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Analysis of the mouse protamine 1 promoter in transgenic mice

(transcription/spermatid specific)

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ABSTRACT **Protamines are small arginine-rich proteins that package DNA in spermatozoa. The mouse protamine 1 (*Prm-1*) gene is transcribed exclusively in post-meiotic spermatids. To identify elements in the *Prm-1* promoter required for spermatid-specific transcription, we generated transgenic mice by microinjection of transgenes containing *Prm-1* 5′ flanking sequences with 5′ truncations or internal deletions of conserved sequences linked to a marked *Prm-1* gene. We also tested *Prm-1* promoter regions with a heterologous human growth hormone reporter gene. We conclude that a 113-bp region can direct spermatid-specific transcription and we have defined sequences within this region that are essential for proper function. These results will facilitate the isolation and characterization of transcription factors essential for post-meiotic gene expression.**

Spermatogenesis is the sequence of developmental events by which spermatogonial stem cells give rise to functional spermatozoa. During this developmental process, spermatogonial stem cells proliferate through several mitotic divisions followed by two meiotic divisions resulting in haploid round spermatids. During spermiogenesis, the spermatids undergo extensive cytological and biochemical changes leading to the formation of mature spermatozoa (for review, see ref. 1).

One of the changes that takes place during spermiogenesis is the replacement of histones, first by transition proteins and, subsequently, by the protamines (for review, see ref. 2). Protamines are small arginine-rich proteins that are important for the packaging of sperm DNA into highly compact side-by-side linear arrays (3, 4). The mouse contains two protamine genes, *Prm-1* and *Prm-2* (5), that are first transcribed in round spermatids (6, 7). Both protamine mRNAs are under translational control and can be stored for up to 8 days before being translated (8). Sequences that direct proper Prm-1 translational control reside within the 3′ untranslated region (9).

Comparison of the two mouse protamine gene sequences has revealed little sequence identity in the coding region, but a number of short conserved sequences have been identified in the promoter regions (10). The protamine genes from a number of other species contain some of the same sequences within their promoters (11–14). It has been suggested (10) that these sequences may be important for transcriptional regulation, but no functional analysis has been performed to demonstrate whether any of these sequences function as cis-acting regulatory elements.

The mutational analysis of promoters is often carried out in tissue culture cells that transcribe the gene of interest, but there is no adequate cell culture system in which to study haploid-specific gene expression. For this reason, studies of protamine transcription regulation have relied on transgenic mice and assays *in vitro*. Transgenic approaches have been used to demonstrate that as little as 465 bp of *Prm-1* 5′ flanking sequence is sufficient to confer spermatid-specific expression on a marked *Prm-1* gene (15, 16). Likewise, when 859 bp of *Prm-2* promoter sequence was fused to a c-*myc* gene, transcripts from this construct were detected only in round spermatids (17). Recently, *in vitro* transcription studies have been carried out on both mouse protamine genes (18, 19).

We have used transgenic mice to analyze *Prm-1* regulation because they allow a comprehensive analysis of cell-specific expression and can also be used to study the onset of expression in the context of normal development. As a first step in understanding the regulation of post-meiotic gene transcription, we set out to identify the sequences that regulate spermatid-specific transcription of the mouse *Prm-1* gene.

MATERIALS AND METHODS

Constructs and Transgenic Mice. Constructs were made using standard cloning techniques (20). The marked protamine gene contains a 20-bp oligonucleotide inserted at position +95 to mark the transcript and 237 bp of simian virus 40 sequence to mark the transgene (15). For making internal deletions, the *Prm-1* gene was cloned into M13 and oligonucleotide-directed mutagenesis was performed (21). Restriction fragments containing the constructs were separated from plasmid sequences on agarose gels, transferred to NA45 paper (Schleicher & Schuell), eluted in 1.5 M NaCl, precipitated with EtOH, and resuspended in 10 mM Tris Cl, pH 8.0/1.0 mM EDTA. Transgenic mice were generated and identified as described (22).

