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Transcription inhibition of the somatic-type phosphoglycerate kinase 1 gene *in vitro* by a testis-specific factor that recognizes a sequence similar to the binding site for Ets oncoproteins

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ABSTRACT

To elucidate the mechanism by which transcription of the somatic-type phosphoglycerate kinase 1 gene is inactivated during mammalian spermatogenesis, we examined the presence of specific transcription inhibitor(s) in the testis by a cell-free transcription system. Transcription of the mouse phosphoglycerate kinase 1 gene using nuclear extracts of the rat liver was significantly inhibited by the addition of testis extracts, whereas brain extracts had little effect. Transcription inhibition required the binding of a testis-specific factor, designated TIN-1, to the region between positions −268 and −259 relative to transcription initiation site at +1. This region had the sequence 5′-AGGAAGTTCC-3′ that includes an inverted repeat of the binding motif, 5′-GGAA-3′, for the oncoprotein Ets. A UV-crosslinking experiment revealed that 43- and 45-kDa polypeptides present in testis extracts bind to that sequence. These results suggest that a testis-specific transcription inhibitor TIN-1 inactivates the phosphoglycerate kinase 1 gene in the mammalian spermatogenic pathway.

INTRODUCTION

Mammalian spermatogenesis is a complex pathway in which spermatogenic cells present in the seminiferous tubules undergo differentiation giving rise to spermatozoa (Ref. 1 for a review). A variety of biological events occur during spermatogenesis, such as meiotic division of spermatogonia, production of sperm-specific proteins, chromosome condensation, and morphogenesis of mature sperm. Morphological change of spermatogenic cells during these events has been well characterized, but the molecular mechanism which controls spermatogenesis remains to be clarified.

The expression of many genes varies during mammalian spermatogenesis, including those coding for sperm-specific proteins and others whose function is unknown, such as the products of proto-oncogenes (Refs. 2–4 for reviews). We have been studying the mechanism which controls selective gene transcription during this process. A glycolytic enzyme, phosphoglycerate kinase (PGK), has two isozymes, somatic-type PGK-1 and testis-specific PGK-2, that are encoded by different genes (5–10). Our previous studies have suggested that the isozyme pattern of mouse PGK switches at the mRNA level from PGK-1 to PGK-2 at the pachytene spermatocyte stage (11,12). The inactivation of *PGK-1* gene expression might be necessary to prevent imbalance of the PGK concentration among haploid cells because the *PGK-1* gene is located on the X chromosome. The autosomal *PGK-2* gene is likely to be induced to compensate for the lack of PGK. To elucidate the mechanism which regulates differential transcription of the two *PGK* genes should be important for improved understanding of selective gene expression in the mammalian spermatogenic pathway. We studied whether the inactivation of *PGK-1* gene transcription is caused by inhibitor(s) present in the testis.

MATERIALS AND METHODS

Nuclear extracts preparation, plasmid DNA, and oligo DNA

Nuclear extracts were prepared from the liver, testis, and brain of 10-week-old rats according to the described procedure (13,14). Extracts were dispensed into small aliquots and stored at −80°C. Plasmids P1Sh and P1Se contained DNA fragments, inserted at the upstream of the chloramphenicol acetyltransferase (CAT) gene in pBLCAT3 (15), that corresponded, respectively, to the regions between positions −410 and +50, and −200 and +50 with respect to the most distal transcription initiation site of the mouse *PGK-1* gene at +1 (12). Oligo dsAG had the sequence 5′-TAG-

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TCAGGAAGTTCCCCCCC-3′ corresponding to the region between positions −273 and −254 of the *PGK-1* gene. Oligo AP1 had the sequence 5′-GTGACTCAGCGCG-3′ flanked by an additional sequence 5′-GATC-3′ at the 5′-ends of both strands. Oligo AP4 had the sequence 5′-CCAGCTGTGGAATG-3′, that corresponded to the region between nucleotide positions 263 and 276 of the simian virus 40 DNA, flanked by an additional sequence 5′-GATC-3′ at the 5′-ends of both strands. Oligo AP1 and AP4 included the binding sites for transcription factors AP-1 (16) and AP-4 (17), respectively.

