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Separate Elements in the 3′ Untranslated Region of the Mouse Protamine 1 mRNA Regulate Translational Repression and Activation during Murine Spermatogenesis

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The mouse protamine mRNAs, *Prm-1* and *Prm-2,* are translationally repressed for several days during male germ cell differentiation. The translational delay of mouse *Prm-1* mRNA has previously been shown to be dependent upon *cis*-acting elements that reside in the last 62 nucleotides of the *Prm-1* 3′ untranslated region (3′ UTR). We have previously identified a 48/50-kDa protein that binds the 3′ UTRs of both *Prm-1* and *Prm-2* mRNAs in a sequence-specific manner, is present in cytoplasmic fractions of postmeiotic round spermatids where the protamine mRNAs are translationally silent, and is markedly reduced in elongated spermatids where the protamine mRNAs become activated for translation. Surprisingly, the binding site for this activity maps to a region of the *Prm-1* 3′ UTR not contained within the functional 62 nucleotides described above. In this report we show that the binding site for the 48/50-kDa protein can also delay translation of a reporter RNA *in vivo,* suggesting that the 48/50-kDa protein can repress the translation of *Prm-1* mRNA during murine spermatogenesis. This observation proves that two separate regions of the *Prm-1* 3′ UTR are sufficient to repress *Prm-1* translation. In addition, immunocytochemistry and polysome analysis have revealed that this transgenic reporter mRNA fails to undergo proper translational activation. These results suggest that an additional region of the *Prm-1* 3′ UTR is required for proper translational activation and that *Prm-1* translational repression elements can be separated from those involved in translational activation.

INTRODUCTION

Many examples exist in a variety of organisms where regulating protein synthesis at the translational level provides an efficient and sometimes necessary means of responding to environmental or morphogenetic cues. During early metazoan development and gametogenesis translational control plays an especially prominent role in regulating gene expression (reviewed by Wickens *et al.*, 1996; Curtis *et al.*, 1995; Davidson, 1986). In a number of cases the 3′ untranslated region (3′ UTR) is the functional part of the mRNA responsible for regulating protein synthesis in this manner. The 3′ UTRs of mRNAs can play critical regulatory roles in diversified processes such as mRNA transport, localization, stabilization, and translation. The possibility that *trans*-acting RNA binding proteins interact with *cis* elements in the 3′ UTR to facilitate this type of control mechanism has recently been demonstrated for some mRNAs (Dubnau and Struhl, 1996; Ostareck Lederer *et al.*, 1994; Rivera Pomar *et al.*, 1996).

During murine spermatogenesis the developing haploid genome becomes transcriptionally inactive well before differentiation events remodel the overall morphology of the spermatid (Kierszenbaum and Tres, 1975; Monesi, 1964). This property of mammalian spermatogenesis necessitates translational control in that it permits changes in protein levels to occur in the absence of new transcription. One of the morphological transformations that accompanies spermatid differentiation is the condensation of the haploid nucleus during spermiogenesis (Dooher and Bennet, 1973;

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Fawcett *et al.*, 1971). The genes that mediate nuclear compaction during murine spermatogenesis include the two transition proteins, *TP1* and *TP2*, and the protamines, *Prm-1* and *Prm-2* (Balhorn *et al.*, 1984). These mRNAs are synthesized in round spermatids and stored as translationally silent mRNA ribonucleoprotein particles (mRNPs) for several days until they become activated for translation in later stages of spermiogenesis (Hecht, 1989; Kleene *et al.*, 1984; Kleene and Flynn, 1987; Yelick *et al.*, 1989). Translational repression of *Prm-1* is imposed by a 3′ UTR mediated mechanism and is essential for normal spermatid differentiation (Braun *et al.*, 1989; Lee *et al.*, 1995). However, the molecular details by which the *Prm-1* 3′ UTR inhibits its translation are unknown. Presumably proteins interact with sequences in the 3′ UTR and prevent translation initiation from occurring at the 5′ end of the message. Defining the *cis*- and *trans*-acting components involved is a necessary first step in understanding the molecular nature of this 3′ UTR mediated translational control mechanism.