RNA Isolation and Analysis. Tissues were removed from transgenic mice and homogenized in guanidinium isothiocyanate, and RNA was precipitated with LiCl (23). For Northern blot analysis, RNA samples were denatured in a formaldehyde/formamide buffer and electrophoresed on 1.5% agarose gels. RNA was then transferred to nitrocellulose and probed with a ^{32}P-end-labeled oligonucleotide specific for the marked *Prm-1* transgene or with random-primed human growth hormone (hGH) or actin probes. Blots probed with oligonucleotides were hybridized and washed as described (15).

Primer-extension analysis was performed as described (15) using a ^{32}P-end-labeled oligonucleotide that hybridizes to the Prm-1 transcript between nt +99 and +119. After electrophoresis on 8% polyacrylamide/8 M urea gels, endogenous and transgene transcript products were excised from the gel and the amount of ^{32}P label incorporated into each band was measured by scintillation counting.

RNA dot hybridizations were carried out as described (24). RNA samples (10 μl) were mixed with 2 μl containing 100 mM

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Abbreviations: hGH, human growth hormone; CRE, cAMP response element; ACE, angiotensin-converting enzyme.

$Na_2B_4O_7$ (pH 8), 400 mM Na_2SO_4, 40 mM CH_3Hg, and 5% (vol/vol) ethanol. RNA was then spotted onto nitrocellulose filters, hybridized, and washed under the same conditions as the Northern blot hybridizations. Filters were exposed to film and radioactivity in spots was measured in a scintillation counter.

RESULTS

Analysis of 5′ Truncations of the Marked *Prm-1* Gene. To study the transcriptional regulation of the *Prm-1* gene, 14 gene constructs were analyzed in transgenic mice (Fig. 1). Constructs 1–5 contain increasing 5′ promoter truncations directing the expression of a marked *Prm-1* gene. The marked *Prm-1* gene contains a 237-bp simian virus 40 fragment insertion to facilitate identification of transgenic animals by dot hybridization and a 20-bp oligonucleotide insertion in the 5′ untranslated region of the first exon at position +95 for identification of transgene transcripts by primer extension (15). These constructs were used to determine the minimal promoter required for testis-specific transcription.

Transgenic mice were made with each of these constructs by microinjection into the male pronucleus of fertilized mouse eggs. To determine whether the constructs retained proper testis-specific regulation of transcription, transgene mRNA was measured in adult testes and a variety of other tissues. The tissues were removed from founder males or the male offspring of female founders at a minimum age of 6 weeks. Expression of all marked *Prm-1* constructs (constructs 1–9) was analyzed by primer extension using an end-labeled oligonucleotide that hybridizes to Prm-1 transcripts 3′ of the 20-bp oligonucleotide insertion and yields a 119-nt extension product from the endogenous mRNA and a 139-nt extension product from the transgene encoded mRNA. These two products are readily separated on a denaturing polyacrylamide gel (15), and transgene expression can be compared to endogenous levels by measuring the amount of label in the two bands.

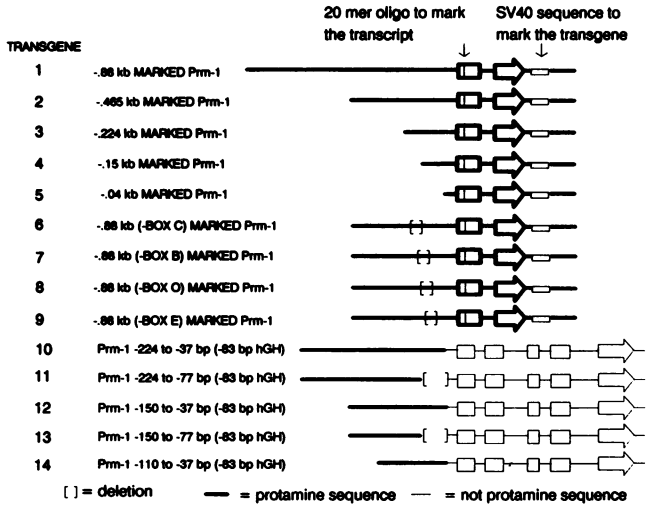

FIG. 1. Transgenes tested. Constructs 1–5 contain various lengths of *Prm-1* 5′ flanking sequence directing the expression of a marked protamine gene. Constructs 1–5 contain 5′ flanking sequence from bp −880, bp −465, bp −224, bp −150, and bp −40, respectively. Constructs 6–9 contain internal deletions of the elements conserved among protamine promoters (boxes C, B, O, and E) in the context of construct 2. Constructs 10–14 contain *Prm-1* sequences (bp −224 to −37, bp −224 to −77, bp −150 to −37, bp −150 to −77, and bp −110 to −37, respectively) directing the expression of the hGH proximal promoter and structural gene at bp −83. Open arrows and boxes represent *Prm-1* and hGH exons.