Cell-free transcription and primer extension reactions

Cell-free transcription was conducted in a 20 μl reaction mixture containing 25 mM Hepes, pH 7.6, 50 mM KCl, 6 mM $MgCl_2$, 0.8 mM DTT, 0.06 mM EDTA, 0.62 mM ATP, GTP, CTP, and UTP, 9% glycerol, 20 units of RNase inhibitor (Takara Shuzo, Japan), 0.09 pmol of covalently-closed circular DNA as the template, and about 40 μg protein of nuclear extracts. The template DNA and nuclear extracts were first incubated on ice for 10 min before other components were added, and transcription reaction was performed at 25°C for 45 min. 280 μl of stop solution (20 mM Tris-HCl, pH7.5, 0.25 M NaCl, 1% SDS, 5 mM EDTA, 70 μg/ml yeast tRNA, and 40 μg proteinase K) was then added and the mixture was further incubated at 37°C for 10 min. RNA transcripts were purified by phenol/chloroform (1:1) extraction and precipitated with ethanol in the presence of a ^{32}P-labeled primer containing the CAT gene sequence, 5′-TCTCGCCAAGCTCCTCGA-3′. Primer extension reaction was performed as described (18,19), and extended products were separated on a 6% polyacrylamide-8.3 M urea gel followed by autoradiography. In transcription competition experiments, nuclear extracts and competitor oligo DNAs were first incubated on ice for 10 min. DNA template was then added and the mixture was further incubated on ice for 10 min. Transcription reaction was finally carried out at 30°C for 45 min, after the mixture was supplemented with other components.

DNase I footprint and gel shift assays

DNA fragments corresponding to the region between positions −410 and +51, and positions −450 and −200 of the *PGK-1* gene were labeled with ^{32}P at the 5′-ends at positions −410 and −200, and used as probes in a footprint assay for coding and non-coding strands, respectively. About 40 μg protein of nuclear extracts were first incubated on ice for 15 min in a 17.5 μl mixture containing 17.5 mM Hepes, pH 7.6, 43 mM KCl, 0.8 mM DTT, 0.8 mM EDTA, 0.42 μg pBR322 DNA, and 10% glycerol. The ^{32}P-labeled probe was then added and the mixture was further incubated on ice for 10 min. The mixture was then digested with 0.7 units DNase I at 30°C for 0.5−1.5 min followed by the addition of 380 μl solution containing 0.5% SDS, 50 mM CH_3COONa, 10 mM EDTA, and 70 μg/ml yeast tRNA. After incubation at 37°C for 10 min, DNA was purified by phenol/chloroform extraction and analyzed on an 8% polyacrylamide-8.3 M urea gel followed by autoradiography.

For a gel shift assay, 30 μg protein of nuclear extracts were incubated in the absence or presence of competitor oligo DNA in a 9 μl reaction mixture containing 17 mM Hepes, pH 7.6, 42 mM KCl, 0.76 mM DTT, 0.75 mM EDTA, 9.6% glycerol, and 1 μg poly(dA-dT)-poly(dA-dT) on ice for 15 min. About 0.08 pmol of ^{32}P-labeled oligo dsAG was then added and incubation was continued on ice for 10 min. The mixture was subsequently loaded on a 6% polyacrylamide gel and electrophoresed in a buffer of 50 mM Tris-borate, pH 8.3 and 1 mM EDTA at 8 volt/cm at room temperature followed by autoradiography. In a reaction with liver extracts, 1 μg poly(dI-dC)-poly(dI-dC) were included in addition to poly(dA-dT)-poly(dA-dT).

UV-crosslinking

A 9 μl reaction mixture containing 17 mM Hepes, pH 7.6, 42 mM KCl, 0.76 mM DTT, 0.75 mM EDTA, 9.6% glycerol, 2 μg poly(dA-dT)-poly(dA-dT), 2 μg poly(dI-dC)-poly(dI-dC), competitor oligo DNAs, and 30 μg protein of testis extracts was incubated on ice for 15 min. About 0.08 pmol of ^{32}P-labeled oligo dsAG were added and the mixture was further incubated on ice for 10 min. The mixture was then exposed to 254 nm UV on ice for 45 min at a distance of 5 cm and electrophoresed on a 12% SDS-polyacrylamide gel followed by autoradiography.

