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Transcription of the testis-specific mouse protamine 2 gene in a homologous *in vitro* transcription system

(nuclear extract/temperature optimum/positive and negative promoter elements)

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ABSTRACT **Transcriptionally active nuclear extracts were prepared from mouse testes to study the transcription of the testis-specific mouse protamine 2 (*Prm-2*) gene *in vitro*. The testicular system is unique among mammalian *in vitro* transcription systems in regard to its temperature optimum. In extracts made from prepuberal testes, the temperature optimum for *in vitro* transcription of *Prm-2* is 30°C, similar to somatic *in vitro* systems. However, in adult testis extracts, the optimum temperature for *Prm-2* transcription is 20°C. The different temperature optima seen *in vitro* for prepuberal and adult testes extracts parallels *in vivo* physiological temperature sensitivities of the differentiating male germ cells. The testis system also differs from other *in vitro* transcription systems in its divalent metal cation and ionic strength requirements for optimal transcription. The mouse *Prm-2* gene is maximally transcribed at a $MgCl_2$ concentration of 3–5 mM and over a KCl concentration range of 40–100 mM. By using the testis *in vitro* transcription system to study the *Prm-2* gene by deletion analysis, we have determined that positive promotion for the gene lies within the region −170 to −82 from the start of transcription. This region contains a putative Sp-1 binding site. Additional upstream sequences appear to repress *Prm-2* transcription in a heterologous transcription system.**

The process of spermatogenesis represents a unique cellular differentiation pathway. Starting at the stem cell stage, spermatogenesis progresses through a series of defined cell types. The male germ cells undergo mitosis, meiosis, and morphological restructuring of the terminal cell stages to produce mature spermatozoa (1). During this process, many testis-specific genes and testis-specific variants of somatic genes are differentially expressed (2–6). The changes in stage-specific gene expression involving somatic and testis-specific forms of genes make spermatogenesis an exceptionally desirable system in which to define transcriptional control mechanisms governing the activation or inactivation of temporally expressed testicular genes.

The study of gene expression in male germ cells has been hampered by the lack of a permanent spermatogenic germ cell line and the short life span (3–4 days) of primary cells in culture, making a testis-derived *in vitro* transcription system an attractive alternative. *In vitro* transcription systems produced from protein extracts of isolated nuclei are useful tools to study the events involved in the specific initiation of transcription and to identify both cell type and tissue-specific factors involved in promoter recognition (7–13).

In a dissociated population of cells from the adult testis, ≈80% of the nucleated cells are postmeiotic germ cells (14). In the dissociated cells obtained from isolated seminiferous tubules, the postmeiotic fraction of germ cells can be increased to ≈90% (14, 15). This makes the adult mouse testis a practical source of cells for producing an *in vitro* transcription system appropriate for studying the transcription of postmeiotically expressed genes such as the protamines (2). The protamines are small highly basic proteins that elicit compaction of the DNA in the nucleus of the spermatozoon (reviewed in refs. 16 and 17).

Analyses of the trout protamine promoter by *in vitro* transcription in a HeLa cell system have defined the TATA box as a required element for transcription (18). Additional studies, by using a hybrid HSV-TK trout protamine promoter transfected into Cos-1 cells, have suggested that GC boxes downstream from the TATA box play a negative regulatory role in heterologous cells (19). From these studies, Dixon and colleagues have proposed a similar negative regulatory role for these trout protamine sequences prior to their expression in spermatocytes (19). The conservation of a number of upstream sequence elements within mammalian protamine genes suggests that additional upstream sequences are involved in the regulation of protamine gene expression (20, 21). Evidence for this has been suggested by transgenic mouse studies. For mouse protamines 1 and 2 (*Prm-1* and *Prm-2*), it has been shown that all the information needed for tissue- and cell-type-specific transcription resides within a 465- and an 859-base-pair (bp) sequence, respectively (22–24). These regions share all but two conserved sequences present in the promoters of *Prm-1* and *Prm-2* (21).

To examine the regulation of testis gene expression, and specifically the temporal regulation of postmeiotic genes, we have developed a highly active *in vitro* transcription system from testis nuclear extracts. In this report, we present a characterization of the system and its ability to transcribe the *Prm-2* gene.

MATERIALS AND METHODS

Animals. All mice used were CD-1 strain (16 or >60 days old) from Charles River Breeding Laboratories.