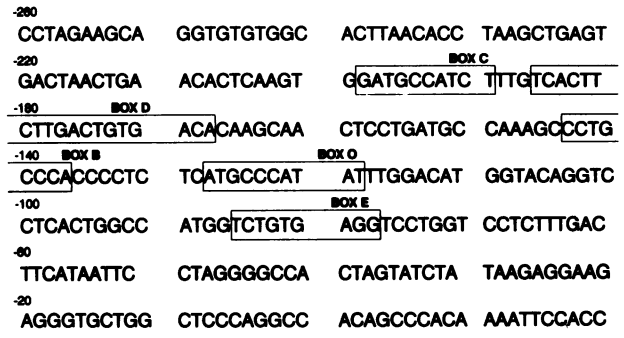

FIG. 2. *Prm-1* promoter sequence and conserved elements. *Prm-1* sequence from bp −260 to +20 is shown with the conserved boxes marked by rectangles and labeled.

Previous studies revealed that truncations of the *Prm-1* promoter to bp −880 and −465 (constructs 1 and 2) result in promoters that retain their ability to confer spermatid-specific regulation on the marked *Prm-1* gene whereas truncation of the *Prm-1* promoter to bp −40 (construct 5), which removes virtually all sequences 5′ of the "TATA box" element, results in a construct that is not expressed in any tissue (16). We tested two further 5′ truncations of the *Prm-1* promoter to bp −224 and −150 (constructs 3 and 4) and both retained testis-specific transcription. The sequence of this functional region of the *Prm-1* promoter is shown in Fig. 2.

Fig. 3 shows a summary of the testis RNA levels in all the animals examined for each of the 5′ promoter truncations. Although all the truncated constructs, with the exception of the bp −40 construct, allow testis-specific transcription, the level of expression is affected by truncation. There is a trend toward decreasing levels of transcript with increasing promoter truncations. To ascertain whether the bp −150 construct (construct 4) retains spermatid specificity in the testis, we carried out a developmental profile of testis expression on a line of mice expressing the transgene. The synchronous nature of the first round of spermatogenesis in the prepubertal mouse is useful for determining in what cell type a transcript first appears (for review, see refs. 1, 25, and 26). The Northern blot in Fig. 4*A* shows that no transgene mRNA was detectable at day 18 after birth whereas mRNA was

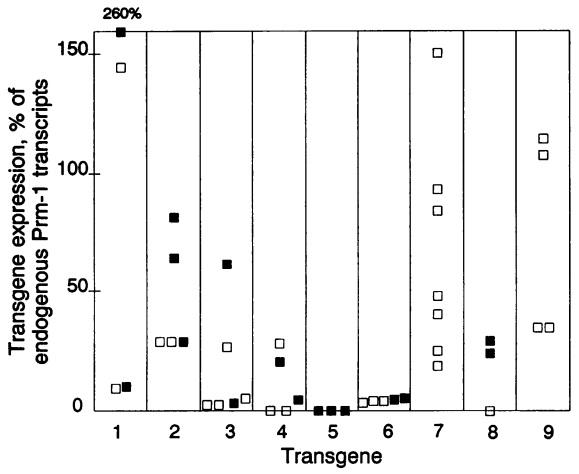

FIG. 3. Summary of marked *Prm-1* transgene expression in testis. This graph summarizes marked Prm-1 transcript levels in the testes of male founders (solid squares) and the male offspring of female founders (open squares). Transcript levels were determined by primer-extension assays and are given as the transgene transcript level as a percentage of endogenous Prm-1 transcript level. Constructs 1–5 retain 0.88, 0.465, 0.224, 0.15, and 0.04 kb of 5′ flank and constructs 6–9 refer to deletions of Boxes C, B, O, and E, respectively.