RESULTS

Inhibition of *PGK-1* gene transcription by testis extracts

To examine the possibility that specific inhibitor(s) of *PGK-1* gene transcription is present in the testis, we first set up a cell-free transcription reaction of the *PGK-1* gene using nuclear extracts of the rat liver. The DNA fragment containing the 410 bp 5′-upstream and transcription initiation sites of the mouse *PGK-1* gene was linked to the CAT gene and used as the template in a cell-free transcription reaction. When transcripts were analyzed by primer extension, an extended product with the expected size was detected which disappeared in a reaction with 1 μg/ml α-amanitin (Fig. 1A). This signal was derived from transcripts initiated at the same sites as *in vivo* transcripts (12) when analyzed on a sequencing gel (data not shown). Smaller products, that were sensitive to α-amanitin, are probably due to the premature termination of reverse transcription. Essentially, the same results were obtained when transcripts were analyzed by S1 nuclease mapping (data not shown). These results indicate that the mouse *PGK-1* gene is faithfully transcribed in a cell-free reaction using nuclear extracts of the rat liver.

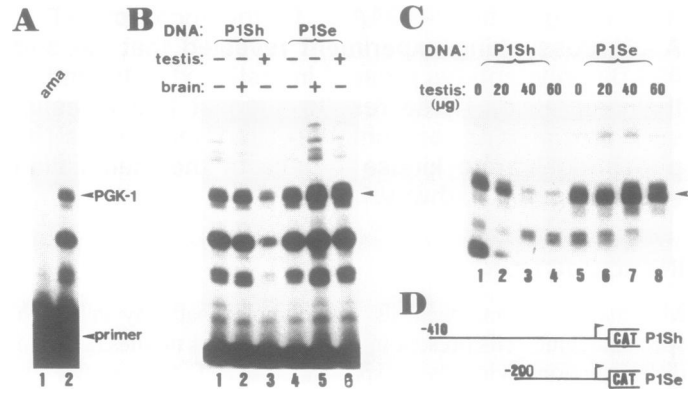

Figure 1. Inhibition of *PGK-1* gene transcription *in vitro* by testis extracts. **(A)** P1Sh DNA was transcribed in liver extracts in the presence (lane 1) and absence (lane 2) of 1 μg/ml α-amanitin. An autoradiogram of a polyacrylamide-urea gel is shown. The arrowheads indicate positions of the primer-extended product with the expected size and a free primer. **(B)** P1Sh and P1Se DNAs were transcribed in liver extracts (40 μg protein) in the presence of testis or brain extracts (40 μg protein) as indicated at the top of the panel. **(C)** P1Sh and P1Se DNAs were transcribed in liver extracts with increasing amounts of testis extracts. **(D)** The structure of P1Sh and P1Se DNAs is shown schematically.

We then added nuclear extracts prepared from the rat testis and brain into the transcription reaction with liver extracts. *PGK-1* gene transcription was significantly reduced by the addition of testis extracts, whereas brain extracts had little effect (lanes 1–3 in Fig. 1B). Testis extracts inhibited *PGK-1* gene transcription in a dose-responding manner (lanes 1–4 in Fig. 1C).

Requirement of a distinct upstream region of the *PGK-1* gene for transcription inhibition by testis extracts

Transcription inhibition by testis extracts was not observed when plasmid P1Se, containing the 200 bp upstream region of the *PGK-1* gene, was used as the template (Fig. 1B and 1C). There was no apparent difference in the effect on transcription between testis and brain extracts using this DNA template (lanes 4–6 in Fig. 1B). The addition of testis extracts at levels that almost completely inhibited transcription from plasmid P1Sh, which contains the 410 bp upstream region, did not affect transcription from P1Se DNA (Fig. 1C). These results indicate that the region between positions −410 and −201 of the *PGK-1* gene was required for testis extracts to inhibit transcription in a cell-free system.

Bunick *et al.* previously reported that transcription of the mouse protamine 2 gene in the mouse testis extracts occurred at a temperature optimum of 20°C and that transcription did not occur at 30°C (20). This suggests that the transcription machinery in testis extracts, which directs transcription of the protamine 2 gene, is sensitive to high temperatures. We thus examined whether or not transcription inhibition of the *PGK-1* gene by testis extracts is observed when the reaction is conducted at 30°C. The results showed that transcription occurred at 30°C slightly more efficiently than at 25°C, and transcription inhibition was clearly observed at 30°C (Fig. 2). *PGK-1* gene transcription was almost turned off in the reaction at 20°C, which temperature resulted in maximum transcription of the protamine 2 gene. These results suggest that factor(s) present in testis extracts functions *in vitro* at 30°C to inhibit *PGK-1* gene transcription, unlike the putative positive transcription factor(s) for the protamine 2 gene. Basal level transcription of these two genes showed different temperature sensitivity in a cell-free system. This might be due to the difference in promoter structure, that is, the protamine 2 gene promoter contains a TATA box whereas that of the *PGK-1* gene does not (12).

