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Multiple Elements Influence Transcriptional Regulation from the Human Testis-Specific *PGK2* Promoter in Transgenic Mice[1]

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ABSTRACT

The *PGK2* gene is expressed in a strictly tissue-specific manner in meiotic spermatocytes and postmeiotic spermatids during spermatogenesis in eutherian mammals. Previous results indicate that this is regulated at the transcriptional level by core promoter sequences that bind ubiquitous transcription factors and by sequences in a 40-base pair (bp) upstream enhancer region (E1/E4) that bind tissue-specific transcription factors. Transgenic mice carrying different *PGK2* promoter sequences linked to the chloramphenicol acetyltransferase (CAT) reporter gene, one containing only the 40-bp E1/E4 enhancer sequence plus the core promoter and two containing 515 bp of *PGK2* promoter but with either the E1/E4 enhancer region or the Sp1-binding site in the core promoter disrupted by in vitro mutagenesis, all showed levels of expression reduced to less than half that of the wild-type 515 *PGK2*/CAT transgene. These results indicate that multiple factor-binding regions normally regulate initiation of transcription from the *PGK2* promoter. The single disruption of any one of these binding activities reduced, but did not abolish, transgene expression. This is consistent with an ''enhanceosome''-like function in this promoter involving multiple bound activator proteins that interact in a combinatorial manner to synergistically promote testis-specific transcription.

INTRODUCTION

In eutherian mammals including humans, the glycolytic enzyme phosphoglycerate kinase (PGK) is encoded by two genes, *PGK1* and *PGK2* [1–3]. Although the two genes produce proteins that are both structurally and functionally similar [4], they differ markedly in the manner in which their expression is regulated [4–8]. *PGK1* is X-chromosome-linked and expressed in all somatic cells, oogenic cells, and premeiotic spermatogenic cells [7, 9]. *PGK2* is autosomal and is expressed specifically in meiotic and postmeiotic spermatogenic cells [7, 10, 11]. Several lines of evidence suggest that the *PGK2* gene arose as a processed duplication of the *PGK1* gene through RNA-mediated retroposition early during mammalian evolution [2, 8]. Additional data suggest that the original *PGK2* retroposon carried a copy of the progenitor ''*PGK1*-like'' promoter that directed ubiquitous expression, and that this promoter subsequently evolved a tissue-specific regulatory function so that it now directs testis-specific transcription [6, 8]. Conservation of a duplicate *PGK* gene expressed uniquely in meiotic and postmeiotic spermatogenic cells was potentially advantageous because it provided an alternate source of PGK to compensate for repressed expression of the *PGK1* gene resulting from X-chromosome inactivation during spermatogenesis [7, 8].

Transcription of the *PGK2* gene is controlled by regulatory sequences located in the 5′ flanking region of the gene [5, 12–15]. This region includes both core promoter sequences and tissue-specific enhancer sequences. Core promoter function has been demonstrated in the first 188 base pairs (bp) upstream from the translational start site in the human *PGK2* gene, including a 70-bp 5′ untranslated region [14]. This core promoter contains a CAAT-box and a GC-box upstream from the single transcriptional start site, but lacks a TATA-box [5]. It has been shown that CAAT- and GC-boxes act as binding sites for the ubiquitous transcription factors, CTF-1 and Sp1, respectively [16, 17]. That this is the case in the *PGK2* promoter was directly demonstrated by gel-shift experiments [13].

Cell line cotransfection experiments indicated that the transcription factor Sp1 is indispensably required for initiation of transcription from the *PGK2* core promoter, and that the core promoter region alone is sufficient to direct basal transcription of a ligated reporter sequence in cultured cells [14]. However, previous studies with transgenic mice showed that the core promoter alone is not sufficient to activate transcription of a reporter gene in vivo [12]. Appropriate expression was achieved with a transgene that included the core promoter plus an additional 327 bp of upstream sequence, indicating the presence of enhancer activities in this upstream region [12]. Tissue-specific protein-binding activities within this region were mapped by gel-shift experiments to a 40-bp subregion (E1/E4) located immediately upstream from the core promoter [13].

These results are consistent with the idea that optimal transcriptional activation of tissue-specific genes requires precisely coordinated interactions between transcription factors and promoter/enhancer sequences. To determine the role each of these binding sequences plays in regulating expression of the *PGK2* gene in spermatogenic cells in vivo, we generated transgenic mice carrying the chloramphenicol acetyltransferase (CAT) reporter gene ligated to subfragments of the human *PGK2* promoter gene promoter, or to the complete promoter with specific factor-binding sites mutated to selectively eliminate individual protein-DNA interactions. Our results indicate that normal transcriptional activation from the *PGK2* promoter involves the binding of multiple transcription factors to multiple sites, and that these factors appear to interact among themselves to form an enhanceosome-like complex [18, 19].

MATERIALS AND METHODS

Transgene Constructs

Five transgenes were generated for microinjection (Fig. 1). The plasmid pSV/PBR/*PGK2*/CAT [12] contains 1.4 kilobases (kb) of the human *PGK2* upstream regulatory re-

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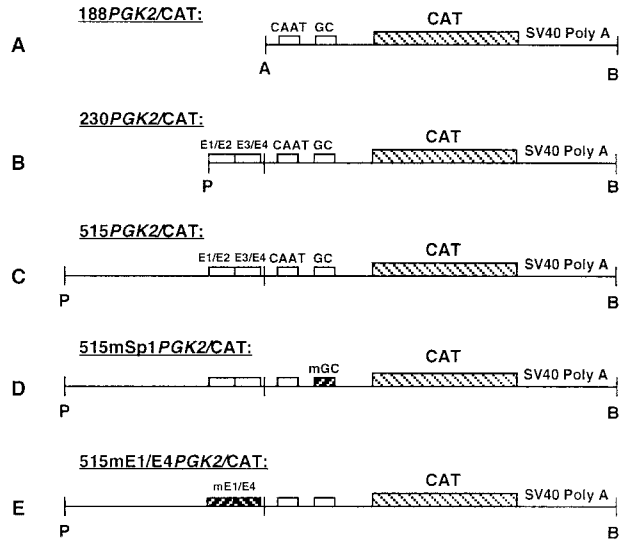

