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A 1.3-kb Upstream 5′ Region of the Rat Phosphoglycerate Mutase m Gene Confers Testis and Skeletal Muscle-Specific Expression in Transgenic Mice

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Spermatogenesis is a complex process that occurs in successive mitotic, meiotic and post-meiotic phases and involves a highly regulated selective gene-expression pattern. However, this process has not been well characterised at the gene expression level due to the absence of germinal cell lines. We previously demonstrated that the rat skeletal muscle-specific gene for the glycolytic enzyme phosphoglycerate mutase is also specifically expressed in meiotic and haploid male germ cells from testis (12). To analyse the promoter elements that regulate the transcription of the phosphoglycerate mutase m gene (*pgam-m*)during spermatogenesis, we developed transgenic mice for a construct containing 1.3 kb from the *pgam-m* promoter linked to the *Escherichia coli LacZ* gene. RNA analysis by retrotranscription and PCR amplification of transgene expression showed transcriptional activity in the testis with a pattern during testis development that was identical to the endogenous gene. The transgene was also active in skeletal muscle but not in the adult heart in all the transgenic lines analysed. Collectively, these studies demonstrate that the 1.3 kb *pgam-m* promoter contains sufficient sequences to specify temporally regulated testis-specific expression as well as skeletal-muscle expression. © 1999 Academic Press

Key Words: **phosphoglycerate mutase; rat gene; lac Z; skeletal muscle; spermatogenesis; testis; transgenic.**

Abbreviations used: ACE, angiotensin converting enzyme; CRE, cyclic AMP response element; CREM, cyclic AMP responsive element modulator; LDHC, lactate dehydrogenase c; P1, protamine-1 gene; PDHA-2, testis pyruvate dehydrogenase E1-α subunit; PGAM, phosphoglycerate mutase; *pgam-m*, phosphoglycerate mutase m gene (DNA) encoding muscle-specific phosphoglycerate mutase; PGK-2, testis-specific phosphoglycerate kinase; RT-PCR, retrotranscription and polymerase chain reaction.

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Spermatogenesis is a complex cellular process that involves proliferation and differentiation. Although the cellular progression is relatively well understood, the biochemical and genetic mechanisms responsible for spermatogenesis have been poorly characterised.

Currently, the gene activation network that accounts for this process is the subject of intense research (1–4). During spermatogenesis a large number of new proteins appear. Some of these proteins are encoded by genes only expressed during spermatogenesis (for instance protamines). Others have functions and catalytic activities, already described in somatic tissues, but are encoded by different genes with testis-specific expression. Examples include several testis-specific metabolic isozymes: lactate dehydrogenase c (LDHC) (5), phosphoglycerate kinase-2 (PGK-2) (6) and testis pyruvate dehydrogenase E1- subunit α (PDHA-2) (7). The molecular bases for the testis-specific expression are diverse: some proteins are encoded by distinct genes (for instance, *pgk-2, pdha-2*) while others originate from alternative promoters or alternative splicing sites, such as testis ACE or CREM τ (8, 9).

Pgam-m encodes the skeletal muscle-specific subunit (M) of the phosphoglycerate mutase. This subunit is also expressed in heart and testis (10, 11). Previously (12), we demonstrated that the same *pgam-m* gene encoding the muscle-specific subunit is transcriptionally activated during spermatogenesis. The mRNAs for both tissues are almost identical in size except for a longer poly A tail for the testis species. The expression of the gene begins around day 22, when germ cells have started meiosis. Thus the *Pgam-m* gene is included in the growing number of testis and stage-specific genes co-ordinately expressed during spermatogenesis.

