gene, including a portion of the coding sequence, undergoes a specific demethylation event [17, 18].

Spermatogenesis provides an excellent model system for studies of developmentally regulated gene expression in mammals [19, 20]. The ability to isolate relatively pure populations of specific spermatogenic cell types [19, 21] facilitates an analysis of the developmental order of specific regulatory parameters associated with the initiation of spermatogenesis-specific transcription in particular and tissue-specific transcription in general. Here we describe the use of transgenic mice to identify the location and time of action of *cis*-acting regulatory signals that direct tissue-specific demethylation of *Pgk-2* transgenes during mammalian spermatogenesis. Our results indicate that a demethylation signal sequence resides in the core promoter of the *Pgk-2* gene and that it acts in a gene-, tissue-, and developmental stage-specific manner.

## MATERIALS AND METHODS

### *Transgene Constructs*

The plasmid pSV/PBR/PGK2/CAT [15], containing 1.4 kilobases (kb) of the human *PGK-2* upstream regulatory region ligated to the reporter sequence encoding prokaryotic chloramphenicol acetyltransferase (CAT) (Fig. 1A), was digested with *Ava* II, *Pst* I, or *Hind*III at the 5' end, and *Bam*HI at the 3' end, to produce fragments of 1.8 kb ("188 *Pgk-2*/CAT") (Fig. 1B), 2.1 kb ("515 *Pgk-2*/CAT")

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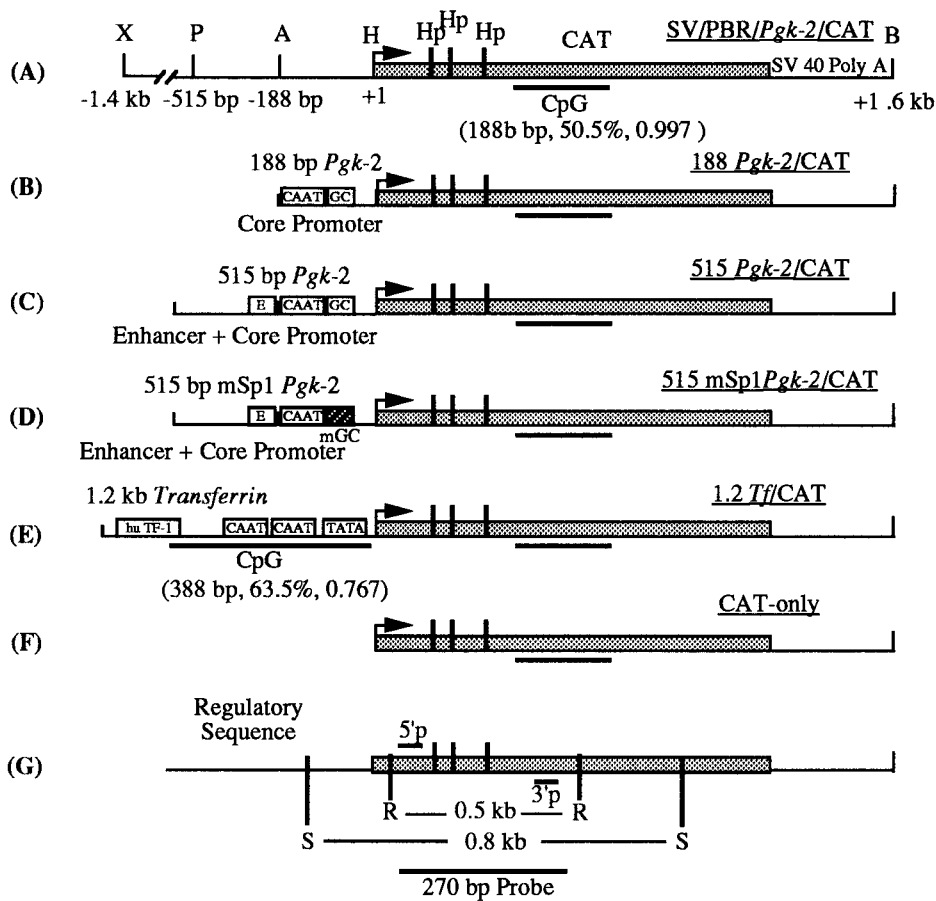


FIG. 1. Promoter-reporter constructs. A) 1.4 kb of the human *Pdk-2* upstream region, B) 188 bp of the human *Pdk-2* regulatory sequence that contains only the core promoter, and C) 515 bp of the human *Pdk-2* regulatory sequence including both the core promoter and enhancer regions, each ligated to the CAT reporter gene followed by a simian virus 40 (SV 40) poly(A)<sup>+</sup> addition sequence (3' UTR). D is identical to 515 *Pdk-2*/CAT except that the Sp1-binding site in the *Pdk-2* core promoter was disrupted by PCR-based in vitro mutagenesis. E) 1.2 kb of the human *transferrin* gene regulatory sequence ligated to the CAT reporter and SV 40 3' UTR sequences [32]. F) CAT reporter plus SV 40 3' UTR sequences alone, without any 5'-regulatory sequences. G) Analysis of methylation of promoter/CAT or CAT-only constructs—positions of *Hpa* II sites (vertical bars), *Rsa* I sites (R), and *Sau* 3A sites (S) is shown, along with the location of the 270-bp probe fragment. The location and size of CpG-island regions are illustrated by horizontal bars labeled CpG below each transgene. The relative strength of each CpG island is measured as the percentage C+G content and the ratio of observed over expected frequencies of CpG dinucleotides within each region. A, *Ava* II; B, *Bam* HI; H, *Hind* III; Hp, *Hpa* II; P, *Pst* I; X, *Xba* I; GC, GC box; CAAT, CAAT box; TF-1, *transferrin* enhancer sequence; bent arrow, transcriptional start point; thick line, transcribed sequence; 5'p, forward primer, and 3'p, reverse primer, used for PCR analyses.