The full-length 156-nt *Prm-1* 3′ UTR is necessary and sufficient to confer *Prm-1*-like translational control on a chimeric reporter human growth hormone (*hGH*) mRNA *in vivo* (Braun *et al.*, 1989, Fig. 1A). Additional transgenic experiments showed that the 3′ most 62 nt (Fig. 1B), but not the 3′ most 23 nt which harbor the *Prm-1* polyadenylation signal (Fig. 1C), are sufficient to mediate this level of control. A candidate translational control factor called *Prbp*, for protamine 1 RNA binding protein, was cloned from an expression screen designed to enrich for *trans*-acting translational repressors of *Prm-1* (Lee *et al.*, 1996). Immunocytochemistry experiments showed that expression of the Prbp protein is restricted to germ cell cytoplasm at a time when *Prm-1* mRNA is under translational repression. Furthermore, the binding site for Prbp has been mapped to a putative stem-loop secondary structure contained within the 3′ most 62 nt shown to be functional *in vivo*, and the protein inhibits reporter mRNAs *in vitro* in a cell-free translation assay. These properties are consistent with Prbp playing a role in the translational control of *Prm-1*.

Using RNA band shift and UV crosslinking assays, we have previously described an RNA binding activity of 48 and 50 kDa that has *Prm-1* translational repressor characteristics (Fajardo *et al.*, 1994). This activity binds *Prm-1* and *Prm-2* 3′ UTRs in a sequence-specific manner, is only present in the cytoplasm of round spermatids when the protamine mRNAs are translationally repressed, and is markedly reduced in elongated spermatids where the protamine mRNAs undergo translation. The binding site for this activity was mapped to a 22-nt region contained within the first 37 nt of the *Prm-1* 3′ UTR. Surprisingly, the 48/50-kDa proteins do not bind to the 3′ most 62 nt of the 3′ UTR that represses translation *in vivo*. This suggests that either the 48/50-kDa proteins are involved in an aspect of *Prm-1* mRNA metabolism other than translational control or there are redundant elements that mediate *Prm-1*-like translational delay. We have tested the binding site for the 48/50-kDa protein in transgenic mice and show that it too can repress translation *in vivo*. Surprisingly, the transgenic mRNA is poorly activated for translation in elongated spermatids, suggesting that separate elements may control translational repression and activation.

RESULTS

Redundant Translational Repression Sequences

To assess whether the *Prm-1* 3′ UTR binding site for the 48- and 50-kDa RNA binding proteins confers *Prm-1*-like translational delay, we constructed a transgene that encodes a chimeric reporter mRNA that contains the *Prm-1* 5′ UTR, *hGH* coding sequences, and a 3′ UTR containing the first 37 nt of the *Prm-1* 3′ UTR fused to the last 23 nt of the *Prm-1* 3′ UTR (Fig. 1D). The first 37 nt of the 3′ UTR contains the binding site for the 48/50-kDa protein, and the last 23 nt contains the polyadenylation signal.

In the mouse, spermatogenesis starts in a synchronous manner at birth and takes about 35 days to complete the first round (Nebel *et al.*, 1961). This property of mouse spermatogenesis can be utilized to study developmental gene regulation by analyzing samples from prepubertal animals of different ages. For example, at day 25 cells undergoing the first cycle of spermatogenesis are well into the haploid round spermatid stage, and the endogenous *Prm-1* gene is known to be transcribed but not translated (Kleene *et al.*, 1984). Two lines of mice, lines 11246 and 11303, were derived carrying the above described transgene and prepubertal animals were sacrificed at day 26, 28, and 32. Testes from these animals were used to evaluate mRNA and protein expression. The prepubertal accumulation profile for the transgenic mRNA (Fig. 2) was identical to that observed for the endogenous *Prm-1* mRNA (data not shown). This result was expected given that the transgene contained the transcriptional regulatory elements of the *Prm-1* gene (Peschon *et al.*, 1987). Transgenic *hGH* mRNA was detected in all prepubertal samples; however, hGH protein was not observed until day 32 (Fig. 2). This prepubertal hGH profile is indicative of *Prm-1*-like translational delay, compared to previously published results evaluating similar transgenes. In transgenic mice that contain the full-length *Prm-1* 3′ UTR fused to the *hGH* reporter, hGH is not detected until day 32. Conversely, hGH is detected at day 28 in transgenic animals where the *hGH* reporter is fused to its own 3′ UTR (Braun *et al.*, 1989).

