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A factor stimulating transcription of the testis-specific *Pgk-2* gene recognizes a sequence similar to the binding site for a transcription inhibitor of the somatic-type *Pgk-1* gene

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ABSTRACT

The glycolytic enzyme phosphoglycerate kinase (PGK) consists of two isozymes, somatic-type PGK-1 and testis-specific PGK-2. The isozyme switch from PGK-1 to PGK-2 occurs during spermatogenesis at the mRNA level. The distal upstream region of the gene encoding mouse PGK-2 (*Pgk-2*) possesses a silencer-like negative *cis* element. In the present study, a positive *cis* element located in the proximal upstream region and factor(s) bound to it were analyzed *in vitro*. Cell-free transcription using nuclear extracts of rat organs demonstrated that the region between nucleotide positions −82 and −64, relative to the most distal transcription initiation site at +1, stimulates transcription in testis extracts. The *cis* element did not act on the promoter of the thymidine kinase gene, suggesting that it stimulates *Pgk-2* transcription in a promoter-specific manner. The *cis* element bound a nuclear factor(s), which we designated TAP-1. Introducing various base substitutions within the *cis* element revealed that TAP-1-binding to the element requires the sequence 5′-GGAA-3′, which is the binding motif for Ets oncoproteins. We previously reported that TIN-1, a transcription inhibitor of *Pgk-1*, binds to a sequence similar to the Ets-binding site. The addition of an oligo DNA containing the TIN-1-binding sequence of *Pgk-1* prevented TAP-1 from binding to the *Pgk-2 cis* element, and vice versa. These results suggest that both TIN-1 and TAP-1, which are presumably involved in transcription regulation of the two *Pgk* genes, recognize DNA sequences related to the Ets-binding motif.

INTRODUCTION

Mammalian spermatogenesis is a complex pathway consisting of a variety of distinct biological events, including proliferation and meiotic division of spermatogonia, chromosome condensation, production and displacement of sperm-specific proteins, as well as morphogenesis of mature sperm (Ref. 1 for a review). Although the processes of these events are histochemically well understood, the molecular basis for the spermatogenic pathway remains to be elucidated. Since many biological reactions participate in the regulation of mammalian spermatogenesis, differing perspectives should be investigated to reach a conclusion. One important approach should be to clarify the mechanism of differential gene expression during spermatogenesis. Expression of a variety of genes is activated or inactivated as the spermatogenic pathway continues (Refs. 2–4 for reviews). To understand the mechanism for the expression of spermatogenesis-specific genes is important, since these genes may include those coding for the proteins necessary for the progress of spermatogenesis.

The gene encoding a glycolytic enzyme phosphoglycerate kinase (PGK) is a spermatogenic gene. PGK exists as two isozymes, somatic-type PGK-1 and testis-specific PGK-2. The isozyme switch from PGK-1 to PGK-2 occurs at the mRNA level during the stage of the pachytene spermatocyte (5). The transcription switch of the two *Pgk*, that is, inactivation of *Pgk-1* and activation of *Pgk-2*, is a suitable model with which to study spermatogenic gene expression. We previously suggested the involvement of a testis-specific transcription inhibitor, designated TIN-1, in the cessation of *Pgk-1* transcription in the testis (6). In addition, we recently found a silencer-like negative *cis* element in the distal upstream region of mouse *Pgk-2*, which may be responsible for the repression of *Pgk-2* transcription in somatic tissues (7). We report here, a positive *cis* element in the proximal upstream of *Pgk-2* and a factor(s) that binds to it.