(Fig. 1C), or 1.6 kb (“CAT-only”) (Fig. 1F), respectively. Polymerase chain reaction (PCR)-based in vitro mutagenesis was used as previously described [22] to specifically modify the GC box in the *Pdk-2* core promoter of the 515 *Pdk-2*/CAT construct such that Sp1-binding activity was eliminated (mSp1 515 *Pdk-2*/CAT) (Fig. 1D).

*Transgenic Mice*

Transgenic mice were produced by injection of fertilized eggs from superovulated (C57BL6/J-DBA2J) F<sub>1</sub> females as described previously [23]. Pups were screened by PCR with CAT-specific primers (5'-ACGTTTCAGTTTGCTCATGG-3' and 5'-AGCTAAGGAAGCTAAAATGG-3') [24]. Positives were confirmed by Southern blot hybridization using a 1.6-kb fragment of the CAT coding sequence as a hybridization probe. All investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

*Isolation of Spermatogenic and Somatic Cell Types from the Testis*

Type T<sub>1</sub> prospermatogonia and Sertoli cells were recovered from male fetuses at 18.5 days postcoitum (dpc) using a mini-Sta Put (Johns Scientific, Toronto, ON, Canada) gradient system as previously described [14]. Pachytene spermatocytes and round spermatids were obtained from testes of adult mice using a standard Sta Put gradient also as described previously [14, 25]. Purities of all isolated germ cell or Sertoli cell types were determined by analysis of cellular morphology under phase optics. Populations of

pachytene spermatocytes and round spermatids were consistently > 90% pure, while populations of 18.5 dpc prospermatogonia or Sertoli cells were ≥ 85% pure.

*Analysis of DNA Methylation*

Genomic DNA was prepared as described elsewhere [26]. Each sample was first digested with either *Sau* 3A or *Rsa* I to produce 0.8-kb or 0.5-kb fragments, respectively, each spanning the three methyl-sensitive *Hpa* II sites in the transgenic CAT coding sequence (Fig. 1G). These digested DNA samples were then divided into either two or three aliquots; one received no further digestion (to serve as a control for DNA uncut at the *Hpa* II sites), while the other one or two were digested with the methyl-sensitive restriction enzyme, *Hpa* II, or its isoschizomer, the methyl-insensitive restriction enzyme, *Msp* I, respectively. The digested DNA samples were electrophoresed through 1.0% agarose and blotted to GeneScreen Plus membrane (Dupont NEN, Boston, MA) according to manufacturer's instructions; they were then probed with a PCR-synthesized, 270-bp fragment spanning the three *Hpa* II sites (Fig. 1G) labeled by random priming to a specific activity of > 1 × 10<sup>9</sup> dpm/μg using a kit from Amersham (Megaprime System; Arlington Heights, IL) according to manufacturer's instructions. It should be noted that some of the digestion products on the Southern blots were small (less than 200 bp) and either were run out of the gel or did not hybridize well with the probe. In the case of *Pdk-2*/CAT, the biggest of the digestion products was 419 bp (which can be seen on the Southern blot), and the rest of the fragments were less than

240 bp. For the CAT-only gene, the biggest digestion product was less than 300 bp.

For PCR analysis of DNA methylation in 18.5 dpc spermatogonia and adult pachytene spermatocytes, 0.1-mg aliquots of genomic DNA were either digested with *Hpa* II or left undigested; they were then PCR amplified using 0.2 mM of each of the CAT-specific primers (5'-TCA-GCTGGATATTACGGCCT-3' and 5'-TAACACGCCA-CATCTTGCGA-3') flanking the three *Hpa* II sites (Fig. 1G) using a kit from Perkin-Elmer (Irvine, CA). After 2 min of denaturation at 95°C, 20 cycles of 1 min at 95°C, 1.5 min at 55°C, and 40 sec at 72°C were performed, followed by a final extension/annealing step at 72°C for 7 min. The PCR conditions and number of cycles for this experiment were determined on the basis of a serial analysis of various cycle numbers to determine where the maximal difference in amount of PCR product occurs between reactions templated by methylated or unmethylated DNAs (data not shown). PCR products were run on a 1.0% agarose gel, transferred to GeneScreen membrane (Dupont NEN), and hybridized with a probe spanning the *Hpa* II sites in the CAT reporter gene as described above.

#### Quantitation of Southern Blot Data

For quantitative analysis of methylation, Southern membranes hybridized with <sup>32</sup>P-radioactively labeled probes were exposed to a Kodak Storage Phosphor Screen (Eastman Kodak, Rochester, NY) for 4–8 h. The exposed screen was then scanned using a Molecular Dynamics Phosphor-Imager (model 445SI; Sunnyvale, CA), and the resulting digital images were quantitatively analyzed. The percentage of methylation is expressed as the proportion of the intensity of the band representing undigested fragment remaining after digestion with the methyl-sensitive *Hpa* II enzyme compared to that of the same fragment not digested with *Hpa* II. Animals from two separate lines of transgenic mice were examined for each transgene, and the results for each were averaged to yield the values represented in Figure 8.

#### Analysis of CpG-Island Sequences

A computer program for analyzing CpG islands was developed based on criteria described by Gardiner-Garden and Frommer [9]. CpG islands were defined as regions of DNA sequence of at least 100 bp in length in which the GC content (percentage G+C) is greater than 50%, and the ratio of observed/expected (O/E) CpG dinucleotides is greater than 0.6 (where expected = the number of CpG dinucleotides expected based on the G+C content in the window, assuming equal representation of G+C and A+T in all permutations in that sequence).

#### Detection of CpG Islands in Specific Genes

The *transferrin* promoter sequence was found to contain a 388-bp CpG-island region from -388 to +1 relative to the transcriptional start point, with a G+C percentage of 63.5% and an O/E ratio of CpG dinucleotides of 0.767 (Fig. 1E). The *Pgk-2* promoter sequence does not contain any CpG-island sequence. However, we did identify a small, 188-bp region in the CAT reporter sequence from +506 to +695 (Fig. 1) that possesses characteristics of a CpG island, including a G+C percentage of 50.5% and an O/E CpG ratio of 0.997.