Templates Used/Transcript Sizes. Runoff transcripts of different sizes were produced by restriction enzyme digestions of the following clones: mouse *Prm-2*, *Pvu* I (470 bp), *Bam*HI (285 bp), or *Fok* I (415 bp); γ-fibrinogen (γ-Fib), *Nco* I (597 bp); P-Glemp (an adenovirus 2 major late promoter subclone), *Nar* I (481 bp); PmP2-64 (a *Hae* III subclone of *Prm-2*), *Hae* II (444 bp), *Hin*fI (401 bp); PmP2-64c is PmP2-64 plus the CAAT sequence absent in PmP2-64 (see Fig. 5).

Preparation of Testis Extract. This procedure is based on an adaptation of several previously published procedures (11, 25, 26). The testes from 50 adult or 200 16-day CD-1 mice were isolated, decapsulated, finely minced, and placed in a Potter homogenizer containing 40 ml of homogenization buffer 1 (11). Nuclei were prepared according to Schibler (11) except that Hepes was used instead of Tris·HCl. The pelleted nuclei were rinsed two times with nuclei buffer [10 mM Hepes, pH 7.9/10%

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(vol/vol) glycerol/1.5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride] and then resuspended in nuclei buffer (1.5–2 ml for the nuclear pellet from 50 adult animals). KCl was added to a final concentration of 0.3 M, the sample was rocked gently for 1 hr, and then centrifuged at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was removed and dialyzed for 3 hr against dialysis buffer [20 mM Hepes, pH 7.9/20% (vol/vol) glycerol/100 mM KCl/0.2 mM EDTA/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride]. After dialysis, the extract was clarified by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 10 min, aliquoted, and stored at −70°C. Prepared in this manner, the extracts remain active for at least 8 months. We routinely obtain protein concentrations of 16–18 mg/ml as determined by optical absorbance (27).

HeLa Cell Extract. HeLa cell extract was prepared by the method of Dignam *et al.* (25).

Transcription Reactions. All transcription reactions were performed and analyzed as detailed (25). HeLa cell reactions were carried out at 30°C, and testis reactions were done at 20°C unless otherwise noted.

RESULTS

Protamine Transcription. In general, the testis-derived nuclear extract behaves as do other *in vitro* systems in that it initiates transcription correctly and produces runoff transcripts of the expected size (8, 11, 13, 25). Fig. 1 shows representative runoff transcripts produced from the *Prm-2* promoter, which was digested at various downstream sites. By using a *Hae* III digest of ϕX174 as positional markers in the gels (lane 5), the sizes of the runoff RNAs were found to be consistent with the expected initiation site as determined from sequence data (21). S1 nuclease analysis of transcripts establishes that the system initiates at the predicted start site of the promoter (data not shown). Inhibition of transcription by α-amanitin (1 μg/ml) confirms that the transcripts are generated by RNA polymerase II (lane 4). Typical of *in vitro* systems, the extract has a total DNA dependence with optimal transcription at template concentrations between 0.8 and 1.2 μg per 20-μl reaction mixture. Optimal transcription is achieved at 3–5 mM $MgCl_2$ and 40–100 mM KCl (data not shown). We have standardized our assay conditions at 4 mM $MgCl_2$ and 60 mM KCl.

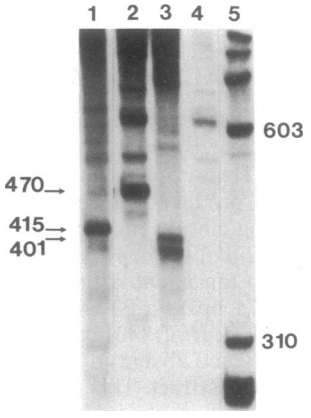

FIG. 1. Runoff transcripts from the *in vitro* transcription of the *Prm-2* gene in a testicular nuclear extract. Lanes: 1 and 2, 415-nucleotide (*Fok* I) and 470-nucleotide (*Pvu* I) RNA runoff transcripts; 3, 401-nucleotide PmP-64 (*Hinf*I) transcript (upper band of doublet); 4, α-amanitin (1 μg/ml) control for lane 2; 5, *Hae* III-digested ϕX174 ^{32}P-end-labeled DNA marker. Arrows indicate location of the transcript bands. Additional bands are due to nonspecific kinase and polymerase activities in the extract.