MATERIALS AND METHODS

DNA

Various portions of the 5′-upstream region of mouse *Pgk-1* and *Pgk-2* (5) were inserted into pBLCAT3 (8) at the *Xho*I site, and the resulting DNAs were used as templates for cell-free

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transcription. The nucleotide positions on the left of *Pgk* DNA are indicated in the figures, and those on the right end were +51 and +60 for *Pgk-1* and *Pgk-2*, respectively. OligoP2 had the sequence, 5′-CTGGATTTCCTTTCAAATT-3′, corresponding to the region between nucleotide positions −82 and −64 of *Pgk-2*. The oligoKB sequence, 5′-TGGGGATTCCCCA-3′, contained an NF-κB-binding site present in the promoter of the major histocompatibility complex class I gene (9). The oligoAP4 sequence, 5′-CCAGCTGTGGAATG-3′, and the oligoKBII sequence, 5′-GGGGACTTTCCC-3′, contained the AP-4 (10) and NF-κB (11) sites respectively, within the enhancer of simian virus 40 DNA. OligoAG had the sequence 5′-TAGTCAGGAA-GTTCCCCCCC-3′, which corresponds the region between nucleotide positions −273 and −254 of *Pgk-1* (6).

Nuclear extract preparation and cell-free transcription

Nuclear extracts were prepared from the liver and testes of 10-week-old rats according to the method of Gorski *et al.* (12). Cell-free transcription proceeded in a 20-μl reaction mixture containing 0.21 pmol of a DNA template and 40 μg extract protein (6). RNA transcripts were analyzed by primer extension with the ^{32}P-labeled primer, 5′-TCTCGCCAAGCTCCTCG-A-3′, as described (6). In a transcription competition assay, 0.1 μg pBLCAT3 DNA was included in the reaction mixture.

Gel-shift, DNase I footprint, and UV-crosslinking assays

For the gel-shift assay, 10 μg of proteins from nuclear extracts were incubated in a 9-μl mixture containing 17 mM Hepes (pH 7.6), 42 mM KCl, 0.76 mM DTT, 0.75 mM EDTA, 9.6% glycerol, 2 μg of heat-denatured salmon testis DNA for 15 min on ice. A ^{32}P-labeled oligo DNA (0.025 pmol) was then added and the mixture was further incubated for 10 min on ice. The mixture was subsequently loaded onto a 6% polyacrylamide gel and electrophoresed in 22.5 mM Tris-borate (pH 8.3) containing 0.5 mM EDTA at 8 volt/cm at 4°C followed by autoradiography. For the DNase I footprint assay, a DNA fragment corresponding to the region between nucleotide positions −138 and +61 of *Pgk-2* was labeled at the end with ^{32}P and used as a probe. Forty micrograms of extract proteins and 0.8 ng of the probe were incubated and digested with DNase I as described (6). The final reaction products were separated on an 8% polyacrylamide/8.3 M urea gel followed by autoradiography. The UV-crosslinking assay was performed in a mixture containing 40 μg extract proteins and 0.025 pmol of ^{32}P-labeled oligoP2 as described (6). The mixture was exposed to 254 nm UV for 45 min on ice at a distance of 5 cm, then resolved on a 12% SDS-polyacrylamide gel (13) followed by autoradiography.

RESULTS

Identification of positive *cis*-acting elements in the proximal upstream region of *Pgk-2*

Nuclear extracts prepared from the liver and testes of adult rats were used for cell-free transcription of the two *Pgk* (Fig. 1). The somatic-type *Pgk-1* was efficiently transcribed in liver extracts with no apparent template activity in testis extracts (lanes 2 and 3). Differential transcription of *Pgk-1* in the two extracts was probably provided by the testis-specific transcription factor, TIN-1, that specifically inhibits *Pgk-1* transcription in testis extracts (6). On the other hand, the testis-specific *Pgk-2* promoter, that drives transcription from multiple initiation sites (14), seemed to be more active in liver, than in testis extracts (lanes 1 and 4). This can be explained as follows. The region between nucleotide positions −1567 and −681 contains a *cis*-acting