FIG. 1. Transgene constructs. All transgenes were composed of different human *PGK2* promoter fragments ligated to the CAT reporter gene plus the simian virus 40 (SV40) 3′UTR poly(A) addition sequences. **A**) 188*PGK2*/CAT: 188 bp of *PGK2* regulatory sequence containing only the core promoter, including a CAAT-box and a GC-box; **B**) 230*PGK2*/CAT: 230 bp of *PGK2* regulatory sequence containing both the core promoter and the E1/E4 putative enhancer region; **C**) 515*PGK2*/CAT: 515 bp of *PGK2* regulatory sequence including both the core promoter and the entire upstream enhancer region; **D**) 515mSp1*PGK2*/CAT: 515 bp of *PGK2* regulatory sequence with a mutagenized Sp1 binding site (mGC) in the core promoter; **E**) 515mE1/E4*PGK2*/CAT: 515 bp of the human *PGK2* regulatory sequence with the E1/E4 binding region mutagenized. A, *Ava*II; B, *Bam*HI; P, *Pst*I.

gion ligated to the CAT coding sequence. This plasmid was used as a source of transgenes containing wild-type *PGK2* promoter/enhancer sequences. Two modified *PGK2*/CAT constructs were generated from this plasmid by polymerase chain reaction (PCR)-based in vitro mutagenesis. The following specific binding sequences were disrupted in each case: 1) the Sp1 binding site in the *PGK2* core promoter (515mSp1*PGK2*/CAT; Fig. 1D), and 2) the putative E1/E4 enhancer binding site (515mE1/E4*PGK2*/CAT; Fig. 1E). A transgene construct carrying 230 bp of *PGK2* promoter (core promoter + E1/E4 region) (230*PGK2*/CAT; Fig. 1B) was also generated by PCR from the plasmid pSV/PBR/*PGK2*/CAT using primers R and S listed in Table 1. Plasmids carrying each construct were purified from overnight cultures of *Escherichia coli* using the Wizard Maxiprep DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The 515*PGK2*/CAT transgenes (wild-type [Fig. 1C] and mutated [Figs. 1D, 1E]) were produced by digesting the pSV/PBR/*PGK2*/CAT plasmid with *Pst*I and *Bam*HI. The 188*PGK2*/CAT transgene (Fig. 1A) was generated by digesting the pSV/PBR/*PGK2*/CAT plasmid with *Ava*II and *Bam*HI. Mice carrying the wild-type 515 *PGK2*/CAT and 188 *PGK2*/CAT transgenes were generated in a previous study [12] but were subsequently destroyed, and so they had to be regenerated for this study.

In Vitro Site-Directed Mutagenesis by Recombinant PCR

The recombinant PCR technique was used to mutagenize specific sequences in the *PGK2* promoter as described [20] with minor modifications. Targeted sequences were mutagenized by introducing sequence substitutions through two chimeric primers. Overlapping primary fragments produced in this fashion were purified from 1% Nusieve GTG (Ge-

TABLE 1. Oligonucleotide Sequences of PCR Primers.

Primers	Sequences*	Description
A	CAGCATTCCCACCACGTCCC	A and B were used to synthesize secondary fragments of mutagenized constructs from pairs of primary fragments produced as described below.
B	GTCTTTCATTGCCATACGGA	
C	CACTTGACAGCATGCATTGAAGATTCCAACTTCCTGG	Primer pairs used for synthesis of primary fragments: A/C–B/D and A/E–B/F (mE1/E4); A/H-B/I (mSp1). pSV/PBR/*PGK2*/CAT was the template for synthesis of all primary fragments except mE1/E4, for which pSV/PBR/mE1/E2*PGK2*/CAT was used as template.
D	ATGCATGCTGTCAAGTGGATCGAGATTGACAGGACCA	
E	ATCAGTCGACATTGATGAACTGTGTTGCTAGGTGA	
F	TCATCAATGTCGACTGATGACCATGAGCCAATCACAAA	
G	ATCAGTCGACATTGACACTTACAGCATGCATTGA	
H	GTCCCTATCGCTTTCTTCTCCTACTGTCTC	
I	GCGATAGGACAAGGGCAAAGGCGTTAG	
J	GTTCTTCA CCTACCCAA GT	J was used to sequence all mutagenized constructs to confirm mutagenesis.
K	AGAGCCAGAAGCGGCGCACA	K and L were used to synthesize gel-shift probes containing either wild-type or mutated enhancer fragments for gel-shift assays. M and N were used to synthesize gel shift probes containing either wild-type or mutated Sp1-binding sites in the core promoter. Templates for PCR reactions were the wild-type pSV/PBR/*PGK2*/CAT plasmid or corresponding mutated plasmids.
L	TTGTGA TTGGCTCATGGTCC	
M	GGACCATGAGCC AATCACAA	
N	AGCTTGACAATATAAAGACA	
O	ACGTTTCAGTTTGCTCATGG	O and P were used to screen transgenic mice for the CAT reporter sequence.
P	AGCTAAGGAAGCTAAAATGG	
Q	GCGGACTGTTACTGAGCTGCGT	Q and R were used to detect actin sequences as a control for the RT-PCR studies.
R	GAAGCAATGCTGTCACCTTCCC	
S	TGGAATCTTCACCTAGCAAC	S and T were used to generate the 230*PGK2*/CAT transgene construct.
T	GGGCATCCAGACATGATAAG	

* All primer sequences are listed in the 5′ to 3′ orientation. Underlined sequences indicate those mutagenized from the corresponding wild type sequence.