The study of transcriptional regulation of germ cell-specific genes is hampered by the absence of male germ cell lines or primary cultures. Some cultures and co-cultures from Sertoli and germ cells have been de-

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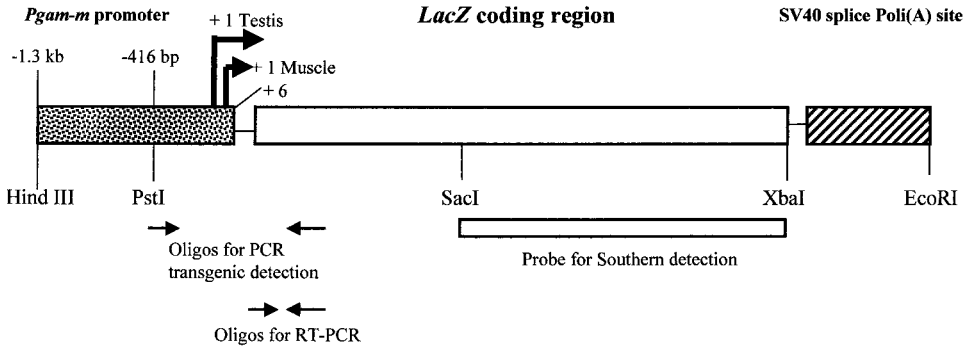


FIG. 1. Structure of the microinjected *pgam-m-LacZ* fused gene. Diagram of the transgene showing the different fragments corresponding to the *pgam-m* promoter (−1,300 bp to +6, referred to the transcription start point for skeletal muscle), the *lacZ* gene, and the splicing and polyadenylation sites from SV40. The position of the oligonucleotides used for transgenic identification by PCR and for RT-PCR detection of mRNA are shown by arrows. The *LacZ* cDNA fragment used as probe for Southern experiments is shown as a bar. Transcription start point for testis and skeletal muscle are also indicated.

scribed that differentiate partially (13–15) although there are no reports on efficient transfection using these cultures. Other approaches have attempted to study transcription by *in vivo* gene transfer into mouse spermatogenic cells *in vivo* (16, 17). Here, in attempt to identify the promoter sequences responsible for the tissue-specific expression of the *pgam-m* gene, we generated transgenic mice with a 1.3-kb genomic fragment of the 5′ regulatory region fused to the *Escherichia coli lacZ* gene. We report the expression pattern of the transgene in several transgenic lines.

MATERIALS AND METHODS

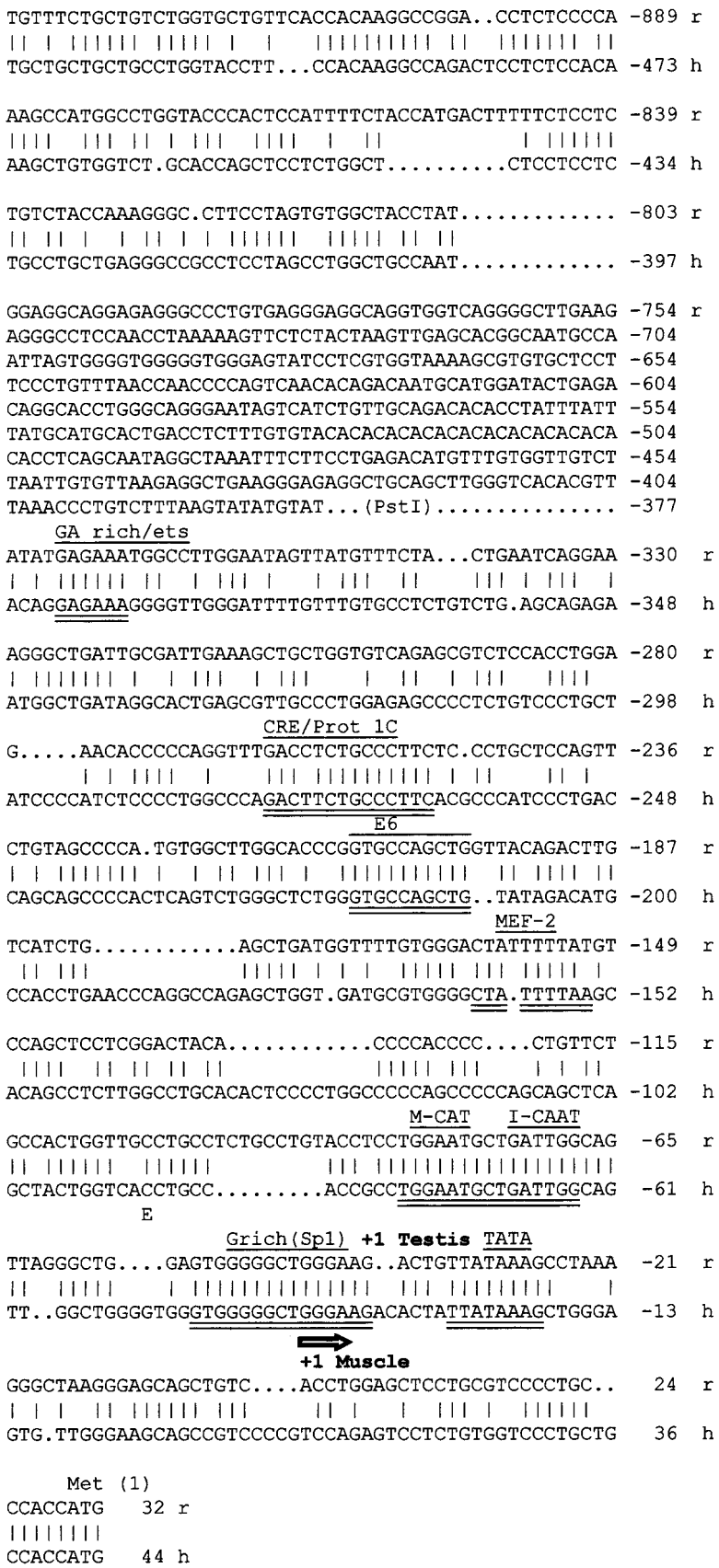
Determination of the transcriptional start point of rat pgam-m gene in testis. The transcription start point for rat *pgam-m* in testis was determined by primer extension. A synthetic oligonucleotide corresponding to a sequence from nt 43 to 73 within the rat *pgam-m* cDNA coding region (18) (5′-AGCCACAGAAACGGTTCTCTTGGTTCCA-TGA-3′) was end-labelled with T4 kinase. The primer was annealed to 20-30 μg of total RNA isolated from rat testis and skeletal muscle and primer extension was performed according to Sambrook *et al.* (19). Extended fragments from skeletal muscle and testis, together with a DNA molecular weight marker were electrophoresed on a 7 M urea 6% polyacrylamide sequencing gel.