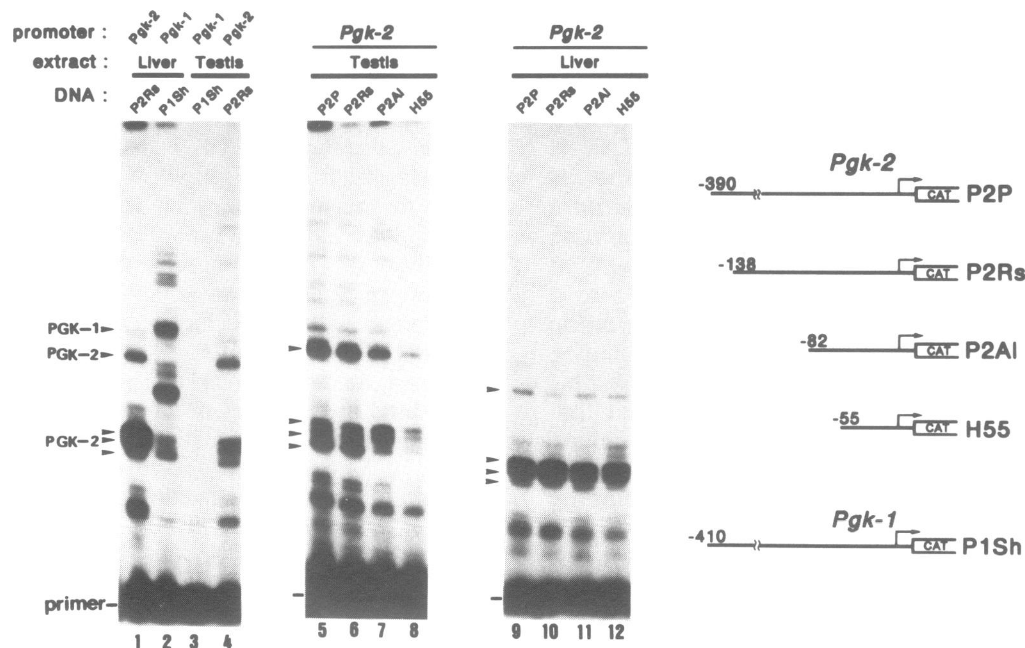

Figure 1. Identification of DNA regions stimulating *Pgk-2* transcription *in vitro*. Various portions of the *Pgk* upstream regions, including transcription initiation sites, were linked to the chloramphenicol acetyltransferase gene, and the resulting DNAs (shown at the right) were used as templates in cell-free transcription using nuclear extracts of adult rat testes and liver. Autoradiograms of polyacrylamide/urea gels containing primer extended products are shown. Extracts and DNA templates are indicated at the top of the panels. The arrowheads show the positions of primer extended products derived from accurate transcripts from *Pgk* promoters.

negative element(s) that represses *Pgk-2* transcription *in vitro* using liver extracts (7). A DNA lacking this negative *cis* element (P2Rs) was used here as a template, since we intended to analyze the *cis* elements located in the proximal upstream region of *Pgk-2*. Nevertheless, tissue-specific transcription of the two *Pgk* was reproduced in a cell-free system using testis extracts.

Under the above premise, we examined the effects of the 5′-upstream region on *Pgk-2* promoter activity in a cell-free system. Using testis extracts, *Pgk-2* transcription was slightly reduced with a loss of the region between nucleotide positions −138 and −83, and a substantial decrease in transcription was observed when the adjacent 27-bp region was deleted (lanes 5−8). Transcription from multiple initiation sites was similarly affected by the deletions, suggesting the presence of a common regulation. These results indicate that the regions between nucleotide positions −138 and −83, and −82 and −56 contain *cis* elements stimulating *Pgk-2* transcription. The contribution of the *cis* element located more proximally to the promoter seemed to be much greater than that of the other. These deletions did not appear to affect *Pgk-2* promoter activity in liver extracts, and a DNA containing only a 55-bp upstream region still showed strong template activity (lanes 9−12). This suggests that the *cis* elements are not functional to affect *Pgk-2* promoter in liver extracts. However, it is conceivable that the basal promoter of *Pgk-2*, which is highly active in liver extracts, obscured the effect of the upstream *cis* elements.