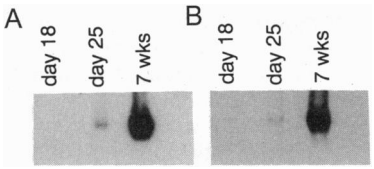

FIG. 4. Developmental Northern blot hybridizations. Northern blots were used to demonstrate the spermatid-specific expression of constructs 4 (*A*) and 12 (*B*) during prepubertal development. Equivalent amounts of total testis RNA (20 μg) were loaded into each lane and the blot was probed with an oligonucleotide probe specific for the marked Prm-1 transcript (*A*) or a hGH probe (*B*). Testis RNA samples were isolated at 7 weeks, day 25, and day 18. The blots were rehybridized with an actin probe to demonstrate the RNA integrity (data not shown).

clearly present at day 25. This pattern of expression is consistent with spermatid-specific transcription. These results suggest that although sequences between bp −150 and −40 are sufficient for spermatid-specific expression, sequences 5′ of bp −150 are required for high levels of transcription. In no case were transcripts found in any other tissue analyzed, including kidney, brain, pancreas, skeletal muscle, heart, spleen, liver, and thymus.

Analysis of Constructs with Deletions of Conserved Elements. Constructs 6–9 consist of a set of internal deletions (see Fig. 2, boxes C, B, O, and E) within the bp −880 marked *Prm-1* gene (construct 1). Since the bp −880 construct was expressed at a high level, we reasoned that deletion of any essential element would eliminate expression whereas deletion of auxiliary elements would reduce expression. The sequences deleted in these constructs are the sequences that have been conserved within the functional mouse *Prm-1* and *Prm-2* promoters [boxes B, C, and E (10)] as well as two sequences conserved in position and sequence among the human, bovine, and mouse (11, 14, 15) *Prm-1* promoters (boxes E and another sequence we have called box O). The four deletions include all three conserved elements that have been identified within the bp −150 construct (construct 4), which directs spermatid-specific transcription. These deletion constructs were used to investigate the functional importance of the conserved elements.

Transgenic mice containing deletions of the conserved elements within the context of the bp −880 promoter all retained testis-specific transcription as demonstrated by primer extension and Northern blot hybridization (data not shown). Deletions of box B or E had no detectable effect on expression levels as roughly half of the resulting mice had transgene mRNA levels that were equivalent to endogenous levels or higher (Fig. 3). Removal of box O resulted in decreased levels of transcript; however, too few animals were produced to be certain of this result. Deletion of box C resulted in consistently low levels of transcript. All five of the mice with the box C deletion expressed between 3 and 5% of endogenous levels of the transgene transcript (Fig. 3). These results indicate that none of the conserved elements we tested was essential for testis-specific transcription, but box C appeared to be required for high-level transcription.

Analysis of hGH Constructs. Another set of transgenes (Fig. 1, constructs 10–14) contains various portions of the *Prm-1* 5′ flanking region fused upstream of the hGH reporter gene with 83 bp of the hGH promoter. These constructs retain the hGH TATA box element but eliminate the *Prm-1* TATA box element. This hGH gene has been used to identify other tissue-specific enhancers (27, 28) and by itself it is never expressed in the testis (unpublished observations).

Transcript levels from the hGH transgenes were analyzed by Northern blot and RNA dot hybridization. Tissues for RNA isolation were removed from male founders or male offspring of female founders. Transgenic mice bearing construct 10 (bp −224 to −37, directing the hGH reporter gene) displayed testis-specific transcription of the hGH structural gene. Fig. 5 shows a Northern blot with RNA obtained from various tissues of three founder males expressing construct 10. Although the transgene was clearly expressed in the testis (lane 12), there was no detectable transcript in other tissues (lanes 1–11). After a 7-day exposure, a very faint band was detected in the spleen sample from one mouse (lane 10). The blot was subsequently probed with an actin fragment to confirm that the RNA was intact (data not shown). These results demonstrate that the bp −224 to −37 *Prm-1* region can function as a testis-specific promoter when placed upstream of the hGH proximal promoter and structural gene.