The developmental regulation of a transgene can be studied in the testis from a single adult mouse because spermatogenesis is ongoing in the adult testis. Germ cells at different stages of development can be identified histologically by their morphological characteristics and predictable associations with cells at other stages of development. Translational regulation of transgenic mRNA D (Fig. 1) was examined using immunocytochemistry on testis sections from each of the two derived lines (Fig. 3) and from three founder males (data not shown). Immunocytochemical analysis re-

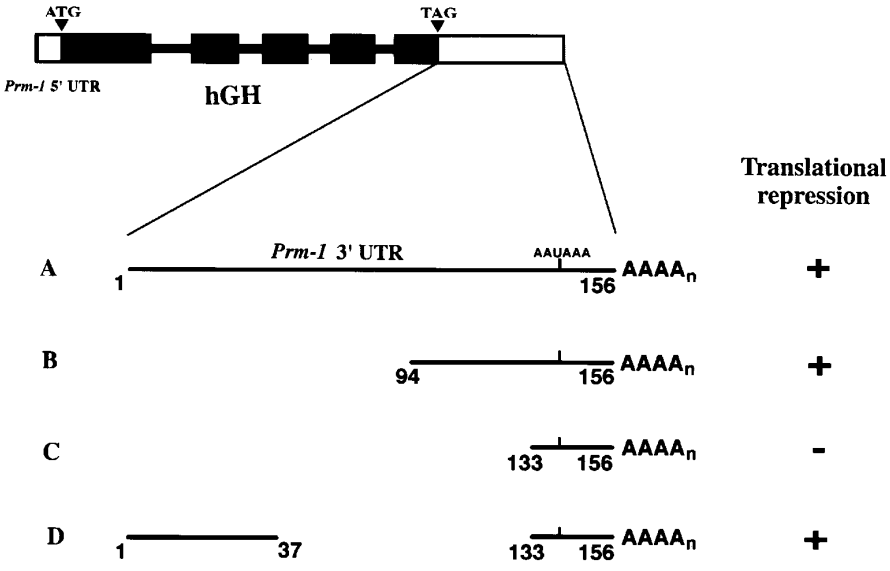

FIG. 1. Summary of chimeric mRNAs assayed for *Prm-1*-like translational control in transgenic mice. The *Prm-1* promoter and enhancer region were used for correct germ cell-specific expression (Peschon *et al.*, 1987). All transgene constructs generate mRNAs with heterologous 5′ UTRs of 159 nt, labeled *Prm-1* 5′ UTR, and represented as an open box at the beginning of the reporter contruct. It is known that *Prm-1* translational control is not dependent on specific 5′ UTR sequences (Braun *et al.*, 1989). The 5′ UTR used in these studies contains 91 nt of *Prm-1* 5′ UTR, 7 nt of linker, and 61 nt of *hGH* 5′ UTR. Each transgenic mRNA contains the complete *hGH*-coding sequence and introns represented as thick and thin solid black boxes, respectively, with translational start and stop codons labeled (DeNoto *et al.*, 1981). The full-length *Prm-1* 3′ UTR, or specific deletion variants of the *Prm-1* 3′ UTR, were linked to *hGH* as represented by the open box at the end of the reporter construct. The fusions (A–D) generate 3′ UTRs that contained the first 7 nt of the hGH 3′ UTR and (A) the full-length 156-nt *Prm-1* 3′ UTR, (B) the 3′ most 62 nt of the *Prm-1* 3′ UTR, (C) the 3′ most 23 nt of the *Prm-1* 3′ UTR, or (D) the first 37 nt of the 5′ end of the *Prm-1* 3′ UTR fused to the 3′ most 23 nt of the *Prm-1* 3′ UTR. All chimeric mRNAs were evaluated for *Prm-1*-like temporal translational control using either or both immunocytochemistry and prepubertal Western blot analysis. A (Braun *et al.*, 1989), B and C (Braun, 1990), D (this study).

vealed that hGH protein was predominately in elongated spermatids (Figs. 3A and 3B); however, some hGH protein was detected in the acrosome of developing round spermatids (Fig. 3C, arrow, see Discussion). These data reveal that the binding site for the 48- and 50-kDa proteins, when fused with the *Prm-1* polyadenylation signal, is sufficient to confer *Prm-1*-like translational delay on a heterologous reporter mRNA.