netic Technology Grade) agarose gel (FMC BioProducts, Rockland, ME). These fragments were then mixed in equal molar concentrations to form heteroduplex templates for a second PCR amplification to produce secondary fragments with the targeted wild-type sequence replaced by the mutagenized sequence. Each secondary fragment containing a mutagenized sequence was isolated and subcloned back into the pSV/PBR/*PGK2*/CAT plasmid. All PCR reactions were conducted using the DNA PCR kit from Perkin-Elmer (Irvine, CA). Reaction conditions for synthesis of the primary fragments consisted of an initial denaturation step at 94°C for 3 min, followed by 30 amplification cycles of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C. Synthesis of the secondary fragments was accomplished using similar conditions, except that the extension step at 72°C was maintained for 2 min during each cycle. Primers used to generate each construct are shown in Table 1. Primer pairs used for synthesis of primary fragments were A/C and B/D, A/E and B/F (mE1/E4), and A/G and B/H (mSp1). Primers A and B were used to synthesize all of the secondary fragments. Subcloning of fragments into pSV/PBR/*PGK2*/CAT was carried out by ligation of 0.5 μg of linearized vector and 0.1 μg of insert fragment in a total volume of 10 μl at 16°C for 6 h in the presence of 1 U T4 DNA ligase (Gibco BRL, Gaithersburg, MD).

DNA Sequencing

Successful mutagenesis of putative factor-binding sequences was confirmed by dideoxy sequencing of wild-type and mutagenized constructs. The CircumVent thermal cycle dideoxy DNA sequencing kit (New England BioLabs Inc., Beverly, MA) was used according to the manufacturer's instructions. Sequencing primer I (Table 1) was designed to anneal to a specific region upstream from both the enhancer region and the Sp1-binding site. In each case, DNA sequencing confirmed that the putative binding site had been selectively modified (data not shown).

Gel-Shift Assay

For analysis of the *PGK2* enhancer region, a 200-bp fragment to be used for gel-shift experiments was synthesized by PCR as previously described [13], using primers J and K (Table 1), and the appropriate plasmid as template (pSV/PBR/*PGK2*/CAT = wild-type; 515mE1/E4/*PGK2*/CAT = mutated E1/E4 region). These fragments were then digested with *Hha*I and *Ava*II to yield a 170-bp probe. Probe fragments were separated by electrophoresis in 1% Nusieve GTG agarose gel, recovered by β-agarose digestion, and purified by organic extraction and ethanol precipitation. For analysis of the *PGK2* core promoter region, fragments were synthesized using primers L and M (Table 1), and the appropriate plasmid as template (pSV/PBR/*PGK2*/CAT = wild-type; 515mSp1*PGK2*/CAT = mutated Sp1 site). Probes used in gel-shift assays were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase, and purified on NucTrap probe purification columns (Stratagene, La Jolla, CA). Testes of adult (> 60-day-old) CD-1 white Swiss mice (Charles River Breeding Labs., Wilmington, DE) were used as a source of nuclear extracts. Testes from 50 mice were dissected, decapsulated, and homogenized in buffer (10 mM HEPES [pH 7.6], 10% glycerol, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose) using an anaerobic tissue processor (B/I SciTech, San Diego, CA) in four full-speed 10-sec bursts. Further preparation of extracts was conducted as described [13, 21]. Freshly prepared proteinase inhibitors were added to all buffers [13]. Isolated nuclear extracts were dialyzed in 0.5-ml microcollodion bags (Sartorius SM 13202, Edgewood, NY) at 4°C for 3 h. The final protein concentration of each extract preparation was determined using a protein assay kit (II) from Bio-Rad (Richmond, CA). Protein concentrations were approximately 5 mg/ml in each case. Protein-DNA binding assays were performed as described [13].

Each assay included 2 μl (10 μg) of testis nuclear extract, 1 μl of ^{32}P-labeled probe fragment (approximately 0.5 ng labeled to a specific activity of $1–4 \times 10^6$ cpm/μg), and 1 μl (0.1 μg) of double-stranded poly(dI-dC):poly(dI-dC) (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting protein-DNA complexes were analyzed by electrophoresis on 4% native polyacrylamide gels. These gels were run in a recirculating, low-ionic-strength buffer (50 mM Tris-HCl, 1 mM EDTA [pH 8.0]) at 60 mA for 1–4 h. Gels were dried on Whatman (Clifton, NJ) 3 MM paper and exposed to x-ray film for analysis.

Transgenic Mice

Superovulated (C57BL6/J-DBA2J) F1 hybrid females were mated to F1 males to produce fertilized eggs. These eggs were isolated and microinjected with each of the six transgenic constructs. Surviving zygotes were then reimplanted into pseudopregnant females of the same hybrid strain, essentially as described [22]. CAT-specific primers (N and O, Table 1) were used to screen genomic DNA samples from tail tissue of putative transgenic mice as described [23]. PCR-positive mice were further confirmed as founders by Southern blot hybridization to genomic tail DNA. This hybridization used a ^{32}P-labeled 1.6-kb CAT-coding sequence fragment as the probe. Subsequent generations of transgenic mice were bred to homozygosity, which was confirmed by Southern blot analysis.

Isolation of Spermatogenic Cells

Populations of pachytene spermatocytes, round spermatids, and residual cytoplasmic bodies were obtained from testes of adult mice using a standard StaPut gradient [7, 24, 25]. Purities of the isolated germ cell types were determined by light microscopic analysis of cellular morphology under phase optics. Populations were consistently > 90% pure for the desired cell type.

RNA Preparation

Tissues and purified cell populations were homogenized in guanidinium thiocyanate-containing solution using a Polytron homogenizer (Brinkman Instr., Westbury, NY)) for three bursts of 5 sec as described [7, 26]. The homogenate was then centrifuged through a cushion of 5.7 M CsCl at 35 000 rpm for 17 h at 18°C in an SW 50.1 ultracentrifuge rotor (Beckman Instr., Palo Alto, CA) to yield total RNA. RNA pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated, ribonuclease-free water, and precipitated in ethanol. The pellet was recovered by centrifugation and resuspended in DEPC-treated water. RNA concentration was determined by UV-spectrophotometry.