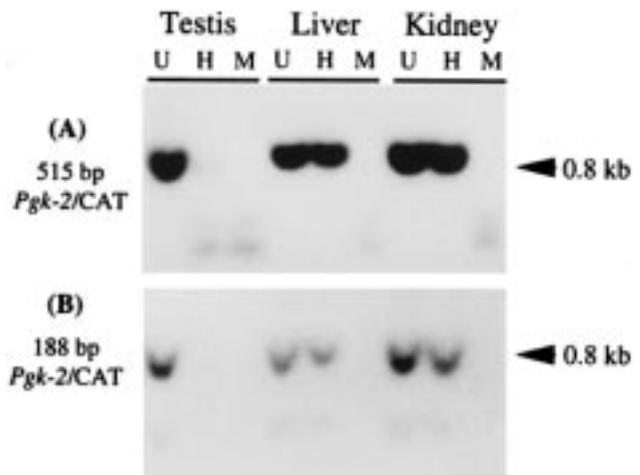


FIG. 2. Testis-specific demethylation of *Pgk-2*/CAT transgenes. **A**) The 515 *Pgk-2*/CAT transgene was analyzed by Southern blot for demethylation in testis tissue, where this transgene is expressed, and in liver and kidney tissues, where the transgene is not expressed. Genomic DNA was prepared from each tissue from transgenic mice and digested with *Sau* 3A, and each sample was divided into three aliquots and further processed as described in *Materials and Methods*. The three *Hpa* II sites probed were found to be completely unmethylated in testis and fully methylated in somatic tissues. **B**) The 188 *Pgk-2*/CAT transgene, which is not expressed in any tissue including the testis, was also examined by Southern blot for tissue-specific methylation. Genomic DNA was isolated from testis, liver, and kidney tissues of 188 *Pgk-2*/CAT transgenic mice and analyzed as described above (see also *Materials and Methods*). These sites were also found to be completely unmethylated in testis and fully methylated in somatic tissues. U, Uncut control; H, *Hpa* II; M, *Msp* I

## RESULTS

### *Pgk-2*/CAT Transgenes Underwent Tissue-Specific Demethylation

Both the endogenous *Pgk-2* gene and the enhancer-bearing 515 *Pgk-2*/CAT transgene have previously been shown to demonstrate exclusively testis-specific expression in the mouse, while the 188 *Pgk-2*/CAT transgene was not expressed [14, 15]. In the present study, two additional lines of mice carrying 188 bp of *Pgk-2* promoter sequence (core promoter only) (188/12 and 188/21), and two additional lines carrying 515 bp of *Pgk-2* promoter sequence (core promoter plus the upstream enhancer region) (515/4, 515/8) (Fig. 1), were established. All lines showed tandem-arrayed, multiple-copy integration except one of the 515 *Pgk-2*/CAT lines (515/4), which showed single-copy integration. Testis-specific expression of the 515 *Pgk-2*/CAT transgenes was confirmed by reverse transcription (RT)-PCR and Northern blot analysis of total RNAs prepared from adult testis and somatic liver and kidney tissues from transgenic mice (data not shown). These same techniques were used to confirm an absence of any expression of the 188 *Pgk-2*/CAT and reduced expression of the mSp1 515 *Pgk-2*/CAT transgenes.

Three methyl-sensitive *Hpa* II sites in the 5' half of the CAT reporter sequence (Fig. 1G) were examined for methylation status of each transgene in various tissues. These sites are analogous in position to the 5' sites in the endogenous mouse *Pgk-2* gene that were previously shown to exhibit specific demethylation during spermatogenesis [17, 18]. Southern blot analysis showed that these three sites were unmethylated in both lines carrying the expressed 515 *Pgk-2*/CAT transgene in testis DNA but methylated in liver and kidney DNAs (Fig. 2A), thus mimicking the pattern

shown by the endogenous *Pgk-2* gene. Surprisingly, both lines carrying the nonexpressed 188 *Pgk-2*/CAT transgene also demonstrated testis-specific demethylation at these same *Hpa* II sites in the CAT reporter sequence (Fig. 2B), indicating that the demethylation event can be uncoupled from active transcription.

#### *Pgk-2*/CAT Transgenes Underwent Cell Type-Specific Demethylation in the Testis

A comparison of the methylation status of the endogenous *Pgk-2* gene in normal seminiferous tubule cells (of which approximately 97% are spermatogenic cells [27]) and that in seminiferous tubule cells from the *W/W<sup>v</sup>* mutant mouse, which include only somatic cells, indicates that demethylation of the endogenous *Pgk-2* gene is cell type specific, occurring only in spermatogenic cells within the testis (Fig. 3B). Analysis of *Pgk-2*/CAT transgene methylation was conducted on DNA isolated from purified populations of pachytene spermatocytes and round spermatids from adult mice carrying either the 515 or 188 *Pgk-2*/CAT transgenes. Results shown in Figure 3C demonstrate that both transgenes were hypomethylated in both spermatogenic cell types examined, suggesting that, like that of the endogenous *Pgk-2* gene, demethylation of the *Pgk-2*/CAT transgenes occurs specifically in spermatogenic cells within the testis.

#### A *Transferrin*/CAT Transgene Was Ubiquitously Unmethylated

To determine whether testis-specific demethylation of *Pgk-2*/CAT transgenes is uniquely signaled by the *Pgk-2* promoter, we examined the methylation pattern of a transgene consisting of the same CAT reporter gene sequence linked to a different promoter sequence, that from the *transferrin* gene. *Transferrin* is produced in many tissues, including liver and brain, where it is expressed at relatively high levels [28–30]. In the testis, the endogenous *transferrin* gene is expressed in somatic Sertoli cells, but not in spermatogenic cells [31]; and 1.2 kb of promoter sequence from the human *transferrin* gene was sufficient to direct similar cell type-specific expression of a CAT reporter gene [32].