Important sequences were delineated further by making 5′ and 3′ deletions from the bp −224 to −37 *Prm-1* region and testing them with the hGH reporter gene. To test for testis-specific expression, Northern blot hybridizations were carried out on at least eight tissue RNA samples from at least two lines bearing each construct. Dot hybridizations were also done on testis RNA samples to quantitate the level of transgene expression in testis of each animal (Fig. 6).

Fig. 6*A* shows the hGH expression levels in testes of transgenic mice bearing the bp −150 to −37 region (construct 12). Northern blot hybridizations demonstrated testis-specific expression, although after a 7-day exposure, a faint band was detected in the brain RNA sample from one mouse (data not shown). The expression level in the five animals was clearly lower than for animals that contained construct 10, suggesting that there are elements between bp −224 and −150 required for high-level transcription, but not essential for testis-specific transcription. When the bp −110 to −37 *Prm-1* region was tested (construct 14), only one of five mice examined expressed hGH in the testis. Northern blot hybridization revealed that the transcript was larger than expected and it was expressed in all nine tissues examined. None of the other mice expressed hGH in any tissue (data not shown). It is likely that this aberrant transcript is the result of a chromosomal insertion effect. These data imply that sequences between bp −150 and −110 are required for testis-specific transcription.

Fig. 6 *B* and *C* shows the testis expression levels as a result of trimming DNA from the 3′ end of the *Prm-1* region. Deletion of the sequences between bp −37 and −77 was tested using sequences extending to both bp −224 (construct 11) and bp −150 (construct 13). In both cases, the expression level in the testis was extremely low. In addition, the fraction

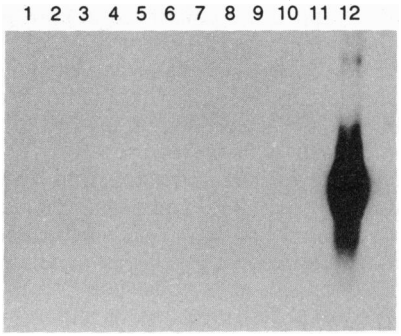

FIG. 5. Sample Northern blot hybridization for *Prm–hGH* mice. Northern blot analysis was used to demonstrate the testis specificity of hGH mRNA in construct 10 transgenic mice. Equivalent amounts of RNA (10 μg) were loaded into each lane and the blot was probed with a hGH-specific probe. RNA samples were obtained from multiple tissues of three adult transgenic founder males expressing construct 10. Lanes: 1, 4, 7, and 11, heart, kidney, liver, and spleen RNA, respectively, from founder 21-2; 2, 5, 6, and 8, spleen, heart, kidney, and liver RNA, respectively, from founder 21-6; 3, 9, 10, and 12, kidney, liver, spleen, and testis RNA, respectively, from founder 22-6. The blot was rehybridized with an actin probe to demonstrate the integrity of the RNA (data not shown).

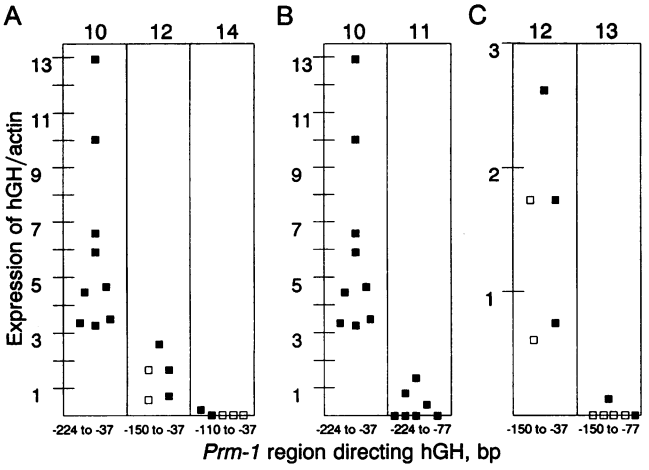

FIG. 6. Summary of testis expression of *Prm–hGH* constructs. Expression of hGH mRNA was examined in the testes of construct 10–13 mice as indicated at the top. The transgene transcript levels were quantitated relative to actin RNA levels by RNA dot hybridization. The 5′ truncations of the *Prm-1* region are summarized in *A* while *B* and *C* summarize the 3′ truncations of the *Prm-1* region. Open squares indicate male offspring of female founders and solid squares indicate male founders.