Gene construction, production and analysis of transgenic mice. A 1.3 kb fragment corresponding to the promoter 5′ regulatory region of the rat *pgam-m* gene was excised by restriction digestion from a genomic clone (20) and subcloned into the vector *pUC19/AUG β-gal* (kindly provided by M. Perry). The resulting construct contained the 5′ regulatory region from the *pgam-m* gene in front of the *E. coli LacZ* gene together with the splicing and polyadenylation signals from SV40 (Fig. 1). The construct was linearized by digestion with EcoRI and HindIII and the 5-kb linear restriction fragment was microinjected by DNX Company according to standard protocols and 50 mice were obtained from embryos. Transgenic founders and the transgenic copy number of the different lines were detected by Southern blot of genomic DNA isolated from mice tails according standard protocols (19, 21). The identification of transgenic animals from successive generations was performed by PCR screening of genomic DNA from tail biopsies. Two primers were used, which spanned a 687 bp fragment containing 416 bp of the 5′ flanking region of *pgam-m* gene and 271 bp of the *lacZ* coding region (upper primer: 5′-CTGCAGCTTGGGTCACACGT-3′, lower primer 5′-TGTAGATGGGCGCATCGTAACCGTGCATCT-3′) (Fig. 1). Primers derived from the *H1* gene were used as an internal control of amplification (upper primer: 5′-TCCACGGACCACCCCAAGTATTCA-3′, lower primer: 5′-CTTGGCCAGCCTGAAGGACCCCGA-3′). Amplification was run for 30 cycles (94°C, 1 min; 55°C, 1.5 min; 72°C, 1.5 min) and the products were analysed on 1.5% agarose gels. Four founder mice and their offspring were studied.