Incomplete Translational Activation

In performing the immunocytochemical analyses described above, it became clear that the strength of the hGH signal observed in the cytoplasm of elongated spermatids (Fig. 3A) was much weaker than that observed in animals expressing the reporter mRNA fused to the full-length *Prm-1* 3′ UTR (Braun *et al.*, 1989). Given this observation, we postulated either that the transgenic mRNA (Fig. 1D) was unstable in elongated spermatids or that the transgenic mRNA was not being properly activated for translation. To test these two possibilities we performed RNA *in situ* hybridization and polysome analysis experiments.

Using a ^{33}P-labeled, *hGH*-specific antisense RNA probe to carry out *in situ* hybridization, we found that the transgenic mRNA had an accumulation profile in haploid germ cells similar to that of the endogenous *Prm-1* mRNA (Fig. 4). *Prm-1* mRNA expression is normally detected in late step 7 round spermatids, exhibits peak expression in steps 9–10 elongating spermatids, and remains constant until the message is undetectable in step 15 elongated spermatids (Braun *et al.*, 1989; Mali *et al.*, 1989; Fajardo, unpublished data). The *hGH* reporter mRNA was easily detectable in the cytoplasm of early step 8 round spermatids (Fig. 4A), peaked during step 9–10 elongating spermatids (Fig. 4B), was present in step 12–14 elongated spermatids (Figs. 4C and 4D), and, surprisingly, appeared to persist at a low level into step 15 elongated spermatids (Fig. 4E). These *in situ* expression data suggest that the low-level hGH signal in elongated spermatids observed by immunocytochemistry is not due to premature degradation of transgenic mRNA. Furthermore, these data show that the transgenic mRNA actually persists longer than the endogenous *Prm-1* mRNA. We conclude that the relatively low hGH immunocytochemical signal is not due to message instability.

Improper translational activation predicts that the transgenic mRNA would be poorly recruited onto poly-

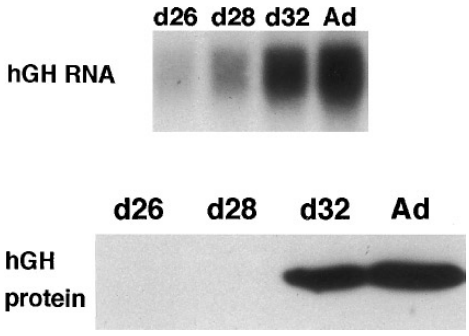

FIG. 2. Northern and Western blot analysis of extracts from prepubertal animals. RNA was isolated from the testes of animals 26, 28, 32, and 42 days old, and Northern blot analysis was performed. The Northern blot membrane was hybridized with a ^{32}P-labeled probe specific for *hGH*-coding sequences. Total SDS-soluble protein extracts from testes were prepared from the same prepubertal animals described above. Equivalent amounts of protein from each prepubertal time point was separated by SDS–PAGE, electroblotted, treated with rabbit anti-hGH antibody and peroxidase-conjugated goat anti-rabbit IgG secondary antibody, as described under Materials and Methods. Purified hGH protein was used as a protein marker (data not shown). These data represent those obtained from the analysis of line 11246 and were reproducible in a second independent line, 11303 (data not shown).