Reverse Transcription (RT)-PCR

One microgram of total RNA was used for RT-PCR amplification of CAT transcripts. Contamination by genomic DNA was eliminated from each RNA sample by pretreat-

ment with deoxyribonuclease I as described [27]. Samples were then subjected to RT-PCR amplification using the RNA Gene-Amp kit from Perkin-Elmer according to the manufacturer's instructions. Controls lacking RNA template or reverse transcriptase were run in all experiments. The cDNA products of each 20-μl RT reaction were divided into two 10-μl aliquots. One aliquot was used to amplify a 453-bp fragment of the ubiquitously expressed mouse β-actin cDNA as a control for the quality of each preparation using 22-mer primers (O and Q, Table 1) [28]. CAT-specific primers (N and O, Table 1) were used to amplify a 320-bp product from the remaining aliquot of cDNA. Fifteen microliters of each PCR product were then combined and run in a single lane on a 1% agarose gel.

Northern Blot Analysis

For Northern blots, 20 μg of total RNA was electrophoresed in each lane of a 1% agarose gel containing 2% formaldehyde as a denaturant, and blotted to GeneScreen membrane (Dupont NEN, Boston, MA) according to the manufacturer's instructions. Each blot was then hybridized with a ^{32}P-labeled CAT-specific probe. Hybridization was conducted overnight at 42°C in a buffer containing 5-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 50% formamide, 5-strength Denhardt's solution (single-strength Denhardt's solution is 1% ficoll, 1% polyvinylpyrrolidone, 1% BSA [fraction V]), 0.5% SDS, 8% dextran sulfate, 500 μg/ml heparin, and 50 μg/ml herring sperm DNA. Results were visualized by autoradiography to x-ray film or by direct analysis using a phosphorimager (see below). To confirm that equal amounts of RNA were loaded in each lane, the membrane was then stripped in 0.1-strength SSC/1% SDS at 100°C for 20 min and reprobed with a ^{32}P-labeled β-actin-specific probe. This probe was synthesized from mouse total RNA by RT-PCR using primers P and Q (Table 1).

For quantitative analysis, the hybridized Northern membranes were exposed to a Kodak Storage Phosphor Screen (Eastman Kodak, Rochester, NY) for 4–18 h, and the exposed screen was then scanned using a Molecular Dynamics Phosphorimager (Model 445SI; Sunnyvale, CA). IPLab Gel software (Signal Analytics Corp., Vienna, VA) was used to quantitate the radioactive bands. Quantitation was first performed on membranes hybridized with the CAT-specific probe and then on the same membranes after they were stripped and reprobed for β-actin to normalize for any unequal loading. Final CAT expression levels were calculated as the ratio of the quantitated CAT signal divided by that of β-actin.

RESULTS

Effects of Site-Specific Mutagenesis on Factor Binding

Previous in vitro gel shift experiments demonstrated that transcription factor Sp1 binds to the wild-type GC-box in the *PGK2* core promoter, and that unidentified tissue-specific factors bind to the E1/E4 enhancer regions of the *PGK2* promoter [13]. To demonstrate that mutagenesis of the GC-box specifically precludes binding of Sp1 factor and that mutagenesis of the E1/E4 region specifically precludes binding of the respective corresponding tissue-specific factors, we performed similar in vitro gel-shift experiments using each of the mutated *PGK2* promoter sequences as probe with nuclear extracts from adult mouse testis tissue (Fig. 2).

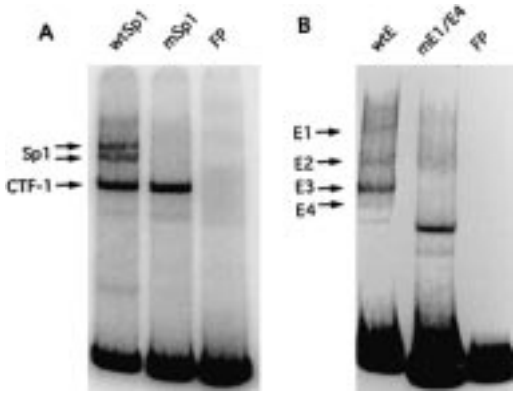

FIG. 2. Effects of mutagenesis of *PGK2* promoter/enhancer regions on protein-DNA interactions. **A**) End-labeled 188-bp *PGK2* core promoter fragments containing either a wild-type GC-box (wtSp1) or a mutated GC-box (mSp1) were incubated with testis nuclear extract. Mutation of the GC-box specifically precluded binding of Sp1 but not CTF-1. **B**) End-labeled 170-bp *PGK2* enhancer-containing fragments, each carrying either a wild-type 40-bp E1/E4 enhancer region (wtE), or a mutated E1/E4 region (mE1/E4), were each incubated with testis nuclear extract before PAGE. Mutation of each region is shown to specifically preclude the corresponding protein-DNA interaction(s). The appearance of new, lower molecular-weight bands in the mE1/E4 lane is believed to represent aberrant binding of ubiquitous factors. Similar results are seen when this construct is incubated with extracts from somatic tissues (data not shown). FP, Free probe.

Analysis of Expression of PGK2/CAT Transgenes

Two lines of mice carrying the 188-bp *PGK2*/CAT transgene (188/12, 188/21) and two lines carrying the 515-bp *PGK2*/CAT transgene (515/4, 515/8) were newly established for this study. RT-PCR was initially used to assay for CAT transcripts in testis, liver, and kidney tissue from each line. Transcription of the CAT reporter gene was detected in adult testis from both lines of 515*PGK2*/CAT mice but not in any of the somatic tissues tested (Fig. 3A). No expression of the CAT gene was detected in any tissues from either of the lines of 188*PGK2*/CAT mice (Fig. 3B). The relative levels of testis-specific expression of the 515*PGK2*/CAT transgene in each line were analyzed by Northern blot hybridization (Fig. 3C). These results confirm those from previous studies [12], indicating that critical elements residing in the 327-bp enhancer region upstream from the 188-bp core promoter in the *PGK2* gene are required to direct testis-specific expression of a reporter gene in vivo.