RT-PCR of mRNA from transgenic mice tissues. Total RNA was extracted from 50-100 mg of frozen adult tissues and from testis at several postnatal ages, using the lithium chloride method described by Auffrey and Rougeon (22). Before cDNA generation the samples were treated with RNase-free DNAse I and heated to 65°C to inactivate DNase I. The RNA was then extracted, ethanol precipitated and resuspended in 20 μl of sterile distilled water. cDNAs were generated from 500 ng of RNA with an RNA RT-PCR kit (Perkin-Elmer) using random hexamer primers. Retrotranscription was stopped by heating the samples to 95°C for 5 min. 10 μl of cDNA products were amplified by PCR for 40 cycles (94°C, 40 s; 55°C, 40 s; 72°C, 40 s) using a commercial kit (Pharmacia). Primers corresponded to a *lacZ* region that amplifies a 310 bp fragment (upper primer: 5′-GGATTCACTGGCCGTCGTTTTACAACGTCG-3′, lower primer: 5′-TGTA GATGGGCGCATCGTAACCGTGCATCT-3′) (Fig. 1). Actin primers were used as a positive control (upper primer: 5′-ATGGATGACGATATCGCTG-3′, lower primer: 5′-ATGAGGTAG-TCTGTCAGGT-3′). RT-PCR products were analysed on 2% agarose gels and stained with ethidium bromide.

RESULTS

Determination of the transcription start point of the rat pgam-m gene in testis. In a previous study we determined the transcription start point for the rat *pgam-m* gene in skeletal muscle (20). This sequence was included in the construction used for the generation of transgenic mice. The construct used for transgenic mice generation also contained 1.3 kb of 5′flanking region with strong homology between rat and human *PGAM-M* promoters, especially in the region of −400 bp (Fig. 2). To assess that our transgenic construct contained sufficient elements to be transcribed accurately we mapped the endogenous transcription start point in rat testis together with the skeletal mus-


GA rich/ets
CRE/Prot 1C
E6
MEF-2
M-CAT
I-CAAT
E
Grich(Sp1) +1 Testis TATA
+1 Muscle
Met (1)

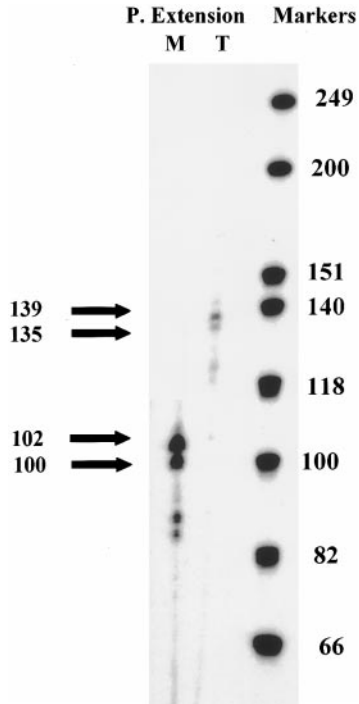


FIG. 3. Determination of the transcription start point for the rat *pgam-m* gene in testis and skeletal muscle. Lanes M and T correspond to extended bands obtained by primer extension with total RNA from skeletal muscle and testis, respectively. Right lane corresponds to DNA molecular weight markers. Numbers indicate the length of the bands (in nt).

cle as a control. Two main extended products of 100 and 102 nt were detected in skeletal muscle, coincident with the transcription start point previously determined (Fig. 3, lane M). In testis, however, two different extended products of 135 and 139 nt were obtained, which mapped upstream of skeletal muscle transcription start point and of the putative TATA box (see Fig. 2). These results establish a different transcription start point in testis than skeletal muscle for rat *pgam-m* and demonstrate that our transgenic construct contained the endogenous transcription start point for testis and skeletal muscle. These result suggest that *pgam-m* gene could be transcribed as a TATA less promoter in testis, although a cryptic TATA box cannot be excluded.

Establishment of transgenic strains and transgenic copy number. Southern blot analysis of genomic DNA isolated from tail biopsies from microinjected mice

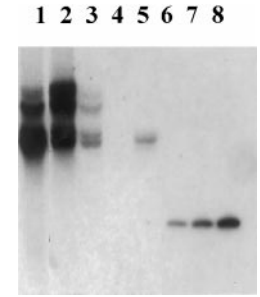


FIG. 4. Southern blot of genomic DNA from different transgenic mouse lines. The DNA was digested with PstI and hybridized to a *lacZ* probe. The radioactive bands were scanned and measured by densitometry with a phosphoimager. Lanes 1, 2, 3, and 5 correspond to 4 transgenic lines (tgPGM lines 3, 4, 2, and 1, respectively). Lane 4 is a negative control from a non-transgenic animal. Lanes 6-8 correspond to different amounts of the *lacZ* probe used and are equivalent to 1, 2, and 4 copy numbers, respectively.