somes. Sucrose gradient sedimentation analysis was used to evaluate the polysome distribution of the transgenic reporter mRNA. Postmitochondrial supernatants from the testes of sexually mature transgenic mice were allowed to sediment on sucrose gradients and were divided into 12 fractions to separate polysomal and nonpolysomal mRNAs (Fig. 5A). Total RNA was isolated from equal aliquots of each sucrose gradient fraction and assayed by Northern blot analysis as described under Materials and Methods. The optical density profile (Fig. 5A) shows noticeable peaks of ribosomal subunits, monosomes, disomes, trisomes, and higher molecular weight polysomes. Nonpolysomal mRNAs are typically found in the ribonucleoprotein (RNP) containing fractions (Fig. 5, fractions 1 and 2). Translationally inactive *Prm-1* mRNPs sediment between 10S and 40S, peaking at approximately 20S (Kleene, 1993). In these assays the endogenous *Prm-1* mRNA was very abundant in the mRNP portion of the gradient (Fig. 5A, fractions 1 and 2). The *hGH* reporter mRNPs appear to be slightly larger than *Prm-1* mRNPs and thus are contained within one fraction (Fig. 5A, fraction 2). When the *Prm-1* mRNA is activated for translation it is mobilized onto small polysomes and becomes heterogeneous in size (Fig. 5A, fractions 3–6). The *Prm-1* mRNA is found on small polysomes because the message only codes for a 50-amino-acid polypeptide. Its heterogeneity is due to changes in the length of the poly(A) tail, ranging from an original 160 to 30 nt, and appears to be a consequence of ongoing translation (Kleene, 1989). In comparison, very little transgenic mRNA is found on polysomes (Fig. 5A), suggesting that this mRNA is poorly activated for translation.

As a control to verify that the hybridization observed in the polysomal portion of the gradient was indeed due to an active association between mRNA and ribosomes, postmitochondrial supernatants were prepared and allowed to sediment in the presence of EDTA (Fig. 5B). This modification in the procedure dissociates mRNA and ribosomes, causing polysomal mRNA to move into the nonpolysomal portion of the gradient (Penman *et al.*, 1968). The presence of EDTA significantly reduced the *Prm-1* mRNA polysomal hybridization, confirming an association between ribosomes and *Prm-1* mRNA (compare fractions 3–6 in Figs. 5A and 5B). Conversely, there was only a slight effect on the polysomal hybridization detected for the transgenic *hGH* mRNA (Fig. 5B). Comparison of the *hGH* signal in gradients prepared with and without EDTA shows that the most significant difference is present in the monosome containing fraction (Figs. 5A and 5B, fraction 3), suggesting that the transgenic mRNA is poorly translated. It is likely that the mRNA that continues to sediment in the polysomal portion of EDTA-treated gradients is due to contamination from nonpolysomal mRNP containing fractions during collection of the gradient. Contamination of the polysomal fractions has also been observed by others (Kleene *et al.*, 1984). In summary, the transgenic reporter mRNA that harbors the 48- and 50-kDa binding site is present in the cytoplasm of elongated spermatids, but fails to be properly recruited onto polysomes.

DISCUSSION

In earlier studies we characterized an RNA binding activity of 48 and 50 kDa that exhibits binding specificity for the 3′ UTRs of *Prm-1* and *Prm-2* (Fajardo *et al.*, 1994). This activity is very abundant in developing germ cells where *Prm-1* mRNA is translationally dormant and practically undetectable in cells where *Prm-1* mRNA is actively translated. Using a transgenic approach we functionally tested the binding site for this RNA binding activity that has properties of a *Prm-1* translational repressor. In performing this analysis, we have observed that this region of the *Prm-1* 3′ UTR is sufficient to execute *Prm-1*-like translational repression, but lacks sequences necessary for *Prm-1*-like translational activation. These results demonstrate that multiple elements in the *Prm-1* 3′ UTR participate in temporal regulation of the mRNA. Two different regions of the *Prm-1* 3′ UTR can repress translation, and a third region appears to be required to activate translation (Fig. 6). Thus, it seems that *Prm-1* translational repression elements can be separated from those involved in translational activation, suggesting that additional interactions between RNA and *trans*-acting factors are required to disengage the repression machinery and mobilize the message onto polysomes. These observations raise questions concerning the evolutionary pressure for two *Prm-1* translational repression ele-