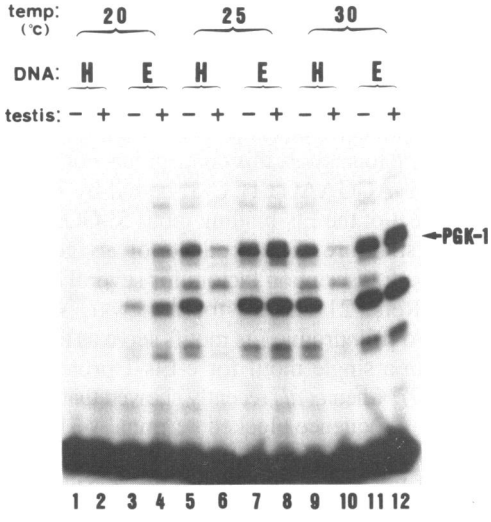

Figure 2. Effect of temperature on *PGK-1* gene transcription *in vitro*. P1Sh (H) and P1Se (E) DNAs were transcribed in liver extracts (40 μg protein) at various temperatures in the presence or absence of testis extracts (40 μg protein) as indicated at the top of the panel.

Detection of factor(s) in testis extracts that binds to the upstream sequence of the *PGK-1* gene

We next performed a DNase I footprint assay using DNA probes containing the region required for transcription inhibition of the *PGK-1* gene by testis extracts. Both testis and liver extracts produced footprints at almost the same position (Fig. 3A). The region, with the sequence 5′-AGGAAGTTCC-3′, located between positions −268 and −259 was protected from DNase attack by the extracts. This region contained an inverted repeat of the sequence 5′-GGAA-3′, that is known as the common binding motif for the products of *ets* gene family (Refs. 21 and 22 for reviews). Footprints were observed symmetrically on both strands around the sequence complementary to this motif.

Since the pattern of the footprints on a non-coding strand caused by the two extracts seemed to be somewhat different, a gel shift assay was next carried out using oligo dsAG as a probe which contained the region between positions −273 and −254 (Fig. 3B). Three shift bands were observed with testis extracts, and the fastest and the slowest ones (C1 and C2) were specifically competed out by the addition of unlabeled probe in excess (lanes 2–6). Liver extracts also showed multiple shift bands with almost the same mobility as those produced by testis extracts, but only the most slowly-migrating band (C3) disappeared in the presence of excess unlabeled probe (lanes 7–9). These results, consistent with those of the footprint assay, suggest that the two extracts