To analyze the developmental stage- and cell-type specificity of expression of the 515*PGK2*/CAT transgene, total RNA was recovered from newborn testis tissue and from purified populations of specific spermatogenic cell types from adult testis tissue, and assayed by either RT-PCR or Northern analysis. CAT gene transcripts were detected in RNA from pachytene spermatocytes, round spermatids, and residual cytoplasmic bodies isolated from adult testis tissue (Fig. 4A), but not in RNA from newborn testis tissue (Fig. 4B). This indicates that, like the endogenous mouse *Pgk2* gene, expression of the 515*PGK2*/CAT transgene is specific to meiotic and postmeiotic spermatogenic cells. Thus, in addition to tissue specificity, appropriate developmental stage and cell-type specificity is also regulated by sequences within the 515 bp of promoter sequence included in this construct.

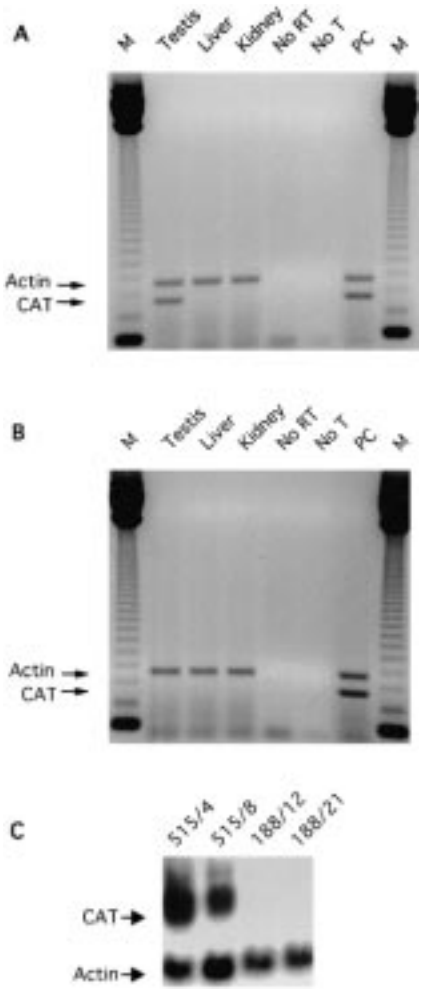

FIG. 3. Testis-specific expression of *PGK2*/CAT transgenes. Expression of the CAT transgene in both 515*PGK2*/CAT and 188*PGK2*/CAT lines was analyzed using total RNA prepared from a variety of tissues. **A**) Expression of the CAT transgene was analyzed in one of two 515*PGK2*/CAT lines (515/8) by RT-PCR using CAT-specific primers. CAT transcript was detected only in testis, and not in liver or kidney RNA samples. Similar results (not shown) were obtained for a second line (515/4) carrying the same transgene. **B**) Expression of the CAT transgene was examined in one of two 188*PGK2*/CAT lines (188/12), and was negative in all tissues examined, including testis. Similar results (not shown) were obtained from a second line (188/21) carrying the same transgene. **A** and **B**) To confirm the integrity of each RNA sample, actin-specific RT-PCR products were included in each lane. No RT, no reverse transcriptase; No T, no RNA template; PC, positive control template; M, 123-bp ladder marker. **C**) Total RNA was prepared from testis tissue from transgenic mice from both lines carrying the 515*PGK2*/CAT construct (515/4 and 515/8), and both lines carrying the 188*PGK2*/CAT construct (188/12 and 188/21), and analyzed by Northern blot hybridization using a ^{32}P-labeled CAT-specific probe. Expression of CAT was detected in testis tissue from the two 515*PGK2*/CAT lines, but not in either of the 188*PGK2*/CAT lines. The membrane was then stripped and reprobed for actin mRNA (bottom) to confirm the integrity and loading of RNA in each lane.

Mutagenesis of the Sp1-Binding Site in the PGK2 Core Promoter Reduced, but Did Not Preclude, Transcriptional Activation

To test whether a functional Sp1-binding site is required in the core promoter of the *PGK2* gene to facilitate transcriptional activation in vivo, we constructed a transgene composed of the CAT reporter sequence ligated to the 515-bp *PGK2* promoter/enhancer sequence in which the Sp1-binding GC-box sequence had been selectively mutated.

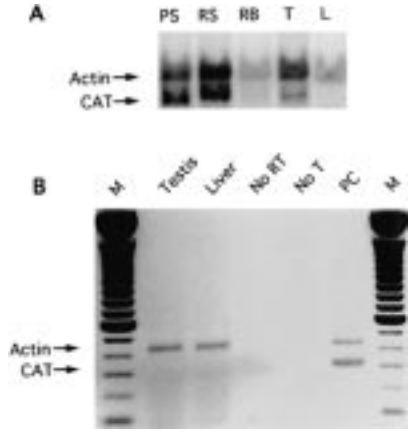

FIG. 4. Cell-type- and developmental-stage-specific expression of *PGK2*/CAT transgenes. **A**) Expression of the 515*PGK2*/CAT transgene in different spermatogenic cell types. Total RNA was prepared from purified populations of pachytene spermatocytes (PS), round spermatids (RS), and residual cytoplasmic bodies (RB), as well as whole testis (T), and liver (L) tissues from adults from one of two 515*PGK2*/CAT lines (515/8) and analyzed on a Northern blot hybridized first with a CAT-specific probe and then with an actin-specific probe. Expression of the *PGK2*/CAT transgene was detected in all cell types tested except liver. Actin transcript was detected in all samples. **B**) Expression of the CAT reporter gene in newborn pups from the same line was analyzed by RT-PCR using total RNA from each tissue. CAT transcripts were not detected in either testis or liver tissues. Actin transcripts were detected in both samples. The relatively low signal for actin RNA in the liver sample in **A** is due to underloading of RNA in this lane. No RT, no reverse transcriptase; No T, no RNA template; PC, positive control template; M, 123-bp ladder marker.