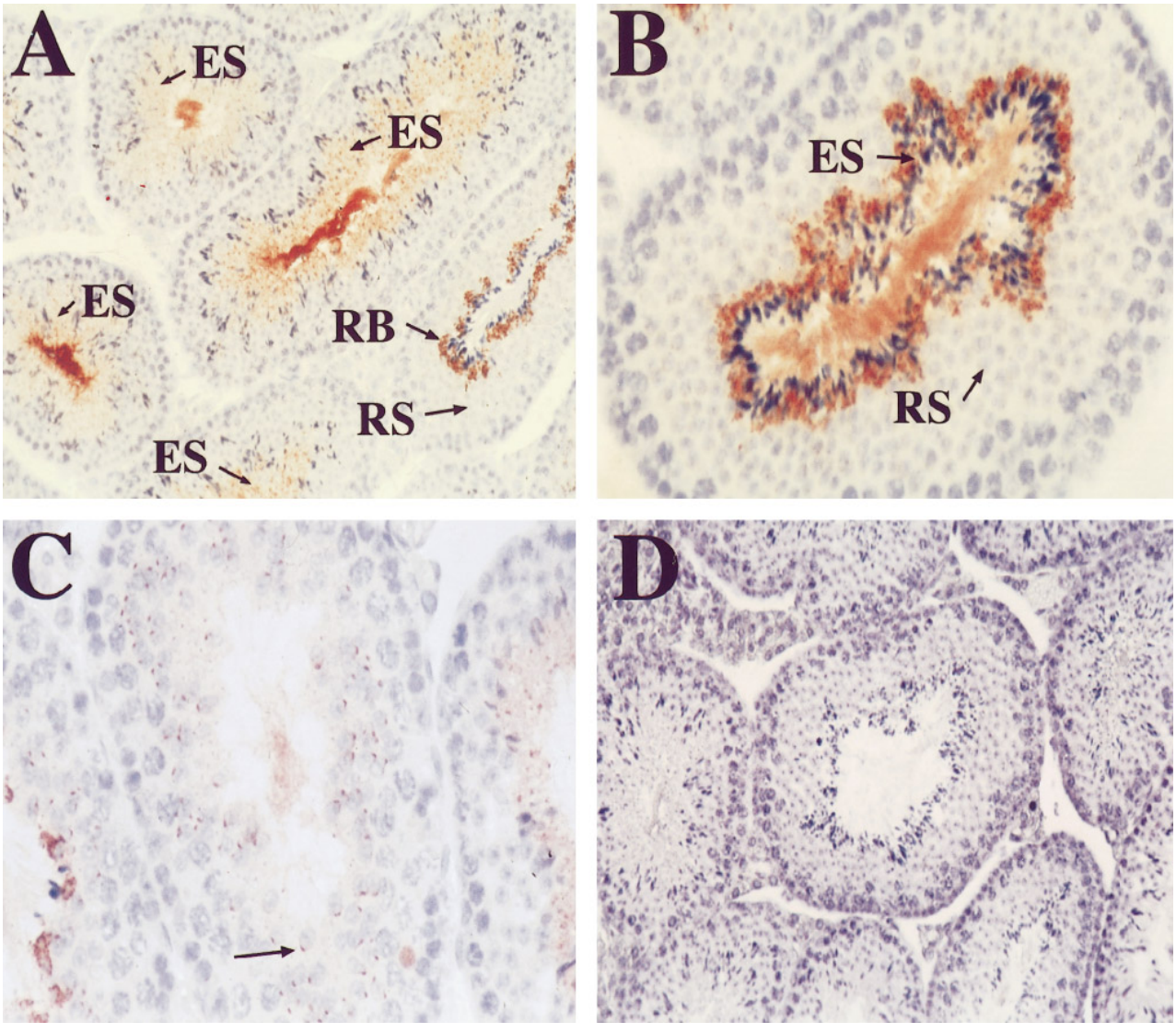

FIG. 3. Immunocytochemical analysis of chimeric reporter mRNAs. Testes from adult transgenic animals were fixed, sectioned, and treated with a rabbit anti-hGH primary antibody and a peroxidase-conjugated goat anti-rabbit secondary antibody, as described under Materials and Methods. Sections were counterstained with hematoxylin. In transgenic mice carrying construct D (Fig. 1), immunoreactivity is observed in residual bodies (RB) of late-stage elongated spermatids, in the cytoplasmic compartment of elongated spermatids (ES), and is weak in round spermatids (RS), (A and B). Sections showed no immunoreactivity when incubated only with the secondary antibody (D). Synthesis of hGH was detected in the acrosome (arrow) in some stage IX tubules indicating that the transgenic mRNA at a low level can escape repression (C). These data (A–D) represent that obtained from line 11246, reproducible data were obtained in evaluating another independent line, 11303, and three founder males 11270, 11273, and 11180 (data not shown). Additional negative controls were performed using both the primary and secondary antibody on testes from nontransgenic animals. Magnification (A and D) ×200, (B and C) ×400.

ments and the process by which *Prm-1* translational activation is achieved.

Other 3′ UTR translational control mechanisms that have been studied in other systems exhibit functional redundancy via multiple *cis*-acting elements in the 3′ UTR. For example, in *Drosophila melanogaster* there are multiple copies of the Bruno response element (BRE) in the 3′ UTR of the *oskar* mRNA (Kim Ha *et al.*, 1995). Additionally, the translational repression of *Drosophila hunchback* is dependent on two copies of a bipartite element in its 3′ UTR called the nanos response element (NRE) (Wharton and Struhl, 1991). Two NRE-like sequences found in the 3′ UTR of *Caenorhabditis elegans Glp-1* mRNA have been implicated in translational control (Evans *et al.*, 1994), and the