Temperature Requirement. It is well known that the mammalian testis operates at a lower temperature than core body temperature (28). To determine whether this physiological temperature difference is maintained in our *in vitro* transcription system, we assayed transcription extracts derived from adult mouse testes, 16-day-old mouse testes (an age at which spermatogenesis has advanced to meiosis), and a heterologous control system—HeLa cells—at several different temperatures. Fig. 2 shows the results of transcribing the *Prm-2* gene in each system at temperatures from 15°C to 30°C. In the adult extract (Fig. 2*A*, *; Fig. 2*B*, lanes 1–5), peak transcription levels are achieved at 20°C with little transcription occurring at 15°C or 30°C. Typical of other mammalian somatic cell-derived transcription systems, the HeLa cell extract (Fig. 2*A*, +; Fig. 2*B*, lanes 6–10) transcribes best at 30°C. The 30°C temperature optimum of the HeLa cell extract is well documented, with transcription falling off sharply at higher temperatures (25). Interestingly, the temperature curve of the 16-day mouse testis extract (Fig. 2*A*, ■; Fig. 2*C*, lanes 1–5) is more similar to the HeLa cell curve than to that of the adult testis. It shows an optimum at 30°C with decreased amounts of transcript produced at lower temperatures.

The adult temperature pattern is also seen when extracts are prepared from isolated seminiferous tubules, thus making it highly unlikely that interstitial cells are the source of the 20°C temperature optimum. While adult seminiferous tubules consist mainly of spermatocytes, spermatids, and Sertoli

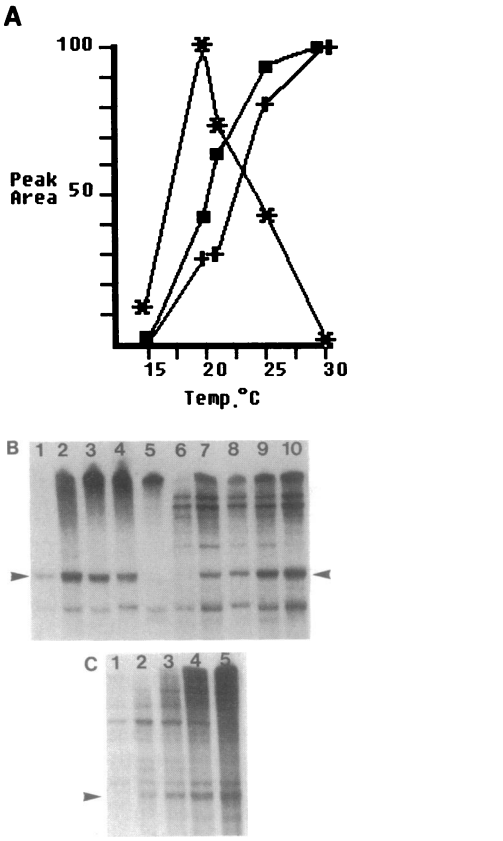

FIG. 2. Temperature optima for *in vitro* transcription in extracts of adult mouse testis, HeLa cells, and prepuberal testis (16 day old). (*A*) The combined densitometry data from the transcriptions of *B* and *C*. Each set of temperature points [adult testis (*), HeLa (+), and 16 days old (■)] was normalized to the highest data point set at 100%. (*B*) *Prm-2 Pvu* I runoff transcripts for adult testis (lanes 1–5) and HeLa cell extracts (lanes 6–10) from incubations at 15, 20, 23, 25, and 30°C, respectively. (*C*) *Prm-2 Pvu* I runoff transcripts using a prepuberal testis extract (lanes 1–5). Arrowheads bracket the RNA transcripts.

cells, the spermatocytes and Sertoli cells only make up a minor percentage of cells present and the Sertoli cells function well at 37°C (28). The lower temperature requirement seen here most likely lies with the spermatids, which are present in adult testis but absent in 16-day testis, and could be the basis of the observed physiological decline of spermatogenesis at elevated temperatures.