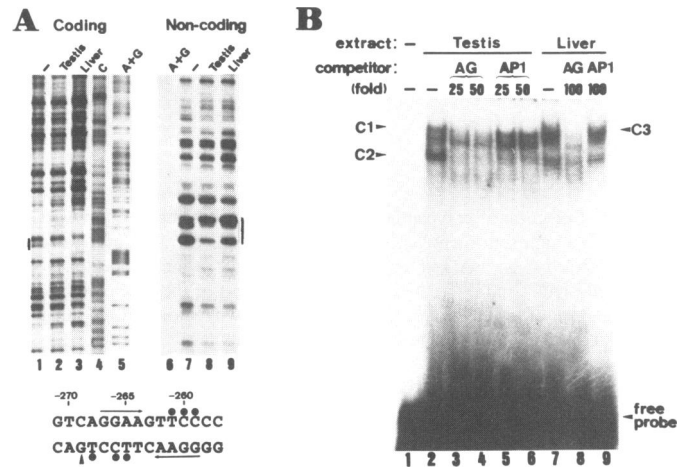

Figure 3. Identification of factor(s) in testis extracts binding to the 5′-upstream sequence of the *PGK-1* gene. **(A)** A DNase I footprint assay was performed with testis or liver extracts (40 μg protein) using the ^{32}P-labeled DNA fragments as probes for coding (lanes 1–5) and non-coding (lanes 6–9) strands. Autoradiograms of polyacrylamide-urea gels are shown. Lanes 4–6 contained the Maxam/Gilbert sequence ladders (44) as markers. The footprint regions are indicated by vertical lines, and their sequences are shown below. Protected bases and a DNase I hypersensitive site in the presence of the extracts are indicated with closed circles and an arrowhead, respectively. The arrows show an inverted repeat of the sequence 5′-GGAA-3′. **(B)** A gel shift assay was conducted using ^{32}P-labeled oligo dsAG as a probe with testis or liver extracts (30 μg protein) in the presence and absence of the competitor DNAs, oligo dsAG (AG) and oligo AP1, as indicated at the top of the panel. An autoradiogram of a polyacrylamide gel is shown. The positions of specific complexes formed between the probe and factors present in testis (C1, C2) and liver (C3) extracts are indicated.

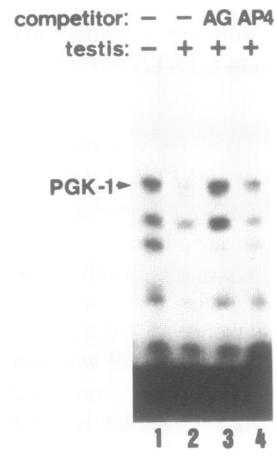

Figure 4. Transcription competition with oligo DNAs. P1Sh DNA was transcribed in liver extracts (40 μg protein) in the presence (+) or absence (–) of testis extracts (40 μg protein). Competing oligo dsAG (AG) and oligo AP4 were added to the transcription reaction at 100-fold excess of the template.

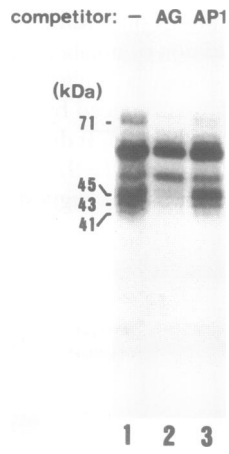

Figure 5. Detection of polypeptides in testis extracts binding to oligo dsAG. A UV-crosslinking experiment was conducted with testis extracts (30 μg protein) and ^{32}P-labeled oligo dsAG in the presence or absence of the competitor DNAs, oligo dsAG (AG) and oligo AP1, at 100-fold excess of the labeled DNA. An autoradiogram of a SDS-polyacrylamide gel is shown. Shown at the left is the molecular mass of polypeptide bands in kilo-daltons.

contained factors which recognize similar sequence but bind to it in different manners resulting in difference in the function to control *PGK-1* gene transcription.

Competition of transcription inhibition with a synthetic DNA oligo dsAG

To examine whether the binding of factor(s) to the sequence 5′-AGGAAGTTCC-3′ is important for transcription inhibition of the *PGK-1* gene by testis extracts, transcription competition was performed by adding excess oligo dsAG into a cell-free transcription reaction with P1Sh DNA as the template (Fig. 4). The addition of oligo dsAG almost completely abolished the inhibitory effect of testis extracts (lane 3), while a control oligo AP4 had a minimum effect (lane 4). These results suggest that interaction between testis-specific factor(s) and the sequence 5′-AGGAAGTTCC-3′ present in the upstream region of the *PGK-1* gene is necessary for transcription inhibition by testis extracts. We designated this testis-specific factor TIN-1 for testis-specific transcription inhibitor 1.

Identification of polypeptides in testis extracts that bind to oligo dsAG

A UV-crosslinking experiment was carried out to directly detect TIN-1 in testis extracts. The labeled oligo dsAG was mixed with testis extracts, and the mixture was exposed to UV prior to separation on a SDS-polyacrylamide gel (Fig. 5). Several polypeptides were detected after autoradiography in the reaction without competitor oligo DNAs, and four of them, 71, 45, 43, and 41 kDa in size, were competed out when the binding reaction was performed in the presence of excess unlabeled probe (lane 2). In contrast, the addition of excess oligo AP1 made 71- and 41-kDa bands disappear with no effect on 43- and 45-kDa bands (lane 3). Polypeptides of 43 and 45 kDa were thus likely to be TIN-1 that inhibits *PGK-1* gene transcription by binding to the sequence 5′-AGGAAGTTCC-3″.