Three lines of mice carrying this construct were generated, and expression of the reporter gene was first analyzed in adult testis and liver tissues from each by RT-PCR using CAT-specific primers. CAT transcripts were detected in testis tissue, but not in liver tissue, from all three lines (data not shown). Levels of expression of this transgene were analyzed on Northern blots. In all three lines, expression of the CAT reporter gene in the testis was less than half that seen from the same transgene carrying a wild-type Sp1-binding site (Fig. 5). This finding indicates that binding of Sp1 to the core promoter is normally involved in the process of transcriptional activation of the *PGK2* gene although disruption of this binding activity does not completely abolish initiation of transcription.

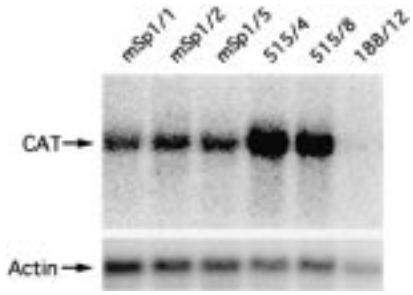

FIG. 5. Expression of a *PGK2*/CAT transgene bearing a disrupted Sp1-binding site. Total testis RNA was prepared from testis tissue from three lines carrying a 515*PGK2*/CAT transgene with a mutated Sp1 binding site in the core promoter (mSp1/1, mSp1/2, mSp1/5), and two lines carrying the wild-type 515*PGK2*/CAT transgene (515/4 and 515/8), and was analyzed on a Northern blot hybridized with a ^{32}P-labeled CAT-specific probe. Total RNA from testis tissue from one 188*PGK2*/CAT line was included as a negative control. The membrane was then stripped and reprobed for actin mRNA to confirm the integrity and loading of RNA in each lane.

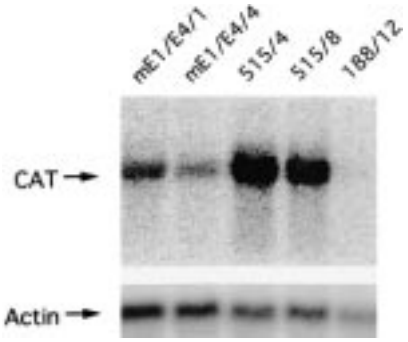

FIG. 6. Expression of a *PGK2*/CAT transgene bearing a disrupted E1/E4 binding site. Total testis RNA was prepared from testis tissue of mice from two lines carrying a 515*PGK2*/CAT transgene with a disrupted E1/E4 binding region (mE1/E4/1 and mE1/E4/4) and two lines carrying wild-type 515*PGK2*/CAT transgenes (515/4 and 515/8), and was analyzed by Northern blot hybridization with a CAT-specific probe. Total RNA from testis tissue from a 188*PGK2*/CAT line was included as a negative control. The membrane was stripped and reprobed for actin mRNA to confirm the integrity and loading of RNA in each lane (bottom).

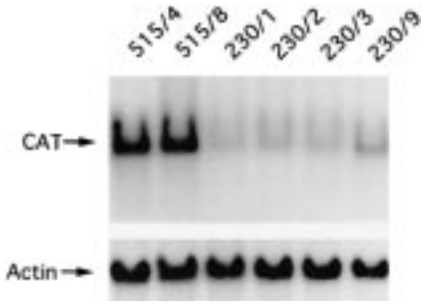

FIG. 7. Expression of a *PGK2*/CAT transgene bearing the core promoter plus the E1/E4 enhancer region. A *PGK2*/CAT transgene carrying 230 bp of *PGK2* promoter sequence (the 188-bp core promoter plus an additional 42 bp of upstream sequence encompassing the E1/E4 putative enhancer region, but no additional upstream sequence) was examined for expression in total RNA isolated from testes of transgenic mice. Expression in four lines carrying the 230*PGK2*/CAT transgene (230/1, 230/2, 230/3, and 230/9) was compared to that in two lines carrying the wild-type 515*PGK2*/CAT transgene (515/4 and 515/8). The membrane was then stripped and reprobed for actin mRNA to confirm the integrity and loading of RNA in each lane (bottom).

Effect of the E1/E4 Enhancer Region on Transcriptional Activation

Previous in vitro gel-shift studies suggested that a 40-bp region immediately upstream from the core promoter is involved in regulating testis-specific *PGK2* transcription. To assess the effects of mutagenesis of the E1/E4 region, we generated two lines of transgenic mice carrying the CAT reporter gene ligated to 515 bp of *PGK2* promoter bearing a mutated E1/E4 region. Total RNA prepared from testis and liver tissues from both lines was initially analyzed for CAT transcripts by RT-PCR (data not shown). Both lines expressed the CAT transgene in a testis-specific manner. However, quantitative analysis by Northern blot indicated that the levels of transgene expression in both of these lines was reduced to approximately 30% of the wild-type 515*PGK2*/CAT transgene expression level (Fig. 6).