The proximal *cis*-acting region contained the sequence 5′-AAAGGAAAT-3′ (positions between −70 and −78) on the

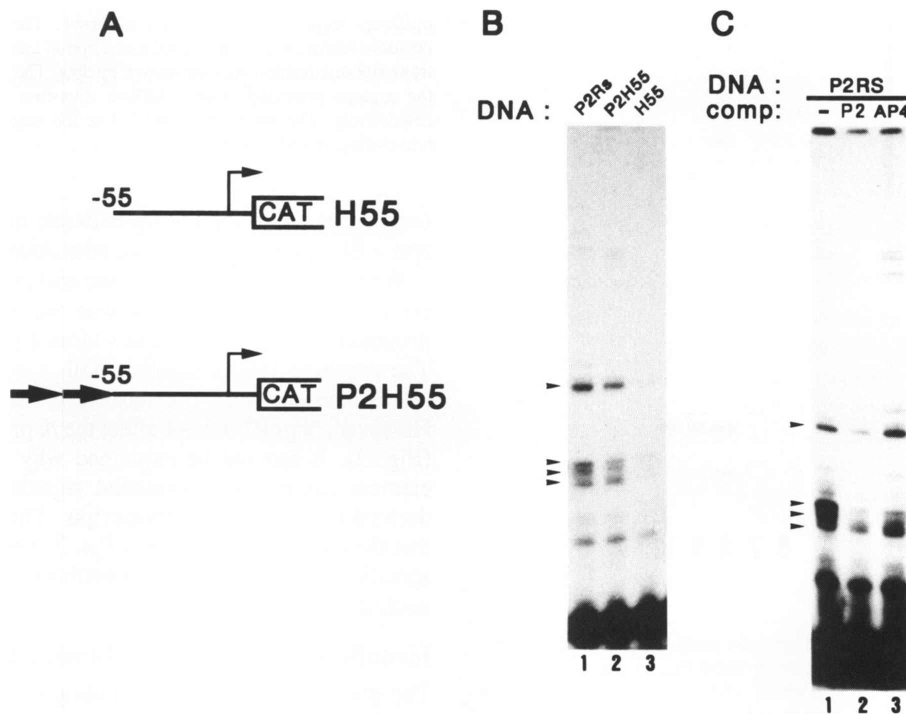

Figure 2. Characterization of the proximal *cis* element. **(A)** The structures of template DNAs are shown schematically. The thick arrow indicates the region between nucleotide positions −82 and −64 of *Pgk-2*. **(B)** The DNAs shown in panel A were transcribed in a cell-free system using testis extracts. Arrowheads indicate the positions of primer extended products derived from accurate *Pgk-2* transcripts. **(C)** Oligo DNAs were added as competitors in the transcription reaction using testis extracts at a 50-fold excess to the template P2Rs DNA.

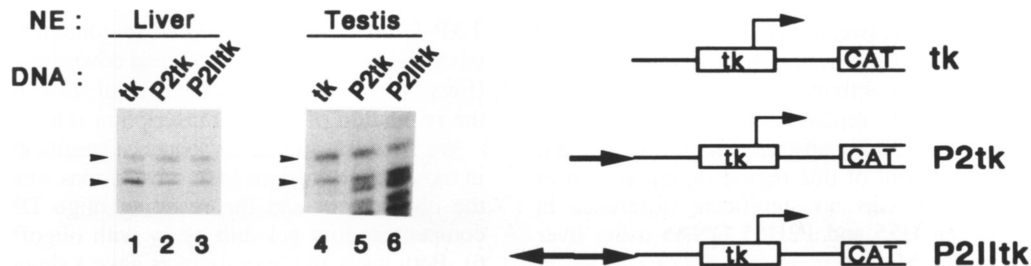

Figure 3. Effect of the *Pgk-2 cis* element on the thymidine kinase promoter. OligoP2 was inserted next to the *tk* promoter of pBLCAT2 (8) as shown at the right. The resulting DNAs (shown at the right) were transcribed in testis and liver extracts, and RNA transcripts were analyzed by primer extension as described for *Pgk-2* transcripts. The extracts (NE) and DNAs used are shown at the top of panels. The arrowheads indicate the positions of the extended products derived from accurate transcripts.