gave 8 animals (5 males and 3 females) that were positive for the transgene. Transgenic lines were established by breeding male founders with non-transgenic females and 4 lines were obtained and analysed. Southern blot analysis of transgenic copy number (Fig. 4) revealed great differences between lines, which ranged from 2 to 4 copies for lines tg PGM-M1 (lane 5) and tg PGM-M2 (lane 3) and 27 to 29 copies for lines tgPGM-M3 (lane 1) and tgPGM-M4 (lane 2), respectively. No signal was obtained with DNA from a non-transgenic animal (lane 4). tgPGM-M1 and tgPGM-M2 showed the highest transcriptional activity and were used for all further studies.

Transcription of the pgam-m/lacZ transgene in adult mouse tissues. To determine the tissue-specific expression and the transcription activity of the transgene, total RNA was prepared from various adult tissues from transgenic lines and a series of RT-PCR experiments were performed. Only testis and skeletal muscle tissues produced an amplification product of the expected size (Fig. 5, lanes 1–2, 5–6). No amplification product was detected with RNA samples obtained from heart and liver from transgenic mice (lanes 3–4 and 7–8, respectively) or in any tissue from non-transgenic mice (lanes 9–12). In addition, no amplified band was obtained from samples subjected to RT-PCR in the absence of reverse transcriptase (lanes 13–16). To exclude problems with the RT-PCR that may result

FIG. 2. Sequence and comparison of 5′-promoter regulatory regions of the *PGAM-M* rat and human genes. 5′ promoter regions of the rat (r) and human (h) *PGAM-M* genes showing nucleotide positions from −936 (r) and −519 (h) to +32 and +44 relative to the transcription start point from skeletal muscle (indicated by open arrow) are aligned. The transcription start point from testis is also indicated. Conserved putative transcription regulatory elements are indicated and double underlined. Met corresponds to the first methionine of the open reading frame. GA rich/ets, a putative binding for ets transcription factors; CRE/Prot 1C, a conserved sequence between human and rat pgam-m promoters containing a putative Cyclic AMP responsive element; E6, a conserved sequence containing an E box; MEF-2, myocyte-specific enhancer binding factor 2; MCAT, muscle CAT responsive element; I-CAAT, Y box, reverse CCAAT sequence motif.

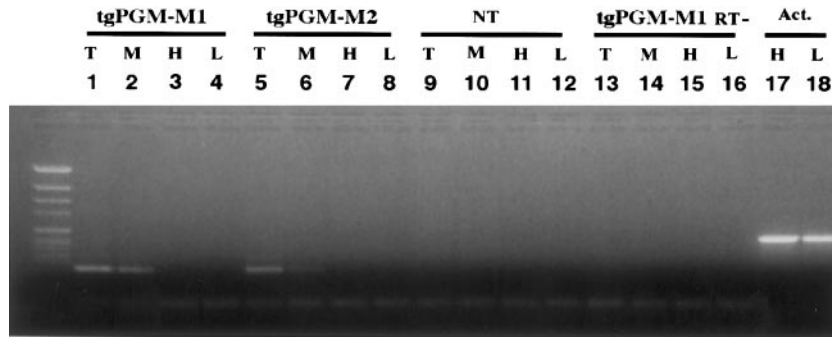


FIG. 5. RT-PCR analysis of *pgam-m-lacZ* transgene mRNA expression in adult mouse tissues. RNA samples were subjected to RT-PCR as described, and analysed in a 2% agarose gel. TgPGM-M1 and TgPGM-M2 correspond to 2 transgenic lines described in Fig. 4. NT, non-transgenic animals. T, testis; M, skeletal muscle; H, heart; L, liver. Lanes 13, 14, 15, and 16 correspond to RT-PCR experiments without reverse transcriptase (RT−). Act, over lanes 17 and 18 indicates RT-PCR with RNA from liver and heart of transgenic animals using actin primers.

from poor RNA integrity, liver and heart RNA from transgenic mice were subjected to RT-PCR using actin primers and an amplified band of the expected size was obtained (lanes 17 and 18). Southern blot analysis of the amplified bands obtained from skeletal muscle and testis samples using a *lacZ* gene probe confirmed the identity of the bands (results not shown). Taken together, these data demonstrate that the transgenic gene is actively transcribed in skeletal muscle and testis but silent in heart and liver. These results agree with the absence of mRNA levels in liver where the endogenous gene is not expressed, but not with the absence of expression in heart (23). Thus, additional regulatory sequences may be required for the expression of *pgam-m* gene in heart (see discussion).