We analyzed methylation of the 5' *Hpa* II sites in the CAT sequence in the 1.2 *Tf*/CAT transgene by digesting genomic DNA from testis, liver, kidney, and brain tissues of transgenic mice with *Ban* I to produce a 1.0-kb fragment spanning the three methyl-sensitive *Hpa* II sites in the CAT coding sequence within the transgene. Each DNA sample was then divided into two aliquots. One was not further digested (uncut control) (–), while the other was digested with the methyl-sensitive enzyme, *Hpa* II (+) (Fig. 4A). Like the *Pgk-2*/CAT transgenes, the *Tf*/CAT transgene was unmethylated in the testis (Fig. 4B). However, unlike the *Pgk-2*/CAT transgenes, the *Tf*/CAT transgene was also unmethylated in all somatic tissues examined, including tissues in which this transgene is not expressed. The *transferrin* promoter sequence contains a large CpG island (Fig. 1E) and is therefore expected to be ubiquitously hypomethylated.

#### A CAT-Only Transgene Was Ubiquitously Methylated

As an additional control, we generated transgenic mice carrying only the CAT reporter sequence, in the absence of any associated regulatory sequences (Fig. 1F). Five lines of

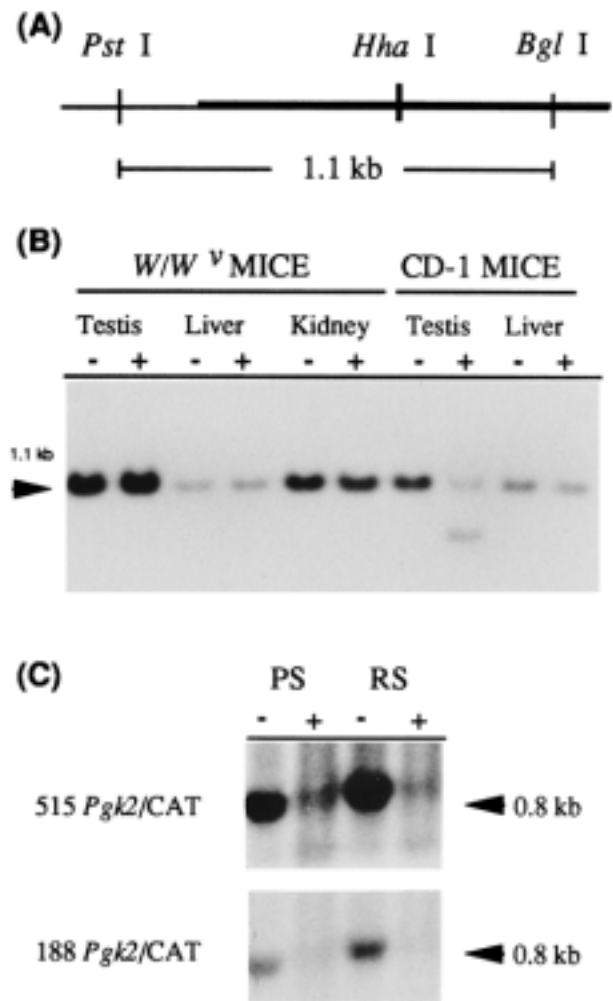


FIG. 3. Cell type-specific demethylation of the endogenous *Pgk-2* gene and *Pgk-2*/CAT transgenes in testis tissue. **A**) The location of a single methyl-sensitive *Hha* I site within the coding sequence of the endogenous *Pgk-2* gene is shown. This site was previously shown to be demethylated in testis tissue but methylated in somatic tissues of normal mice [17, 18]. Methyl-insensitive enzymes (*Pst* I and *Bgl* I) were used to generate a 1.1-kb fragment spanning this site. Each sample was divided into two aliquots, one (+) of which was further digested with *Hha* I and the other (–) of which was not further digested. **B**) Southern blot analysis of DNA from testis, liver, and kidney tissues of adult *W/W<sup>v</sup>* mice, which lack spermatogenic cells, compared to that of CD-1 mice, which are fertile, shows that testis-specific demethylation of the endogenous *Pgk-2* gene is specifically correlated with the presence of spermatogenic cells in this tissue, indicating that the demethylation event occurs only in testicular germ cells and not in testicular somatic cells. **C**) To examine spermatogenic cell type-specific transgene demethylation, purified populations of pachytene spermatocytes (PS) and round spermatids (RS) were isolated from adult testes of 515 *Pgk-2*/CAT and 188 *Pgk-2*/CAT transgenic mice, respectively. Genomic DNA was prepared from each population and analyzed by Southern blot as described in *Materials and Methods*. After digestion with *Sau* 3A, each sample was either further digested with *Hpa* II (+) or not further digested (–). Both the nonexpressed 188 *Pgk-2*/CAT and the expressed 515 *Pgk-2*/CAT transgenes were found to be unmethylated in both spermatogenic cell types, as has been shown to be the case for the endogenous *Pgk-2* gene [17, 18].

CAT-only transgenic mice were analyzed by RT-PCR, and, as expected, no expression of this promoterless construct was detected in any of these tissues (data not shown). Southern blot analysis showed that the three *Hpa* II sites in the CAT coding sequence were heavily methylated in liver and partially to fully methylated (depending on the specific line) in testis tissue (Fig. 5). Importantly, while