of transgenic mice that expressed the transgene dropped from 9 of 9 to 3 of 7 with the bp −224 to −77 region and from 5 of 5 to 1 of 6 with the bp −150 to −37 region. When Northern blot hybridizations were done to examine tissue specificity of expression, no other tissues expressed the transgene except for the brain sample from one construct 13 mouse that expressed it at a very low level (data not shown). These data suggest that the sequences between bp −37 and −77 are required for a high-level transcription and for a high frequency of expression.

The smallest region that could effectively direct testis-specific transcription of the hGH proximal promoter and structural gene was the 113-bp region from bp −150 to −37 (construct 12). To demonstrate that this testis-specific expression was in fact spermatid-specific, testis RNA from a developmental time course was analyzed. The transgene was not expressed in the day 18 testis but was expressed in the day 25 testis (Fig. 4*B*), consistent with spermatid-specific expression.

Since boxes O and E are conserved in both position and sequence in the *Prm-1* promoters of several species and they lie within the region that we have shown is essential for spermatid-specific expression, their ability to direct hGH gene expression was tested. Six copies of box O plus six copies of box E (each box consisted of a 30-bp oligomer with the conserved sequence centered within it) were ligated at bp −83 of the hGH reporter gene. Three of seven male founders expressed this construct in a testis-specific manner (data not shown) although expression level was only ≈0.5% of the average level obtained with construct 12. These results suggest that box(es) O and/or E may be important, but other sequences are clearly required for efficient expression.

DISCUSSION

We have demonstrated that a 113-bp region between bp −150 and −37 of the *Prm-1* gene is sufficient to direct spermatid-specific expression, while sequences 5′ of bp −150 are required for high-level transcription. The importance of conserved elements between bp −150 and −37 was tested by deleting boxes O, B, and E in the context of the bp −880 *Prm-1* promoter. None of these elements was essential for testis-specific transcription in this context. This is surprising considering that boxes O and E are conserved in both position and sequence in the bovine, human, and mouse *Prm-1* promoters. Perhaps redundant elements lie elsewhere within the promoter between bp −880 and −37. This type of redundancy has been seen with the elastase enhancer in which at least two elements must be mutated to abolish expression in transgenic mice (29). Multiple copies of boxes O and E together directed extremely low-level testis-specific expression, supporting a role for these elements in *Prm-1* regulation but implicating other sequences for efficient expression.

The hGH constructs demonstrate that sequences 3′ of bp −37 are not required for spermatid-specific transcription and that the *Prm-1* promoter can function through a heterologous TATA box element to direct a heterologous hGH reporter gene. The 5′ and 3′ truncations of the bp −224 to −37 *Prm-1* fragment directing hGH expression also demonstrate the presence of several sequences that play a role in *Prm-1* transcription. Truncation from bp −224 to bp −150 results in decreased levels of transcription. This construct demonstrates the presence of sequences between bp −224 and −150 that increase transcription in the testis. This region contains box C, whose deletion also produced a decrease in transcription levels. It is likely that box C provides at least some of the function detected between bp −224 and −150. Box C is the only sequence in this region to which we can detect binding of testis nuclear proteins in gel-mobility-shift assays (unpublished data).

Further truncation to bp −110 effectively abolished the spermatid-specific function of the promoter fragment. This suggests that sequences between bp −150 and −110 are essential for spermatid-specific expression. This region between bp −150 and −110 contains boxes B and O that were deleted in the context of the bp −880 promoter without significant effect. Boxes B and O are also the only sequences between bp −150 and −110 to which we can detect the binding of testis nuclear proteins in gel-mobility-shift assays (unpublished data). Perhaps the factors that bind to boxes O and B establish spermatid-specific expression; however, eliminating only one of these binding sites does not inactivate the *Prm-1* gene (at least in the context of the bp −880 promoter). Alternatively, there may be other sequences between bp −150 and −110 that provide cell-specific expression.