Template Preference for Testicular Genes. To determine whether transcription of testis-expressed genes was favored over heterologous genes in the testis extract, we compared template activities in homologous and heterologous transcription systems. Fig. 3*A* shows a comparison of the *in vitro* transcription of the liver-specific gene, γ-fibrinogen, and *Prm-2*. Lanes 1 and 2 show the transcription of the individual genes (γ-fibrinogen and *Prm-2*, respectively) in the HeLa extract. Lane 3 shows the transcription resulting from the combined mixture of the two genes at the same molar ratio as in lanes 1 and 2 but at half the final DNA concentration for each individual template. At this ratio of templates, the fibrinogen promoter is preferentially transcribed in the HeLa extract. However, when the same template mixture is transcribed in the testis extract, the fibrinogen signal is now the weaker of the two (lane 4). This is also true when *Prm-2* and fibrinogen are individually transcribed in the testis extract (lanes 5 and 6, respectively). Fig. 3 *B* and *C* shows bar graph representations of the ratio of transcription levels between the two genes in HeLa (H) and testis (T) extracts, respectively. The ratio of γ-fibrinogen to *Prm-2* is indicated by the hatched boxes and the ratio of *Prm-2* to γ-fibrinogen is shown by the stippled boxes (Fig. 3 *B* and *C*). The above results indicate that the fibrinogen template transcribes better in the HeLa cell extract, whereas *Prm-2* transcribes better in the testis extract. This switch in promoter preference between extracts is also seen when *Prm-2* is compared to the human fetal ε-globin gene (data not shown).

Deletion Analysis of the *Prm-2* Gene. To determine whether the promoter of *Prm-2* contains positive regulatory elements selectively active in the testis extract, we performed a deletion analysis of the promoter. We chose restriction sites that removed known upstream homology elements that exist in mouse *Prm-1* and *-2* genes (21). Fig. 4*A* shows a diagram of the deletion set. A comparison of transcript levels generated by the deletion curve in a testis extract and HeLa extract is shown in Fig. 4*B*. The upper band (upper arrow) is an

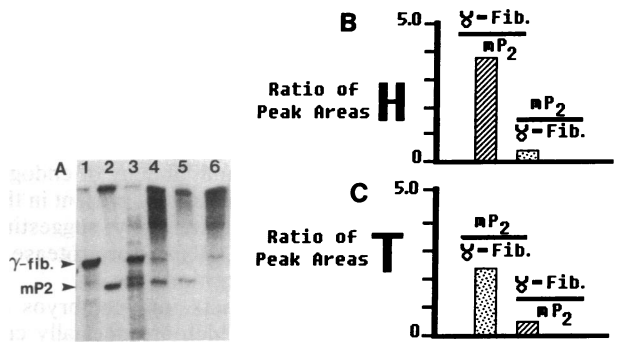

FIG. 3. Comparison of *in vitro* transcription of γ-fibrinogen (γ-Fib) and *Prm-2* (mP2) templates in adult testis and HeLa cell nuclear extracts. (*A*) Transcription of γ-fibrinogen and *Prm-2* in the HeLa cell extract with 0.8 μg of template per reaction (lanes 1 and 2). Transcripts in HeLa cell (lane 3) or adult testis extracts (lane 4) when each template is present in the same transcription reaction at 0.4 μg per reaction. *Prm-2* (lane 5) and γ-fibrinogen (lane 6) template transcription in the adult testis extract with 0.8 μg of template per reaction. (*B* and *C*) Graphs of the densitometry data for the two runoff signals when transcribed together in either the HeLa cell (H) or testis (T) transcription systems. Hatched boxes are the ratio of γ-fibrinogen to *Prm-2*; stippled boxes are the ratios of *Prm-2* to γ-fibrinogen.