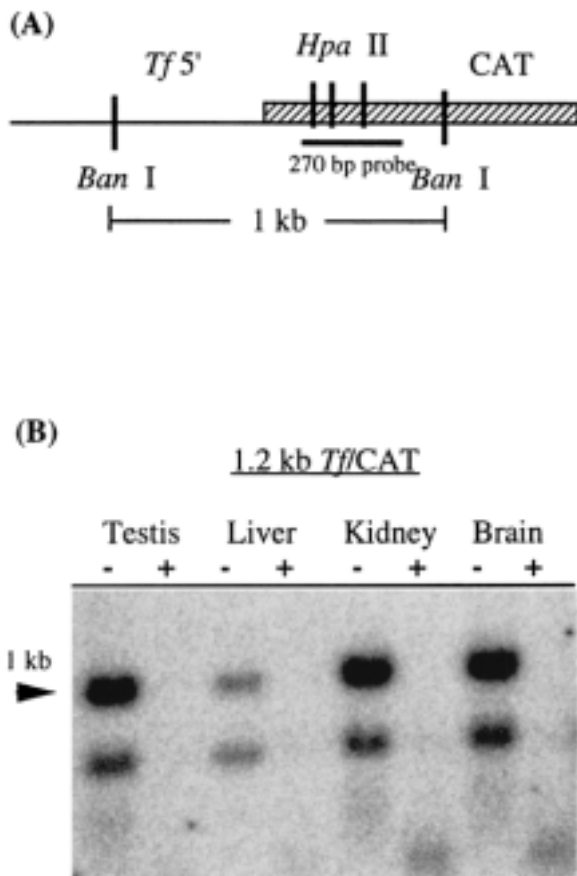


FIG. 4. Ubiquitous hypomethylation of the *transferrin*/CAT transgene. **A)** The widely expressed 1.2 *Tf*/CAT transgene (Fig. 1E) was analyzed for methylation in testis, liver, kidney, and brain tissues of transgenic mice as described in *Results*. **B)** Southern blot analysis was performed using the PCR-synthesized, 270-bp probe that spans the three *Hpa* II sites in the CAT coding sequence. These three sites were found to be completely unmethylated in all tissues tested.

some lines did show partial demethylation in testis cells, we never observed complete demethylation of the CAT sequence in any of the CAT-only lines, whereas we consistently saw this for the *Pgk-2*/CAT transgenes (Fig. 2).

#### Demethylation of *Pgk-2*/CAT Transgenes during Spermatogenesis Was Developmental Stage-Specific

Although CpG island-associated sequences typically remain ubiquitously unmethylated, non-island sequences undergo dynamic changes throughout development [33]. In the developing mouse embryo, changes in DNA methylation in germ cells are distinct in both timing and pattern from those occurring in somatic cells. While many non-island CpG sites in the somatic cell genome undergo de novo methylation in the late blastula, the germ cell genome remains hypomethylated until 15.5–18.5 dpc, when it undergoes a general de novo methylation event as well [33]. Thus the endogenous mouse *Pgk-2* gene is methylated in prospermatogonia at 18.5 dpc. However, it then undergoes a gene- and tissue-specific demethylation event that is complete by 21.5 dpc [18].

To determine whether the gene- and tissue-specific hypomethylation of *Pgk-2*/CAT transgenes observed in testes of adult transgenic mice results from a similar developmentally staged demethylation event, we first used Southern blot analysis to show that the 188 *Pgk-2*/CAT

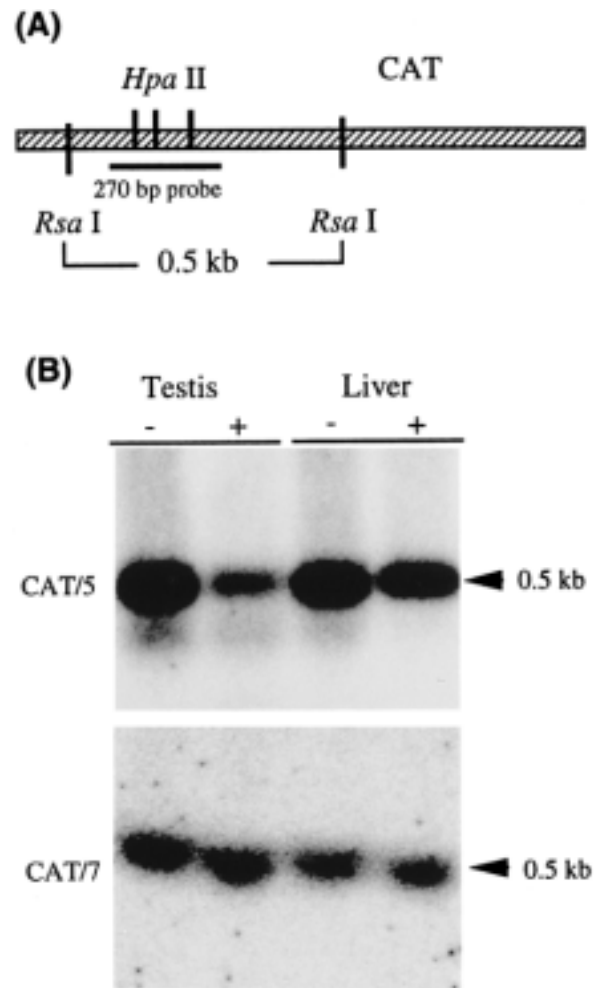


FIG. 5. Ubiquitous methylation of the CAT-only transgene. **A)** The methylation pattern of the CAT reporter sequence alone, in the absence of any influence from an adjacent gene-specific promoter sequence (Fig. 1F), was examined in samples of DNA from testis and liver tissue obtained from five different lines carrying this nonexpressed transgene. Genomic DNA prepared from each tissue was digested with *Rsa* I, as described in *Materials and Methods*. Each sample was divided into two aliquots, of which one was not further digested (uncut control) (–), while the other was digested with the methyl-sensitive enzyme, *Hpa* II. **B)** Southern blot analysis was performed using the PCR-synthesized, 270-bp probe that spans the three *Hpa* II sites in the CAT coding sequence. These three sites were shown to be fully methylated in liver tissue in all five lines. However, the extent of methylation of this transgene in testis tissue varied from fully methylated (CAT/7) to partially methylated (CAT/1, CAT/2, CAT/3, CAT/5) as exemplified by the two examples shown here. Importantly, the CAT-only transgene was not found to be completely demethylated in any tissue in any of the lines examined.