Developmental expression of the pgam-m/lacZ gene during testis maturation. It is well established that in rodents, such as mouse and rat, there is a synchrony in the appearance of germinal cell types during testis development.To study the transgene expression during testis maturation we determined the timing of *lacZ* transgene mRNA appearance. As shown in Fig. 6, a representative RT-PCR experiment with RNA from testis of transgenic mice of several ages, the specific band corresponding to *lacZ* mRNA amplification appeared at day 26 post-natal and continued and increased at day 30 and in the adult (Ad, 60 days old). No band was detected on previous days (15 and 22), when the mouse testis is still immature and very few meiotic cells have appeared. In addition, no specific bands appear with the negative controls made without reverse transcriptase (T RT-) or with non-transgenic mice or liver samples (not shown). Taken together, these results demonstrate that the transgenic *pgam-m-lacZ* gene recapitulates the pattern of expression of the endogenous *pgam-m* gene during testis development. Therefore, the 1.3 kb from the 5′ flanking region of the rat *pgam-m* gene used for the transgenic construct contains sufficient transcription regulatory elements to confer tissue and stage development specificity when located upstream of a heterologous gene as *LacZ*.

DISCUSSION

Gene specific expression during spermatogenesis involves not only selective transcriptional regulation but also accurate post-transcriptional control mechanisms (24). The absence of transfectable spermatogenic cell tissue culture systems has made necessary the use of transgenic mice to study the transcriptional regulation of genes during spermatogenesis. Examples include testis *ACE*, *Pgk-2*, or *Ldh-c* (8, 25–26). We have investigated the molecular mechanisms that control the tissue specific transcription of the *pgam-m* gene by the use of transgenic constructs and microinjection.

The transcription start point for the endogenous *pgam-m* gene in testis (Fig. 3) is located upstream from

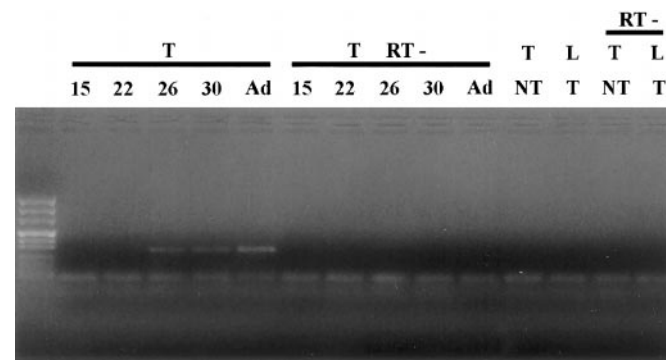


FIG. 6. RT-PCR analysis of *pgam-m-lacZ* transgene mRNA expression during testis development. RNA samples obtained from testis of transgenic mice at several postnatal ages were subjected to RT-PCR as described and analysed in a 2% agarose gel. T, testis tissues; T RT−, corresponds to RT-PCR experiments with RNA from testis without reverse transcriptase. Numbers under the line indicate the age of mice when testis were excised and RNA was prepared. Ad, adult mice (60 days old).

the one in skeletal muscle (25-30 nt) and suggests that *pgam-m* testis transcription is driven by a TATA less promoter, which is often found when the same gene is selectively transcribed in somatic tissues and during spermatogenesis (1). The construct used for these transgenic mice contained both transcription start points (Fig. 1).