DISCUSSION

Testis-specific transcription inhibitor TIN-1

We showed in the present study that a factor present in nuclear extracts of the rat testis inhibits transcription of the somatic-type *PGK-1* gene in a cell-free system. This factor, named TIN-1, has apparent molecular mass of 43 and 45 kDa and binds to the sequence 5′-AGGAAGTTCC-3′ present in the 5′-upstream region of the *PGK-1* gene. TIN-1 seems to be testis-specific protein(s) since its activity was not detected in nuclear extracts of the liver and brain. Our previous study suggested that *PGK-1* gene transcription ceases at the pachytene spermatocyte stage in the spermatogenic pathway (11). This means that the activity or level of TIN-1 should be controlled to allow the *PGK-1* gene to be transcribed until that particular stage. It is thus important to examine stage-specific expression of the TIN-1 gene either by determination of the TIN-1 activity present in the fractionated spermatogenic cells or *in situ* detection of TIN-1 mRNA with the testis sections.

Expression of a variety of genes is altered during mammalian spermatogenesis. Some of these genes code for key proteins with respect to roles in the regulation of gene expression. Those are such proteins as growth factors, zinc-finger proteins, proto-oncogene products, and homeobox proteins. Many of them are expected to play an important role in the spermatogenic pathway, but firm evidence to support this concept has not been obtained. The sequence 5′-AGGAAGTTCC-3′ bound by TIN-1 contains an inverted repeat of the Ets-binding motif 5′-GGAA-3′ forming a palindromic structure. The products of *ets* gene family are considered to be transcription factors for several different genes (23–27, and Refs. 21 and 22 for reviews). Wasylyk *et al.* reported that two palindromic Ets motifs serve as a more effective *cis*-element than a single motif for c-Ets-1 protein to stimulate transcription (27). Chen *et al.* recently showed that antisense oligo DNA to the *ets-2* sequence blocked germinal vesicle breakdown when introduced into Xenopus oocytes, suggesting the involvement of Ets-2 in oocyte maturation (28). Moreover, the *ets-1* and the *ets-2* genes (29), and an *ets*-related gene *elk* (30) seem to be actively expressed in the mouse testis. It is thus possible that TIN-1, a testis-specific transcription inhibitor presumably involved in spermatogenic gene expression, is a product of an *ets*-related gene. TIN-1 appeared to contact the

sequence 5′-TTCC-3′ that is complementary to the Ets-binding motif, whereas the products of *ets*-related genes murine *PU.1* (26) and *Drosophila E74* (31) directly protected the sequence 5′-GGAA-3′. Hence, we presume that TIN-1 and the products of the *PU.1* and the *E74* genes recognize similar sequences in different fashions, even if they are structurally related proteins. Structural analyses of TIN-1 are needed to conclude any relationship between TIN-1 and the Ets oncoprotein.

Inactivation of the *PGK-1* gene during mammalian spermatogenesis

One of the two X chromosomes in female cells becomes genetically silent in early embryo, and this process is called X-chromosome inactivation (Refs. 32 and 33 for reviews). It is also expected that sex chromosomes are entirely inactivated in the testis during spermatogenesis. Since the *PGK-1* gene is located on the X chromosome, the mechanism which makes *PGK-1* gene transcription inactive during spermatogenesis might be related to that controlling X-chromosome inactivation in female cells. It has been suggested that specific DNA methylation and demethylation in the 5′-upstream region of the human (34–36) and mouse (37,38) *PGK-1* gene alters the efficacy of transcription. However, we previously suggested that the methylation state at the 5′-upstream region is not important for the differential expression of the *PGK-1* gene in the mouse liver and testis (11). We now presume, from the results in this study, that the inactivation of *PGK-1* gene transcription at the pachytene spermatocyte stage is caused, at least partly, by the action of a testis-specific transcription inhibitor TIN-1 that directly binds to the *cis*-acting sequence present in the 5′-upstream region of the *PGK-1* gene. However, it is still possible that the stage-specific methylation or demethylation at the *cis*-acting region regulates the binding of TIN-1. Tissue- or cell-type-specific negative DNA elements have been reported to regulate transcription of several genes, such as the neuron-specific SCG10 (39,40), the DNA polymerase β (41), the α T cell receptor (42) and the immunoglobulin κ light chain (43) genes. The negative element for *PGK-1* gene transcription is likely to be testis-specific, since it did not seem to function in liver and brain extracts. *In vivo* experiments should be done to examine whether this element actually has tissue-specificity. It is also important to examine whether or not TIN-1 action is restricted to the *PGK-1* gene by testing its effect on transcription of other spermatogenic genes on the X chromosome.

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