transgene is partially demethylated in DNA isolated from whole testis tissue from newborn mice (Fig. 6A). This partial demethylation is presumed to reflect a combined signal from testicular somatic cells in which the transgene is fully methylated and germ cells in which the transgene is unmethylated. We then used PCR to analyze methylation at *Hpa* II sites in the 188 *Pgk-2*/CAT and *Tf*/CAT transgenes in DNA isolated from purified populations of prospermatogonia at 18.5 dpc and pachytene spermatocytes from adult mice (Fig. 6B). Our results show that *Hpa* II sites in the 5' portion of the CAT coding sequence in the *Pgk-2*/CAT transgene are methylated in prospermatogonia but are unmethylated in adult pachytene spermatocytes. This is in contrast to the CpG island-bearing *Tf*/CAT transgene, which

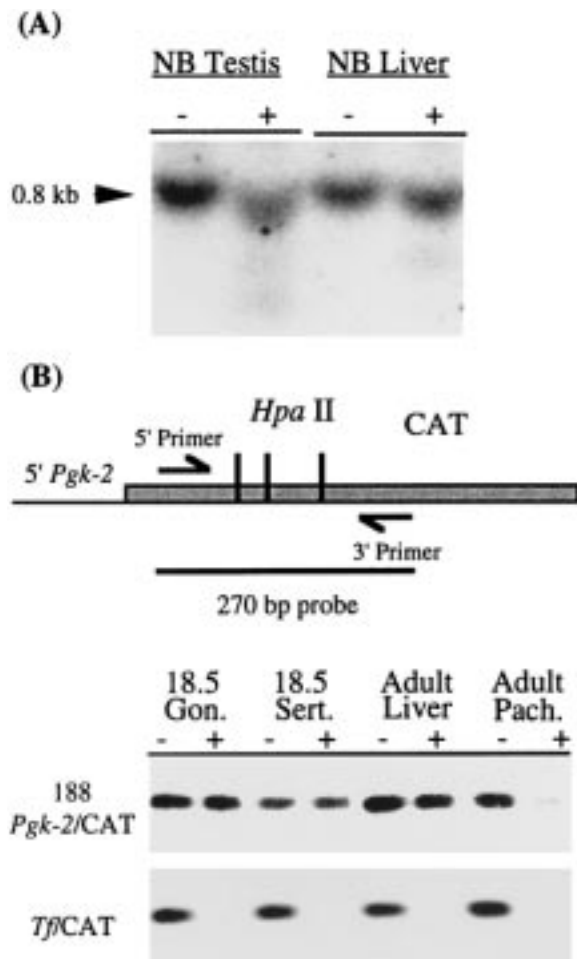


FIG. 6. Stage-specific demethylation of the 188 *Pgk-2/CAT* transgene. **A**) DNA samples from testis and liver tissues from newborn male 188 *Pgk-2/CAT* transgenic mice were analyzed for methylation by Southern blot. After digestion with *Sau* 3A, each sample was divided into two equal aliquots, one of which was further digested with *Hpa* II, and probed as described in *Materials and Methods*. The transgene was shown to be partially demethylated in newborn testis tissue, but fully methylated in newborn liver tissue. This is consistent with demethylation of the transgene specifically in testicular germ cells, which represent less than 15% of the cells in the newborn testis [27]. **B**) Purified populations of type T<sub>1</sub> prospermatogonia (18.5 Gon) as well as somatic Sertoli cells (18.5 Sert.) were recovered from testes of male 188 *Pgk-2/CAT* transgenic fetuses at 18.5 days dpc. Purified populations of pachytene spermatocytes (adult pach.) and liver tissue were obtained from adult male mice bearing the 188 *Pgk-2/CAT* transgene. DNA was prepared from each cell population, digested (+) or not digested (-) with the methyl-sensitive restriction enzyme, *Hpa* II, and then amplified by PCR using primers flanking the three *Hpa* II sites in the CAT coding sequence (schematic diagram). The products were analyzed by Southern blot using the 270-bp PCR-synthesized hybridization probe spanning the three *Hpa* II sites in the CAT coding sequence. The 188 *Pgk-2/CAT* transgene was methylated in somatic 18.5 dpc Sertoli cells and adult liver cells. It was also methylated in 18.5 dpc prospermatogonia, but unmethylated in adult pachytene spermatocytes. This evidence for hypermethylation of the transgene in 18.5 dpc prospermatogonia, along with the evidence in **A** indicating hypomethylation in prospermatogonia in the newborn testis, indicates that this transgene undergoes a stage-specific demethylation event that is exactly analogous to the event the endogenous *Pgk-2* gene undergoes. This is in contrast to the *Tj/CAT* transgene, which was ubiquitously unmethylated at all stages and in all cell types examined.