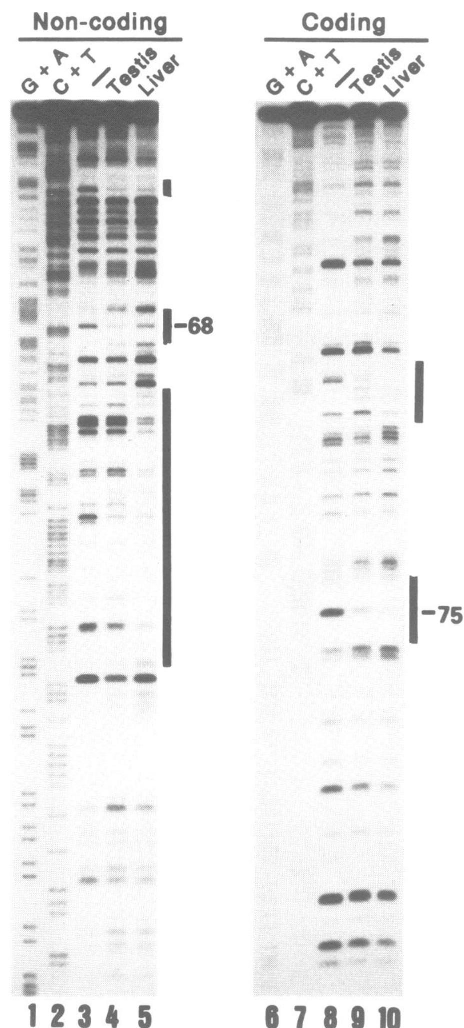

Figure 4. Identification of a factor(s) binding to the positive *cis* element. A DNA fragment corresponding to the region between nucleotide positions −138 and +61 of *Pgk-2* was labeled with ^{32}P at position +61 (for the non-coding strand probe) or position −138 (for the coding strand probe), and used as the probe in a DNase I footprint assay. Nuclear extracts are shown at the top of the panels. − means the reaction with no added protein. Lanes marked by G+A and C+T contained Maxam-Gilbert sequence ladders (20) of the probe as markers. The positions of footprints are shown by bars.

-138 ACCAGGCAAAAAACGTTCCAAATCA

-113 AGATAGACAGGATGGAGAACCAATC

-88 ACAGAGCTGGATTTCCTTTCAAATT

-63 CTACCAATGGCTATTGTGCAGGAGA

-38 CTTTGAACTCACAAAGAAAGGCGGG

-13 GCCAAGACTTAAGCGTTAAAAATCA (+1)

+13 CCACCAAGCCAGCCTCCCAGCAGCA

Figure 5. Summary of DNase I footprinting. The nucleotide sequence of the upstream region of mouse *Pgk-2* is shown. The numbers indicate nucleotide positions relative to the most distal transcription initiation site at +1. The proximal transcription initiation sites are shown by dots. The solid and broken lines indicate the regions protected from a DNase digestion by testis and liver extracts, respectively. The lines above and below the sequence refer to the coding and non-coding strands, respectively.