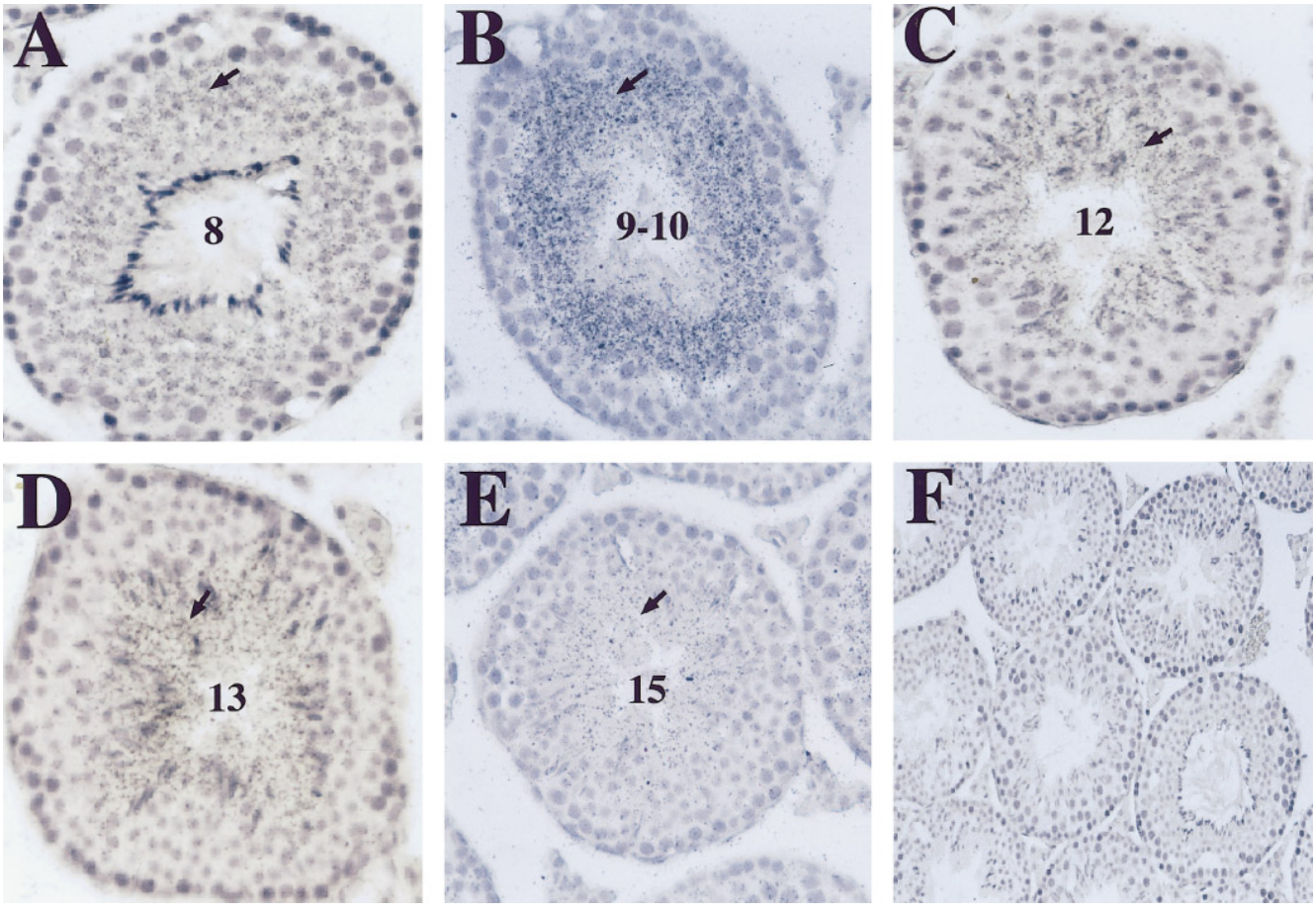

FIG. 4. *In situ* analysis of transgene D RNA (Fig. 1). Testis sections from transgenic mice (line 11246) were hybridized with (A–E) a ^{33}P-labeled *hGH*-specific antisense probe or (F) an *hGH*-specific sense probe, as described under Materials and Methods. Controls included probing with *Prm-1*-specific antisense and sense probes, as well as using the described probes on nontransgenic sections (data not shown). Transgenic mRNA accumulation parallels that of the endogenous *Prm-1* mRNA (Mali *et al.*, 1989). Hybridization of the *hGH*-specific antisense probe (arrow) is observed in (A) the cytoplasmic compartment of step 8 round spermatids, (B) peaked in step 9–10 elongating spermatids, and (C–E) remained detectable through step 15 elongated spermatids. Magnification, (A–E) ×400, (F) ×200.

15-lipoxygenase (*Lox*) mRNA involved in erythrocyte maturation in mammals contains multiple copies of a translational control *cis* element in its 3′ UTR (Ostareck Lederer *et al.*, 1994). As few as two copies of the *Lox* element are sufficient to fully repress a reporter mRNA *in vitro*, whereas a single copy of the element only slightly represses translation (Wickens *et al.