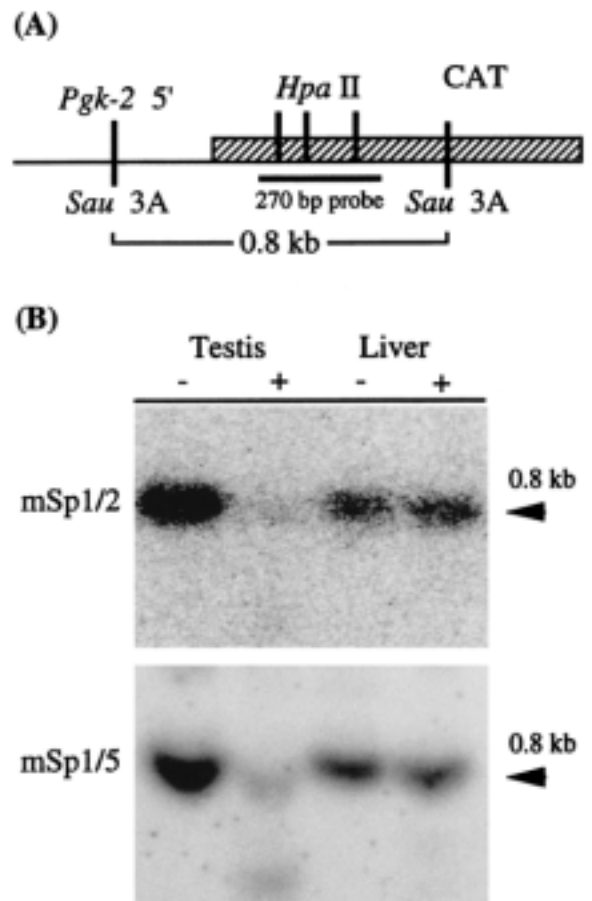


FIG. 7. Tissue-specific demethylation of the mSp1 515 *Pgk-2/CAT* transgene. **A**) Samples of testis and liver tissue were obtained from three different lines carrying the 515 *Pgk-2/CAT* transgene with a disrupted Sp1-binding site (mSp1 515 *Pgk-2/CAT*) and analyzed for demethylation. Genomic DNA was digested with *Sau* 3A, and each sample was then divided into two aliquots, one of which was not further digested (uncut control) (-), while the other was digested with the methyl-sensitive enzyme, *Hpa* II (+). **B**) Southern blot analysis showed that all three sites were found to be fully methylated in liver tissue and completely demethylated in testis tissue, in all three lines examined. Results from two lines are shown here.

was ubiquitously unmethylated at all stages examined. The *Pgk-2/CAT* transgene was also shown to be methylated in testicular somatic cells at 18.5 dpc, as well as in adult liver cells.

#### Disruption of the Sp1-Binding Site Did Not Affect Demethylation of *Pgk-2/CAT* Transgenes

Previous results have shown that in certain CpG island-bearing genes, demethylation is regulated by Sp1-binding sites [34, 35]; and Sp1 is known to be required for normal transcriptional activation from the *Pgk-2* promoter [36]. To determine whether the Sp1-binding site in the non-island-containing *Pgk-2* promoter is involved in regulating testis-specific demethylation of transgenes, we generated three lines of mice carrying a 515 *Pgk-2/CAT* transgene with a mutated Sp-1-binding site (GC box) in the core promoter (mSp1/1, mSp1/2, and mSp1/5) (Fig. 1D). All three lines showed reduced levels of testis-specific expression of the CAT transgene (data not shown), confirming that the Sp1-binding site is important for normal expression of the *Pgk-2* gene. Methylation of this construct in both testis and liver was analyzed by Southern blotting (Fig. 7). Like the 515 *Pgk-2/CAT* and 188 *Pgk-2/CAT* transgenes, the mSp1 *Pgk-*

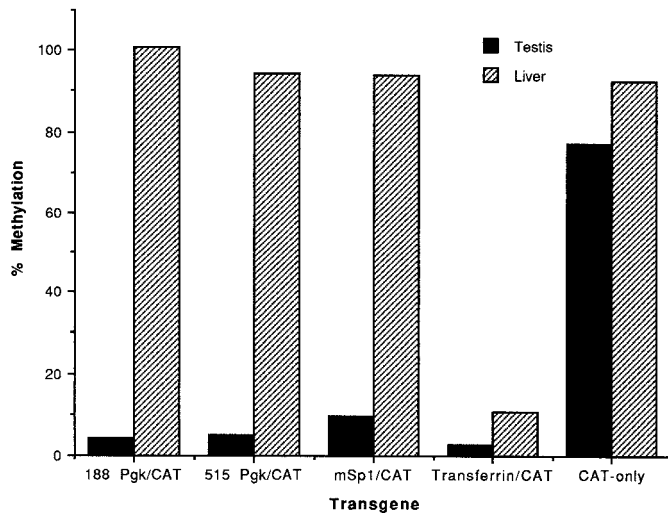


FIG. 8. Quantitative analysis of transgene methylation patterns. Southern membranes hybridized with  $^{32}\text{P}$ -labeled radioactive probes were processed to produce digital images of the bands representing undigested and digested fragments. The percentage of methylation for each transgene in each tissue was determined as described in *Materials and Methods*. In each case the proportion of undigested fragment remaining after digestion with the methyl-sensitive enzyme *Hpa* II, relative to that of the same fragment not digested with *Hpa* II is shown. All of the transgenes except the CpG island-containing transferrin/CAT showed hypermethylation in the liver. All transgenes except CAT-only showed hypomethylation in the testis. This demonstrates the testis-specific demethylation of the *Pgk-2*/CAT transgenes, the ubiquitous hypomethylation of the transferrin/CAT transgene, and the ubiquitous hypermethylation of the CAT-only transgene.

2/CAT transgene was demethylated in the testis and methylated in liver. This suggests that neither the GC box sequence itself, nor binding of Sp1 to that sequence, acts as the signal for testis-specific demethylation of the *Pgk-2*/CAT transgenes.

#### Quantitative Analysis of Transgene Methylation Patterns

To facilitate a comparison of the levels of methylation of the various transgenes in various tissues, we used a PhosphoImager to carry out a quantitative analysis of Southern blot data (Fig. 8). These results, which represent averages of results from two independent lines for each transgene, show that the *Pgk-2*/CAT transgenes consistently demonstrated testis-specific demethylation, while the *Tf*/CAT transgene showed ubiquitous hypomethylation and the CAT-only transgene showed ubiquitous hypermethylation.

## DISCUSSION

Differential expression of tissue-specific genes during mammalian development is achieved through a complex hierarchy of regulatory mechanisms. The well-documented correlation between expression and the degree of hypomethylation of DNA in a large number of tissue-specific genes suggests that demethylation plays a role in this process. Many genomic DNA sequences are at least partially methylated in the genome of the spermatozoon and in the very early (1- to 8-cell stage) embryonic genome [33]. During subsequent stages in the blastula, this methylation is lost, such that a relatively unmethylated "ground state" is attained. This is followed by de novo methylation of most non-CpG island-bearing genes at or just prior to gastrulation [33]. Then, as specific cell lineages become allocated, demethylation occurs in a gene- and tissue-specific manner,

such that many tissue-specific genes become hypomethylated specifically in the cell types in which they are, or will be, expressed [7, 10].