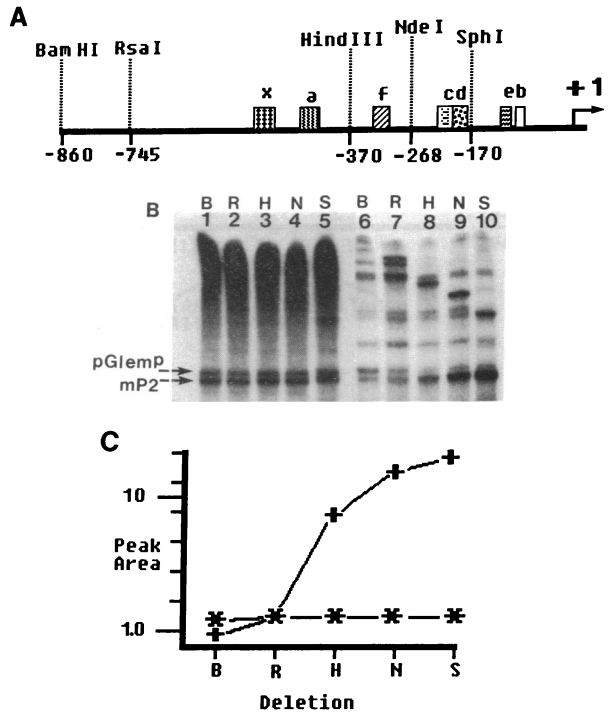

FIG. 4. Deletion analysis of the *Prm-2* (mP2) promoter assayed in adult testis and HeLa cell nuclear extracts. (*A*) Diagram showing locations of the restriction sites used to make the promoter deletions, the positions of the conserved sequence elements of *Prm-1* and *Prm-2*, and the start of transcription (+1). (*B*) Transcription assays using the deletion set. Lanes 1–5, transcriptions in the testis extract. Lanes 6–10, equivalent transcriptions in the HeLa cell extract. Templates generated by the restriction enzymes are denoted as follows: B, *Bam*HI; R, *Rsa* I; H, *Hin*dIII; N, *Nde* I; S, *Sph* I. (*C*) Graph of the densitometry data for each transcription set. The ratio of the deletion template band to the control adenovirus 2 template band is plotted versus the deletion templates.

adenovirus 2 internal control runoff transcript (PGlemp) present in a constant amount in all transcription reactions. The lower band (lower arrow) is the 470-base *Prm-2* runoff transcript. No substantial increase or decrease in transcription level was seen with each template in the testis extract (Fig. 4*B*, lanes 1–5; Fig. 4*C*, *). The shortest template (the *Sph* I restriction digest) retains the conserved e/b element, CAAT box, and TATA box. In the testis *in vitro* system, this template is sufficient for maximal transcription (Fig. 4*B*, lane 5; Fig. 4*C*). In contrast, increased transcription was seen in the HeLa extract as the templates were shortened (Fig. 4*B*, lanes 6–10; Fig. 4*C*, +). The highest levels of synthesis were obtained when the f and c/d element fragments were removed. These results suggest that the upstream sequences are not essential for *in vitro* transcription in either extract and they are acting as negative regulatory elements in the heterologous extract. Since both extracts received an equal amount of the same promoter mixture, the differences are not likely due to disparities in promoter concentrations. This suggests a negative regulatory role for upstream sequences and a mechanism that in part involves release of protamine gene repression in the testis but not in somatic cells.

Deletion of the e/b Element Fragment. To evaluate the role of the promoter region containing the conserved e/b element of the *Prm-2* gene, a construct deleting this sequence was made. Fig. 5*A* shows the *Prm-2* sequence from −170 to +147, the e/b element, CAAT box (c), TATA box (t), and the *Hae* III restriction sites used to produce a subclone lacking the sequences upstream of −64 (designated PmP2-64). To distinguish between the effects on transcription of the CAAT