non-coding strand, which is similar to the binding site for murine Ets-1 (15). We thus synthesized a 19-bp DNA (oligoP2) corresponding to the region between nucleotide positions −82 and −64 and placed it as a direct repeat at nucleotide position −55 (Fig. 2A). The template activity of a DNA (P2H55) was increased by the insertion of the repeat to the same extent as that of P2Rs DNA having the 138-bp upstream region (Fig. 2B), demonstrating the involvement of this region in stimulation of *Pgk-2* transcription. There was no significant difference in template activity between H55 and P2H55 DNAs using liver extracts (data not shown). Moreover, excess oligoP2 added to the transcription reaction using P2Rs DNA caused significant inhibition, whereas a control oligoAP4 showed only a marginal effect on transcription from one of the initiation sites (Fig. 2C). These results suggest that the binding of a factor(s) present in testis extracts, to the region between nucleotide positions −82 and −64, is responsible for the stimulation of *Pgk-2* transcription.

We further examined the tissue and promoter specificity of the *cis* element. OligoP2 DNA was placed just upstream of the promoter of the herpes simplex virus thymidine kinase gene (*tk*). The resulting DNAs together with a control DNA without an oligoP2 insert were transcribed in testis and liver extracts. However, oligoP2 did not affect the *tk* promoter in either extracts (Fig. 3). It can not be explained why the presence of the *cis* element caused extra extended signals that were presumably derived from inaccurate transcripts. These results demonstrated that the *cis* element stimulates *Pgk-2* transcription in a promoter-specific manner, but tissue specificity of the element remained unclear.

Identification of a factor(s) binding to the *cis* element

The presence of a factor(s) binding to the positive *cis* element for *Pgk-2* transcription was examined by a DNase I footprint assay (Fig. 4). The regions around nucleotide positions −68 on the non-coding and −75 on the coding strand were protected from DNase digestion by a factor(s) present in either testis (lanes 4 and 9) or liver (lanes 5 and 10) extracts. This indicates that factors present in the two extracts recognize the same sequence within the *cis* element (Fig. 5). We designated this factor TAP-1 to denote transcription activator 1 for *Pgk-2*. In addition to TAP-1, both extracts appeared to contain binding activities to other regions located upstream and downstream of the *cis* element (Figs. 4 and 5). The involvement of these binding activities in the regulation of *Pgk-2* transcription remains to be elucidated.

We next determined the sequence specificity of TAP-1 binding in more detail. Various base substitutions were introduced within the *cis* element and the resulting oligo DNAs were used as competitors in a gel-shift assay with oligoP2 as a probe (Fig. 6). Both testis and liver extracts gave a single shift-band, which was specifically competed out in the presence of excess unlabeled probe DNA (lanes 2 and 3). Among six oligo DNA competitors containing base substitutions, three (M2, M3, and M4) lost the ability to compete with the probe for binding TAP-1 (lanes 5−7).