We hypothesize that the mechanism regulating tissue-specific demethylation involves at least two components: 1) *cis*-acting, gene-specific signals that distinguish the genes to be demethylated and subsequently expressed [8] and 2) *trans*-acting, tissue-specific factors that interact with the *cis*-acting signals to direct or facilitate gene-, tissue-, and developmental stage-specific demethylation. The results presented in this report provide evidence for the first of these components by showing that signals resident in the 5'-regulatory region of the testis-specific *Pgk-2* gene act to direct gene-, tissue-, and stage-specific demethylation. This is the first report indicating the existence and function of such demethylation signals in an intact, whole animal system; in addition, it provides the first example of a developmental stage-specific demethylation signal. This is also the first example of a specific demethylation signal in a germ line-specific gene.

Demethylation of sites within the 5' half of the coding sequence of the *Pgk-2* gene is presumed to represent the 3' extent of a domain of demethylation that is centered over the 5'-flanking regulatory region. Analogous testis-specific demethylation of transgenes at sites within the coding sequence of the CAT reporter gene linked to either the core promoter alone (188 *Pgk-2*/CAT), or the core promoter plus enhancer region (515 *Pgk-2*/CAT) of the *Pgk-2* gene, indicates that the *cis*-acting signal for testis-specific demethylation resides in the *Pgk-2* core promoter region. That the 188 *Pgk-2*/CAT transgene undergoes demethylation, but is not subsequently expressed, demonstrates that this demethylation signal is distinct from the tissue-specific enhancer signal that has been mapped to a region 5' to the core promoter [16]. This result also shows that demethylation alone is not sufficient to initiate transcription but may be part of a requisite process of potentiation of the gene that normally precedes transcriptional activation.

The *transferrin*/CAT transgene exemplifies the strong effect that CpG islands can exert on flanking sequences to cause them to remain constitutively hypomethylated. Thus, the same sites in the CAT sequence that showed testis-specific demethylation in *Pgk-2*/CAT transgenes showed ubiquitous hypomethylation in *Tf*/CAT transgenes in all tissues tested, including the testis. Hypomethylation of this transgene occurred in the testis despite the fact that this construct is not expressed in spermatogenic cells. This is once again consistent with a role for hypomethylation in potentiating gene expression. Thus genes bearing strong CpG islands appear to remain in a state of constitutive hypomethylation such that they are poised to be expressed in a large variety of tissues, if not all. The specific parameters of different CpG islands that make one stronger or weaker than another with respect to inducing maintenance of hypomethylation of flanking sequences in the testis, or any other tissue, are yet to be defined. Such parameters could include the total size of the CpG island, the total G+C content of each, the frequency of CpG dinucleotides within each, and/or the number of islands and their proximity, if more than one island is present.

Our results indicate the single Sp1 site in the non-island-bearing *Pgk-2* core promoter is not responsible for directing testis-specific demethylation of the *Pgk-2*/CAT transgenes, even though this sequence does play a critical role in transcriptional activation [36]. This conclusion is also supported indirectly by the finding that both the 515 and 188 *Pgk-*

2/CAT transgenes, which carry wild-type Sp1 sites, are methylated in somatic tissues. Thus the mechanism by which Sp1 sites maintain ubiquitous hypomethylation in CpG-island genes is distinct from the mechanism leading to tissue-specific demethylation of the *Pgk-2* gene.

The *Pgk-2*/CAT transgenes underwent the same stage-specific demethylation that the endogenous *Pgk-2* gene undergoes in prospermatogonia. Thus the *Pgk-2* core promoter region appears to contain a unique *cis*-acting signal that confers tissue-, stage-, and cell type-specific demethylation on the CAT reporter gene in a manner exactly analogous to that occurring in the endogenous *Pgk-2* gene. That this signal is unique to the *Pgk-2* promoter is indicated by the absence of any stage-specific demethylation of the *Tf*/CAT transgene (Fig. 6B).

Previous results from studies of cultured cells transfected with either the immunoglobulin *k-chain* gene [13] or the  $\alpha$ -actin gene [12] indicated that demethylation of these genes is also controlled by *cis*-acting signal sequences located in transcriptional regulatory regions. In the case of the *k-chain* gene, a 1.6-kb *cis*-acting fragment was shown to contain all the information necessary to induce demethylation. Whether separate sequences within this 1.6-kb region actually regulate expression and demethylation, as we have shown to be the case in the *Pgk-2* gene, was not determined for the immunoglobulin *k-chain* gene. In the case of the  $\alpha$ -actin gene, multiple sites in the promoter region appear to signal demethylation. Neither of these demethylation activities has been investigated in an intact animal system.

We assume that *Pgk-2* demethylation is related to gene expression, specifically, potentiation of gene expression. Demethylation is the earliest potential regulatory event we have observed prior to the initiation of endogenous *Pgk-2* gene transcription. The appearance in the *Pgk-2* promoter of DNase I-hypersensitive sites [37], tissue-specific protein-DNA interactions [16], and the initiation of transcription itself [14] all occur 5–10 days after the demethylation event [18].

The specific function of demethylation in potentiating gene expression is yet to be established. Possibilities include direct regulation of transcription factor binding, which could be limited to unmethylated sequences [8], or indirect regulation mediated by elimination of a non-sequence-specific methylated DNA-binding protein [6, 38] that, when present, inhibits factor binding. Alternatively, demethylation could be a prerequisite for association of critical promoter sequences with the nuclear matrix where transcription factors and other components of the transcriptional preinitiation complex may be sequestered [39, 40]. That the demethylation signal sequence appears to be distinct from other signal sequences required for initiation of transcription in the *Pgk-2* gene suggests that these events can be experimentally uncoupled. Once delineated, mutagenesis of the demethylation signal sequence in the 515 *Pgk-2*/CAT transgene should allow us to determine whether demethylation is an indispensable requirement for subsequent transcriptional activation *in vivo*.

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