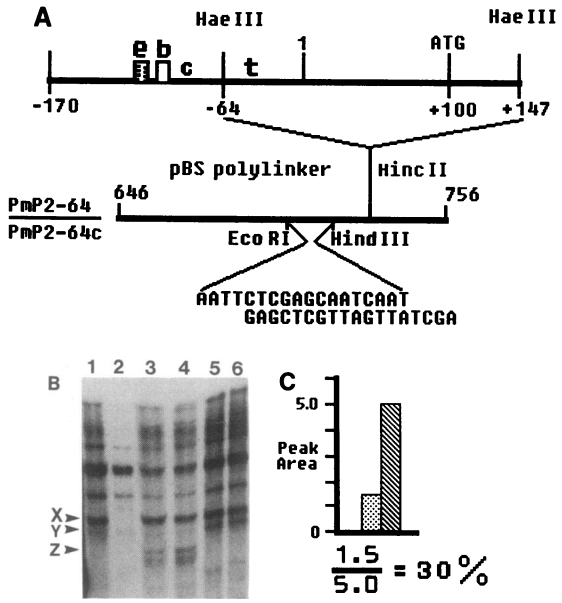

FIG. 5. Transcriptional analysis of a *Prm-2* template lacking the e/b element. (*A*) Diagram of the *Prm-2* promoter from −170 to +147 and the *Hae* III restriction sites used to subclone a promoter lacking the e/b region into the *Hinc*II site of P-Bluescript (PmP2-64). PmP2-64c is the same as PmP2-64 except it contains the *Prm-2* CAAT sequence. (*B*) Competition assays between the *Pvu* I digest of the *Prm-2 Sph* I deletion template (upper arrow X) and the subclones PmP2-64 and PmP2-64c (lower arrows Y and Z) in the adult testis extract. Lanes: 1, *Pvu* I RNA transcript of the *Prm-2 Sph* I template (X); 2, α-amanitin control (1 μg/ml) for lane 1; 3 and 4, *Hin*fI RNA transcripts (Z); 5 and 6, *Hae* II RNA transcripts (Y) of the subclones PmP2-64 and PmP2-64c, respectively. (*C*) Bar graph of the densitometry data for the transcripts from the e/b-less templates (stippled bar) versus the *Prm-2 Pvu* I transcript (hatched bar).

box and the sequences 5′ to the CAAT box, we have reinserted a CAAT box into PmP2-64. An oligonucleotide containing the mouse *Prm-2* CAAT box (CAATCAAT) was cloned into the *Eco*RI/*Hin*dIII polylinker site in PmP2-64 (new clone designated PmP2-64c). Fig. 5*B* compares the two different sized runoff transcripts (*Hin*fI, lanes 1 and 2; *Hae* II, lanes 3 and 4) for both PmP2-64 and PmP2-64c to the control *Prm-2 Pvu* I transcript. Both deletions transcribe more poorly than template containing the e/b element and sequences upstream. It should be noted that in PmP2-64c the flanking sequences in the oligonucleotides and polylinker do not constitute the exact original environment for this CAAT box. Whether these differences would aberrantly affect the functioning of this CAAT sequence in the *in vitro* system is unknown. However, the CAAT sequence does not improve transcription over a template lacking it (PmP2-64). Fig. 5*C* shows a graph of the densitometry data comparing the transcript level of the *Prm-2 Pvu* I transcript (hatched box) to the PmP2-64 promoter runoffs (stippled box). Densitometry data were corrected for differences in labeled nucleotide content between the longer *Pvu* I runoff and the shorter *Hin*fI (+23%) and *Hae* II (+17%) transcripts. The PmP2-64 templates transcribed only 30% as well as the template containing the sequences between −170 to −82, suggesting that the fragment from −170 to −82 contains a positive promoter element that increases transcription from the *Prm-2* promoter *in vitro*. Similar results were seen with the HeLa cell extract (data not shown).

DISCUSSION

To begin to understand the mechanisms and factors regulating gene expression during spermatogenesis, we have developed a testis-derived *in vitro* transcription system. Adult mouse testis extracts have a sharp $MgCl_2$ requirement, a broad KCl optimum, and a uniquely low temperature optimum (20°C). In contrast, a prepuberal testis extract transcribes maximally at 30°C, a temperature optimum characteristic of extracts derived from somatic cells. Such differences most likely reflect the different cellular composition of the prepuberal and sexually mature testes. Initial promoter analysis of the testis-specific *Prm-2* gene in homologous and heterologous systems reveals a small region of positive influence active in both systems and a large region of negative influence, active only in the heterologous system.

Although the extract from the testes of sexually mature mice transcribes exogenous DNA templates similarly to other systems, its temperature optimum is unique among mammalian extracts and may help explain the molecular basis behind the lower temperature requirements of spermatogenesis. Reproductive physiologists have demonstrated that the most temperature-sensitive period of spermatogenesis occurs during the later stages when the majority of testicular cells are spermatids (28). This is consistent with our *in vitro* transcription analyses where we found that the relatively sharp temperature optimum of 20°C in adult extracts differs from the optimum of 30°C in prepuberal extracts. However, since the prepuberal extract is composed of roughly equal numbers of spermatocytes and Sertoli cells [≈39% (15)], we cannot ascribe the higher temperature requirement specifically to early stage germ cells. Yet, if the spermatocytes, of which ≈69% are pachytene stage cells, possess the lower temperature requirement, we might have expected to see more transcription at 20°C with the prepuberal extract.