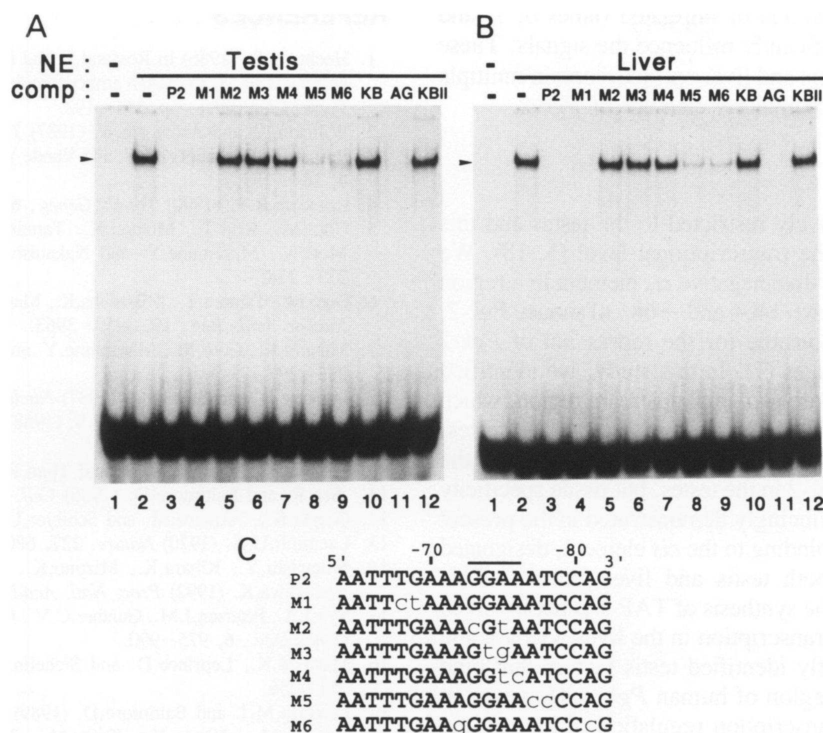

Figure 6. Sequence specificity of TAP-1 binding. Various competitor oligo DNAs containing base substitutions within the TAP-1-binding site were added (at 100-fold excess to the oligoP2 probe) in a gel-shift assay using testis **(A)** and liver **(B)** extracts. The arrowhead shows the position of a specific shift-band. The nucleotide sequences of oligo DNAs are shown on the panel **(C)**. Lower-case letters denote altered bases, and the Ets-binding motif is indicated by a bar.

All three contained base changes within the sequence 5′-GG-AA-3′ on the non-coding strand (Fig. 6C), which is a core sequence of the Ets-binding motif (16). We previously identified a factor named TIN-1 that inhibits transcription of the somatic-type *Pgk-1* (6). TIN-1 binds to the sequence 5′-AGGAAGTT-CC-3′ located between nucleotide positions −268 and −259 of *Pgk-1*. This sequence contains an inverted repeat of the Ets-binding motif 5′-GGAA-3′. We thus used oligoAG, containing the TIN-1-binding sequence, as a competitor in a gel-shift assay. The addition of oligoAG completely abolished the binding of TAP-1 to the oligoP2 probe (lane 11), and oligoP2 competed with the oligoAG probe for binding TIN-1 (data not shown). These results suggest that both TIN-1 and TAP-1 recognize similar sequences that include the Ets-binding motif. On the other hand, the sequence 5′-TGGATTTCC-3′ on the coding strand of *Pgk-2* (between positions −81 and −73) somewhat resembles the binding site for NF-κB (17). We thus used oligoKB and oligoKBII, both of which contained the NF-κB-binding sites as competitors, but neither of them affected the binding of TAP-1 to the oligoP2 probe (lanes 10 and 12). Exactly the same results were obtained using either testis or liver extracts, showing that the DNA-binding activities in the two extracts have quite similar sequence specificity.

Detection of TAP-1 by UV-crosslinking

We directly identified TAP-1 by UV-crosslinking using ^{32}P-labeled oligoP2 as the probe (Fig. 7). Consistent with the results shown in Fig. 6, both testis and liver extracts gave essentially the same signal profile. Multiple signals appeared in the range