Transcription analysis of the *pgam-m/lacZ* fused gene by RT-PCR (Figs. 5 and 6), demonstrated a tissue-specific transcription of the transgene which was restricted to the testis and skeletal muscle. In testis, the expression of the transgene was coupled to development and maturation and started by day 26. These results are consistent with previous detection of the *pgam-m* expression in adult testis and during testis development (11, 12) and with myocyte transfection studies using the same 1.3 kb 5′ upstream fragment as well as a shorter (−416 bp) from rat *pgam-m* promoter attached to the *CAT* reporter gene (20). Transfection of the –1.3 kb and –416 bp constructs into primary cultures of myocytes, hepatocytes and fibroblasts showed CAT reporter activity only in myocytes, demonstrating the tissue-specificity of the rat *pgam-m* promoter. Similar experiments had been performed with the human *PGAM-M* promoter showing its skeletal-muscle specific expression (27). However, the lack of transgenic expression in adult heart disagrees with previous analysis of PGAM isozyme profiles in cardiac muscle (10, 28) where the PGAM-M subunit is clearly detected and with Northern blot analysis of human *PGAM-M* expression (23). Interestingly, experiments performed by Nakatsuji *et al.* (27) demonstrated that large human promoter *PGAM-M* constructs (−2.2 kb to −505 bp) were inactive when transfected into neonatal rat cardiocytes, while shorter constructs (−165 bp) showed reporter activity. Therefore, the results obtained with both human and rat *PGAM-M* promoters studied *in vitro* and *in vivo* suggest that sequences located upstream of the basal *PGAM-M* promoter could act as potent transcription repressors in the heart, thus making the transgene construct silent. In addition, the endogenous *PGAM-M* gene might require further upstream or downstream sequences to be active in cardiac muscle.

Studies on the molecular mechanisms involved in the regulation of male germ-cell specific genes have made use of transgenic animals to characterise the *cis* and *trans* regulatory elements, together with complementary *in vitro* transcription and electrophoretic mobility shift and footprinting experiments. The comparison of the sequences of testis-specific promoters does not indicate significant homologies, with the exception of a CRE (Cyclic AMP Responsive Element) which has been shown to be bound by the specific post-meiotic transcription factor CREM τ (9). Transfection experiments with testis-specific promoters and targeted disruption of CREM suggest that it plays an important role in the expression of meiotic and post-meiotic specific genes (29–33).

A possible clue to the regulatory elements involved in *PGAM-M* testis-specific expression may come from the comparison of rat and human *PGAM-M* upstream flanking sequences since the human *PGAM-M* gene is also expressed in adult testis (Broceño and Pons, unpublished results). Sequence alignment between both promoter regions (Fig. 2) reveals that the homology is essentially reduced to the −400 bp region of the *PGAM-M* genes. This correlates with previous functional studies performed with the *PGAM-M* promoter in skeletal muscle cells showing that the −1.3 kb and −416 bp constructs of the rat gene give full promoter reporter activity (20). Sequence analysis of rat and human promoters reveals that in addition to the TATA, MCAT (CATTCCT sequence motif, muscle CAT-responsive element) and Y box (reverse CCAAT sequence motif) conserved in the proximal sequences of the promoters, there is a conserved MEF-2 (myocyte-specific enhancer-binding factor 2) box and some other additional conserved sequences such as a GA rich segment containing the core for the ets transcription factors (from −376 to −355), an E-box (CANNTG sequence motif, binding site for most of basic helix-loop-helix transcription factors) with conservation of its 5′ flanking sequence (E6 in Fig. 2, from −217 to −200) and a region resembling a CRE site (from −264 to −250). Although ets binding sites have been involved in the transcription of *pgk-2* (34), the CRE elements are the best characterised in testis-specific genes. The CRE-like sequence from the *pgam-m* promoter includes part of the PROT1C regulatory element (TGACCTC), which has been described in several testis-specific genes and it is identical to the one present in the P1 (protamine-1 gene) promoter of various species (see 35 for a review). However, the 3′ half of the CRE sequence diverges completely from P1 genes although it is completely conserved in rat and human promoters (5′-GCCCTTC-3′).

In conclusion, the −1.3 kb 5′ upstream region of rat *pgam-m* promoter contains conserved sequences that are sufficient to confer testis and skeletal muscle-specific transcriptional activity. Further functional studies and DNA-protein binding analysis may clarify the role of the sequence motifs in the testis-specific expression of *pgam-m* gene.

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