The novel testicular temperature optimum reported here is not due to species differences in the transcription apparatus of mouse and human (HeLa cells). Other investigators analyzing *in vitro* transcription in mouse brain extracts, or in rat extracts from liver, brain, and spleen, utilize 30°C to transcribe a variety of genes (11, 29). More importantly, we find a 30°C optimum in extracts prepared from the testes of 16-day-old mice (Fig. 2). Recent studies with extracts from adult rat testes also show the lower 20°C optimum for transcription (data not shown). Furthermore, the optimal temperature for transcription in the testis extracts is independent of the promoter. Both adenovirus major late promoter and γ-fibrinogen show the same temperature profile as seen for the *Prm-2* promoter (D.B., unpublished observations).

We do not believe that the decrease in transcription in the adult testis extract at 30°C is an artifact of RNase or proteolytic activities. As the amount of transcription falls with increasing temperature, the background labeling of endogenous DNA and RNA due to the kinase activity present in the cellular extracts does not decrease substantially, suggesting that a temperature-dependent RNase, DNase, or protease is not responsible for decreased transcription.

Other than mouse testicular extracts, only embryos or tissue culture cells of *Drosophila*, which are normally cultured at 25°C, produce active transcription systems that function between 20°C and 25°C (26). It is possible that the temperature sensitivity of the testis system rests in some common aspect of the transcription process that has been adjusted evolutionarily to maintain the process at lower temperatures.

Studies of *in vitro* transcription systems derived from brain, liver, and spleen (29), as well as *Drosophila* embryos (12), have demonstrated the existence of positive regulatory elements controlling transcription strength of promoters in homologous systems. We have been able to use our *in vitro* system to initially identify two putative regulatory regions of *Prm-2*. The sequence from −170 to −82 contains a positive

promotion element, active in both testis and the heterologous HeLa cell systems. This fragment notably contains two elements conserved between the coordinately expressed *Prm-1* and *-2* genes (21). While finer mapping is needed to define the exact region responsible, three points suggest that the e/b elements are responsible. First, the e element in the mouse *Prm-2* gene overlaps a GC box, which is the consensus sequence for binding of the transcription factor Sp1 (30, 31). This factor functions to increase transcription levels of promoters containing GC boxes. The b element contains a GC sequence interrupted by a single adenosine and potentially represents a cryptic SP1 binding site. Second, the rat *Prm-2* promoter transcribes at only 30% the level of the mouse *Prm-2* promoter in either rat or mouse testis extracts (37). This is the same level at which the e/b-less subclones transcribe relative to *Prm-2* (Fig. 5*C*). The rat promoter, which is highly homologous to the mouse promoter, has an altered GC sequence but otherwise normal e/b elements (32). Third, a double-stranded oligonucleotide containing a simian virus 40 GC box effectively competes transcription. These observations do not preclude the possibility that additional (unique?) factors act on the *Prm-2* flanking region −170 to −82 in homologous or heterologous systems.

Although the competition experiment between templates lacking or containing the CAAT box (Fig. 5) does rule out the possibility that positive or negative interactions occur between the CAAT sequence and other upstream sequences, the similar transcription levels with the two templates suggest a minimal role for a CAAT box directly regulating the level of transcription in the testis *in vitro* system. A second potential upstream regulatory region does not seem to influence transcription in adult testis extracts but does act as a repressor in the heterologous HeLa cell system (Fig. 4). As we deleted 5′ flanking sequence from the *Prm-2* promoter, transcription increased dramatically in the HeLa cell system yet remained constant in the adult testis extract. The highest transcription levels were achieved after deletion of the regions −370 to −268 and −268 to −170. These regions contain the protamine conserved elements f and c/d, respectively (Fig. 4) (21). Strong protein binding in gel shift assays to the f/c/d region of *Prm-2* in HeLa cell and 16-day-old mouse testes extracts, compared to weak binding in adult testis extracts, suggest that this region is binding a factor(s) that acts as a repressor(s) (P.A.J., unpublished observations). Our deletion analysis of the *Prm-2* promoter demonstrates a functional *in vitro* assay for promoter repression, adding to the rapidly growing evidence for repressor or silencer elements modulating transcription (9, 33–36).

Our data support and extend initial promoter analysis for the mouse protamine genes, which have been carried out with transgenic animals (22–24). The *in vitro* transcription data we present suggest a regulatory mechanism in the mammalian testis, which is more dependent on release from repression than activation of positive promotion. The most likely sequence(s) directing positive promotion from the *Prm-2* promoter is the e/b element. Its sequence suggests that an SP1-like factor or another factor that recognizes GC box sequences is present in the mouse testis.

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