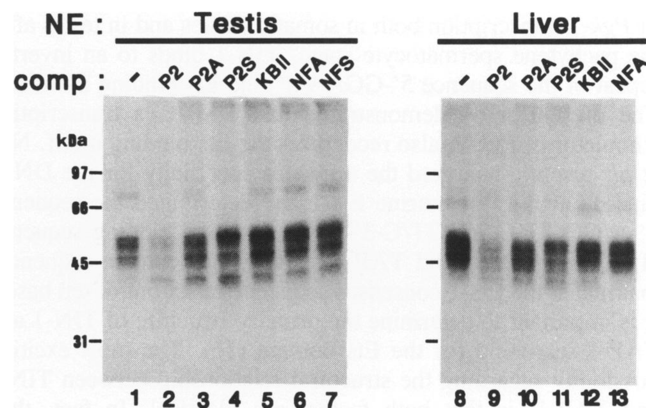

Figure 7. Detection of TAP-1 by UV-crosslinking. A UV-crosslinking assay was conducted using ^{32}P-labeled oligoP2 and nuclear extracts (NE) of testes and liver. Unlabeled double- and single-stranded oligo DNAs were added to the binding reaction as competitors (comp) at 100-fold excess to the probe as indicated at the top of the panel. OligoP2S and oligoP2A are single-stranded DNAs that contain coding and non-coding strands of oligoP2, respectively. OligoNFS and oligoNFA have sequences corresponding to the coding and the non-coding strands of the *Pgk-1* upstream region between nucleotide positions −121 and −153, respectively. The positions of molecular mass markers are indicated at the left.

of 40 to 70 kDa, most of which were competed out when excess unlabeled probe was added to the binding reaction (lanes 1, 2, 8, and 9) with no change in the presence of oligoKBII (lanes 5 and 12). The addition of single-stranded DNAs containing

oligoP2 (lanes 3, 4, 10, and 11) or unrelated (lanes 6, 7, and 13) sequences did not significantly influence the signals. These results suggest that both testis and liver extracts contain multiple factors that bind to the proximal *cis* element of *Pgk-2*.

DISCUSSION

Pgk-2 expression is exclusively restricted to the testes and this seems to be controlled at the transcriptional level (5, 18). We recently identified a silencer-like negative *cis* element in a region between nucleotide positions −1404 and −685 of mouse *Pgk-2*, which appeared to be responsible for the repression of *Pgk-2* transcription in somatic tissues (7). In this study, we identified a positive *cis* element in a more proximal upstream region, which stimulates *Pgk-2* transcription in nuclear extracts of rat testes. It is likely that the proximal *cis* element participates in the augmented expression of *Pgk-2* in the testes, but tissue specificity of the element was not convincingly demonstrated in the present study. A similar protein(s) binding to the *cis* element, designated TAP-1, was detected in both testis and liver extracts. This suggests that regulation of the synthesis of TAP-1 is not involved in the repression of *Pgk-2* transcription in the liver. Gebara and McCarrey (19) have recently identified testis factors that bind to the proximal upstream region of human *Pgk-2*. However, it is not clear whether the transcription regulation of human and mouse *Pgk-2* involves a similar factor(s).

Our previous study (5) suggested that the transcription switch from *Pgk-1* to *Pgk-2*, that is, the cessation of *Pgk-1* transcription and the induction of *Pgk-2* transcription, occurs at the pachytene spermatocyte stage. We identified a factor named TIN-1, which represses *Pgk-1* transcription by binding to the 5′-upstream region (6). We anticipate that TIN-1 is responsible for the inactivation of *Pgk-1* transcription both in somatic tissues and in testes after the pachytene spermatocyte stage. TIN-1 binds to an inverted repeat of the sequence 5′-GGAA-3′, the Ets-binding motif (6). The present study demonstrated that TAP-1, a transcription stimulator of *Pgk-2*, also recognizes the Ets-binding motif. Nye *et al.* recently analyzed the sequence specificity for the DNA-binding activity of murine Ets-1 and determined the sequence 5′-A/GCCGGAA/TGT/C-3′ as a consensus binding sequence (15). Both TIN-1- and TAP-1-binding sites contain sequences that match the Ets-1 consensus sequence in six out of ten bases. It is important to determine the primary structure of TIN-1 and TAP-1 searching for the Ets domain (16). The most exciting possibility regarding the structural relationship between TIN-1 and TAP-1, is that both factors are identical. In fact, they appeared to have similar molecular masses as determined by UV-crosslinking (Ref. 6 and Fig. 7 in this study). If this is so, transcription of the two *Pgk* genes is coordinately regulated by a single factor during spermatogenesis. It will be of interest to determine how a single protein functions in inhibiting and activating *Pgk-1* and *Pgk-2* transcription, respectively. In order to reach any conclusions, molecular probes for TIN-1 and TAP-1 are required.

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