Deletions of sequences between bp −37 and −77 had a similar effect. These sequences appear to be required for both high-level transcription and high frequency of expression. The sequences between bp −37 and −77 include a sequence similar to a cAMP response element that binds a testis-specific nuclear protein called Tet-1 (19). Although it is encouraging that a testis-specific protein binds to this region, our data demonstrate that other sequences between bp −224 and −77 must also provide a testis-specific function, since removal of the fragment between bp −37 to −77, in the two contexts we tested, did not completely abolish testis-specific promoter function.

Of the sequences between bp −224 and −37, we have detected functional importance for three regions. These include the bp −37 to −77, bp −110 to −150, and bp −150 to −224 regions. It remains unclear whether the sequences between bp −77 and −110 provide any promoter function. This fragment contains box E, which appeared to have no essential function when deleted in the bp −880 context. We have also been unable to detect any testis nuclear proteins that bind to this fragment in gel-mobility-shift assays.

We detected no correlation between copy number and transgene expression levels in these studies. Often low-copy-number animals expressed equivalent or greater amounts of the transgene compared to high-copy-number animals, suggesting that the site of transgene insertion has a major effect on expression levels. Although accurate expression levels

cannot be obtained with a few transgenic animals for each construct, trends in expression levels can be inferred. Most of our conclusions concerning the function of specific *Prm-1* promoter regions are based on results from both the marked *Prm-1* and hGH constructs, which serve to strengthen our conclusions. While variability can still be a problem, the ability to study cell-specific gene expression in a normal developmental setting is invaluable.

When *in vitro* transcription was carried out using the *Prm-1* gene (19), no transcription was detected when a construct containing 1.7 kb of *Prm-1* 5′ flanking sequence was used. Transcription was only detected upon truncation of the construct to bp −293 and transcription increased upon further truncation to bp −92. A low level of *in vitro* transcription could also be detected using nuclear extracts from tissues other than testis, including brain. These data are in marked contrast with our transgenic data. Both 4.8 and 1.7 kb of *Prm-1* 5′ flanking region direct the highest levels of spermatid-specific transcription in transgenic mice (ref. 15 and unpublished data). When 5′ truncations were tested in transgenic mice, transcription levels decreased with increasing promoter truncation. If the *in vitro* transcription data were the result of removal of repressor sequences, then one might predict that truncation would result in ectopic expression in transgenic mice. We did not detect ectopic transgene expression with any of the 5′ deletions we tested and we also detected no repressor type function in our studies. Even more importantly, when we truncated to bp −110, we lost promoter function, whereas with *in vitro* transcription (19), the highest level of expression was obtained with 92 bp of 5′ flanking sequence.

A number of genes including transition nuclear protein 1, transition nuclear protein 2, histone H2b, heat shock protein 70.1, smooth muscle γ-actin, and the angiotensin-converting enzyme (ACE) display spermatid-specific expression patterns (30–35). The post-meiotic expression of these genes suggests that they may share common regulatory sequences with the *Prm-1* gene. The ACE gene is regulated by a 698-bp intragenic fragment as determined in transgenic mice (35). Both this ACE fragment and the *Prm-1* gene (within the region of bp −37 to −77) share a sequence very similar to a cAMP response element (CRE) (36). Both CRE-like sequences contain a 7/8-bp identity with the consensus CRE, with the one difference being in the same position in both cases. This one-base difference also lies in a position of the CRE-like element that is not essential for the binding of the testis-specific Tet-1 nuclear protein (19). It remains to be determined what effect removal of this sequence has on the regulation of the ACE gene, but its removal from the *Prm-1* promoter had a significant effect on transcription as described above. Comparison of the functionally important sequences of the *Prm-1* promoter with functionally important sequences of the other spermatid-specific genes as they become available will be instructive.

We have used the transgenic system to define small regions of the *Prm-1* promoter that are functionally important. These regions are all ideal in size for the analysis of DNA–protein interactions *in vitro*. The data described here should facilitate the identification of transcriptional regulatory proteins involved in post-meiotic gene expression.

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