The finding that disruption of the E1/E4 region reduced, but did not completely abolish, transgene expression suggested that E1-E4 are not the only binding activities within the 327-bp upstream enhancer region that normally contribute to expression from the *PGK2* promoter. To determine whether the E1/E4 region is sufficient on its own to direct testis-specific expression from the *PGK2* promoter, without additional portions of the 327-bp upstream region, we generated four lines of mice carrying the CAT reporter gene ligated to a 230-bp *PGK2* promoter sequence consisting of the core promoter plus the E1/E4 enhancer region, but no additional upstream sequence (= 230*PGK2*/CAT transgene). Total RNAs were isolated from testis and somatic tissues from each of these lines and were initially analyzed by RT-PCR. CAT expression was detected exclusively in the testes in all four lines of mice (data not shown). However, quantitative Northern analysis (Fig. 7) indicated that expression of this transgene in testis tissue was consistently diminished to about 20% of the wild-type 515*PGK2*/CAT transgene expression level. This finding, along with the result obtained for the mutated E1/E4 construct, confirmed that the E1/E4 region normally contributes a portion of the protein-DNA interactions involved in initiating testis-specific transcription of the *PGK2* gene. Furthermore, this result demonstrated that the E1/E4 region, in conjunction with the core promoter, is minimally sufficient to direct testis-specific transcriptional activation analogous to that seen with the endogenous *PGK2* gene.

Comparison of Expression Levels Directed by Different PGK2 Promoter Fragments

In order to assess the relative contributions of different *PGK2* promoter elements to transcriptional activation, expression levels of each transgene were quantitated and compared to that of the wild-type 515*PGK2*/CAT transgene (Fig. 8). The results showed that while the core promoter alone was not sufficient to activate transcription in vivo, inclusion of the E1/E4 region with the core promoter (230*PGK2*/CAT) resulted in testis-specific expression of the transgene at about 20% of the level observed for the wild-type 515*PGK2*/CAT transgene. Disruption of the wild-type 515-bp *PGK2* promoter to eliminate either the Sp1-binding site in the core promoter (mSp1*PGK2*/CAT) or the E1/E4 binding sites in the enhancer region (mE1/E4*PGK2*/CAT) resulted in transgene expression levels of approximately 40% or 30%, respectively, of wild-type levels. Thus, these results confirmed the functional status of each of these promoter elements, but they also suggested that additional protein-DNA interactions normally occur within the 285-bp region upstream from the E1/E4 region, and that full-level expression of the transgene is normally achieved through a synergistic effect mediated by factor binding at all of these sites.

DISCUSSION

Previously published data regarding the origin of the autosomal *PGK2* locus via retroposition from a *PGK1*-like progenitor locus [2], and sequence comparisons of this gene with the X-linked *PGK1* gene [2, 5, 6] support the hypothesis that the original *PGK2* retroposon carried a duplicate copy of a *PGK1*-like promoter. Analyses of expression patterns in various marsupial and eutherian mammalian species [8, 9], as well as analyses of promoter function in transcriptional assays [12, 14] including this work, and analysis of protein-DNA interactions [13], all suggest that the *PGK2* gene has retained a core promoter reminiscent of that in the *PGK1* gene, except for the loss of the CpG island still present in the *PGK1* promoter. However, it appears the *PGK2* gene has evolved unique upstream enhancer regions that augment this core promoter to direct testis-specific expression. The results presented in this study indicate that there are one or more upstream enhancer regions, in addi-

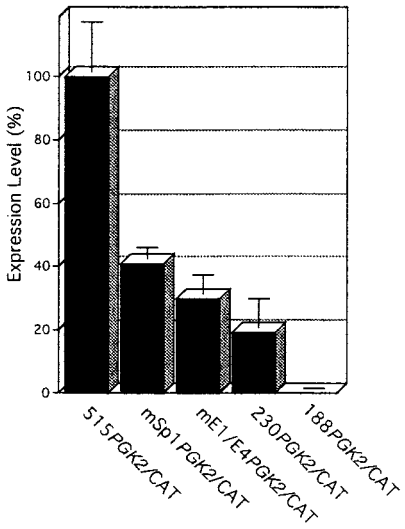

FIG. 8. Expression levels of different *PGK2*/CAT transgenes in testis RNA. To compare the relative contributions of different *PGK2* promoter elements to transcription in the testis, expression levels of transgenes carrying deleted or mutated factor-binding sites were quantitated and compared to that of the wild-type 515*PGK2*/CAT transgene using a phosphorimager. The expression level of the wild-type 515*PGK2*/CAT transgene detected by Northern blot analysis of testis total RNA was set at 100%, and expression levels of all other transgenes are shown relative to that level. Error bars show the extent of variation observed for each transgene among the different lines examined. Characteristics of each transgene construct are described in Figure 1. The data shown represent average expression levels determined from analysis of two different lines carrying wild-type 515*PGK2*/CAT transgenes (515/4 and 515/8), three different lines carrying mSp1*PGK2*/CAT transgenes (mSp1/1, mSp1/2, and mSp1/5), two different lines carrying mE1/E4*PGK2*/CAT transgenes (mE1/E4/1 and mE1/E4/4), four different lines carrying 230*PGK2*/CAT transgenes (230/1, 230/2, 230/3, and 230/9), and two different lines carrying 188*PGK2*/CAT transgenes (188/12 and 188/21).

tion to the previously identified E1/E4 region [13], which appear to direct tissue-specific expression of the *PGK2* gene in eutherian mammals. Surprisingly, our results indicate that these regions can function independently, although all appear to be required to achieve full-level, testis-specific expression of the *PGK2* gene in eutherian mammals.

Specifically, the results of this study confirm previous suggestions that the Sp1-binding site in the core promoter and the immediately upstream E1/E4 enhancer region both contribute to normal transcriptional activation from the *PGK2* promoter [13, 14], and that together these regions are minimally sufficient to direct low-level, testis-specific expression. However, because the 230-bp *PGK2*/CAT construct is expressed at lower levels than the 515-bp *PGK2*/CAT transgene, and because a 515*PGK2*/CAT transgene bearing a mutated E1/E4 region is also expressed, albeit at lower levels than the wild-type 515*PGK2*/CAT transgene, it appears that there are additional enhancer elements upstream of the E1/E4 region that normally contribute to full-level expression from this promoter.

Most surprisingly, these results show that the singular elimination or ablation of protein-DNA interactions at any one of the putative regulatory regions in the *PGK2* promoter, including sequences upstream of the E1/E4 region, within the E1/E4 region itself, or at the Sp1-binding site in the core promoter, fails to preclude the initiation of transcription from this promoter. This indicates an inherent redundancy in the tissue-specific enhancer function of the *PGK2* promoter, and an ''enhanceosome-like'' function [19] involving multiple protein-DNA and protein-protein interactions that normally contribute to testis-specific transcription directed by this promoter.

Our finding that ablation of the Sp1-binding activity in the 515mSp1*PGK2*/CAT transgene reduces transcriptional activity supports the suggestion that binding of Sp1 factor to the core promoter normally contributes to transcriptional activation from the *PGK2* promoter. It was previously suggested that in TATA-less promoters in general [29, 30], and in the TATA-less *PGK2* promoter in particular [14], Sp1 binds to its cognate GC-box sequence and interacts with TATA-binding protein-associated factors, which subsequently act, in the absence of a TATA sequence, to position the TFIID transcription complex, and ultimately RNA polymerase II, at the transcription initiation site. Taken in conjunction with the previous finding that the presence of Sp1 factor is indispensably required for initiation of transcription from the *PGK2* core promoter [14], this suggests that a functional Sp1-binding site normally acts to stabilize this factor in this complex. However, in the absence of an appropriate binding site, it appears that Sp1 can still function, presumably via protein-protein interactions with factors bound to upstream enhancer regions, to organize the necessary transcription complex. Thus we would attribute the reduced level of transcription from the 515mSp1*PGK2*/CAT transgene to a reduction in the stability of the transcription complex due to elimination of one protein-DNA interaction from the group of interactions that normally stabilize this complex. Similar results have been observed for Sp1-binding site mutations in other genes, including the TATA-less human adenosine deaminase (*ADA*) gene [31], the monocyte-specific *CD14* gene [32], the vascular cell adhesion molecule-1 (*VCAM-1*) gene [33], and the multidrug resistance-associated protein (*MRP*) gene [34]. In every case, disruption of an Sp1 binding site reduced, but did not completely abolish, transcription.

An alternative explanation of this result is that, in the absence of a functional Sp1-binding site, the assembly of basal transcription machinery at the initiation site can still be facilitated in a less efficient manner by other elements in the core promoter. In addition to Sp1-binding sites, initiator (Inr) elements located at the transcription start sites of certain TATA-less genes have been found to play an important role in transcriptional initiation [35–37]. The sequence at the transcription start site of the *PGK2* gene shows moderate homology (9/17 bp) to the Inr sequence that was originally identified in the terminal deoxynucleotidyltransferase (*TdT*) gene [35]. However, it is not known if this region actually functions as an Inr element in the *PGK2* gene.

Previous gel-shift experiments mapped tissue-specific protein-binding activities to the first 40 bp of the enhancer region immediately 5′ to the core promoter (E1/E4 region) [13]. Our present results demonstrate that this E1/E4 region (included in the 230 *PGK2*/CAT construct) is indeed sufficient to elicit low levels of testis-specific transcription from the *PGK2* promoter in vivo. However, disruption of the E1/E4 region within the context of the complete 515 bp of the *PGK2* promoter (515mE1/E4*PGK2*/CAT) also resulted in low-level transgene expression in the testis. Thus the E1/E4 enhancer sequence is sufficient to direct testis-specific transcription, but it is not indispensable if additional upstream *PGK2* promoter sequence is present. This indicates that a combination of protein-DNA and protein-protein interactions normally contributes to the assembly of the transcription preinitiation complex at this promoter, and that disruption of any one of the protein-DNA interactions

appears to destabilize, but not completely inhibit, complex formation.

Since the level of transcription driven by the normal promoter with all of the factor-binding sites intact is greater than the sum of those with individual sites deleted or mutated (Fig. 8), it appears that there are normally synergistic interactions among bound activators. This is consistent with the view that enhancers can function by recruiting combinations of activators to create a nucleoprotein assembly called an ''enhanceosome'' that relies on both protein-DNA and protein-protein interactions to localize and stabilize the complex, and to stimulate transcription at optimal levels [19].

The E1/E4 region is the only sequence in 327 bp of upstream enhancer sequence in the 515-bp *PGK2* promoter that was found to possess tissue-specific protein-binding activities in previous in vitro gel-shift assays [13]. Thus, any additional upstream factor-binding sites may bind ubiquitous factors, or they may bind tissue-specific factors that were either not detected in the previous gel-shift experiments or could not be distinguished from ubiquitous factors. Two testis-specific forms of Sp1 factor have been identified that are each distinct from the ubiquitous somatic form and from each other, although all bind similar promoter sequences [38]. One of these is expressed exclusively in meiotic spermatocytes, while the other is expressed in both meiotic and postmeiotic spermatogenic cells, concurrently with *PGK2* gene expression. Interestingly, two potential Sp1-binding sites are present in the 285-bp region upstream from the 40-bp E1/E4 enhancer region.

In summary, results from previous transgenic experiments [12], along with those reported here, clearly indicate that the *PGK2* core promoter alone is not sufficient to direct transcription of a reporter sequence in vivo, even though it is sufficient to direct transient expression in cultured cells [14]. Our present results demonstrate that the core promoter plus the 40-bp E1/E4 region is minimally sufficient to direct tissue-type-, cell-type-, and developmental-stage-specific transcriptional activation similar to that exemplified by the endogenous *Pgk2* gene in the mouse, albeit at reduced levels. Additional upstream enhancer sequences contribute to the normal expression level and can also, in the absence of a normal E1/E4 sequence, direct appropriate tissue-specific transcription. Thus the upstream enhancer regions in the human *PGK2* gene promoter appear to act in conjunction with the core promoter in a combinatorial manner to provide the necessary information to direct regulated transcription of the *PGK2* gene during mammalian spermatogenesis. We assume that factors that bind to each of these regions via protein-DNA interactions also interact with each other, directly or indirectly, via protein-protein interactions to form an enhanceosome-like transcription complex.

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