*, 1996; B. Thiele, cited pers. comm.). This lack of absolute redundancy is also observed for the *Prm-1* 3′ UTR repression elements. However, the distinction between these examples and that of *Prm-1* is that in the *Prm-1* 3′ UTR the *cis* elements involved in repression are not merely repeats of the same element. In this situation it appears two different sites and the their corresponding *trans*-acting factors can repress *Prm-1* mRNA translation. The selective pressure for two *Prm-1* regulatory regions may be revealed by the fact that when only one site is present, translational repression of the transgenic reporter is not complete. This is indicated by the presence of a low-level signal in the acrosome of round spermatids when examining transgenic mRNAs that contain one of the two described *Prm-1* translational repression elements. It has been previously observed that the subcellular localization of hGH in spermatids is dependent on the time of synthesis during spermiogenesis (Braun *et al.*, 1989). Production of hGH early in round spermatids targets the protein to the developing acrosome, whereas delayed synthesis results in intracellular accumulation, but not acrosomal localization in elongated spermatids. The acrosome signal observed in animals expressing transgenic mRNA D (Fig. 1) is not nearly as robust as the acrosomal signal detected in animals carrying the hGH reporter fused to its own 3′ UTR (Braun *et al.*, 1989). This same low-level acrosomal signal is also observed in animals expressing transgenic mRNA B (Fig. 1, Braun, 1990, unpublished observations). Thus, the hGH acrosomal signal

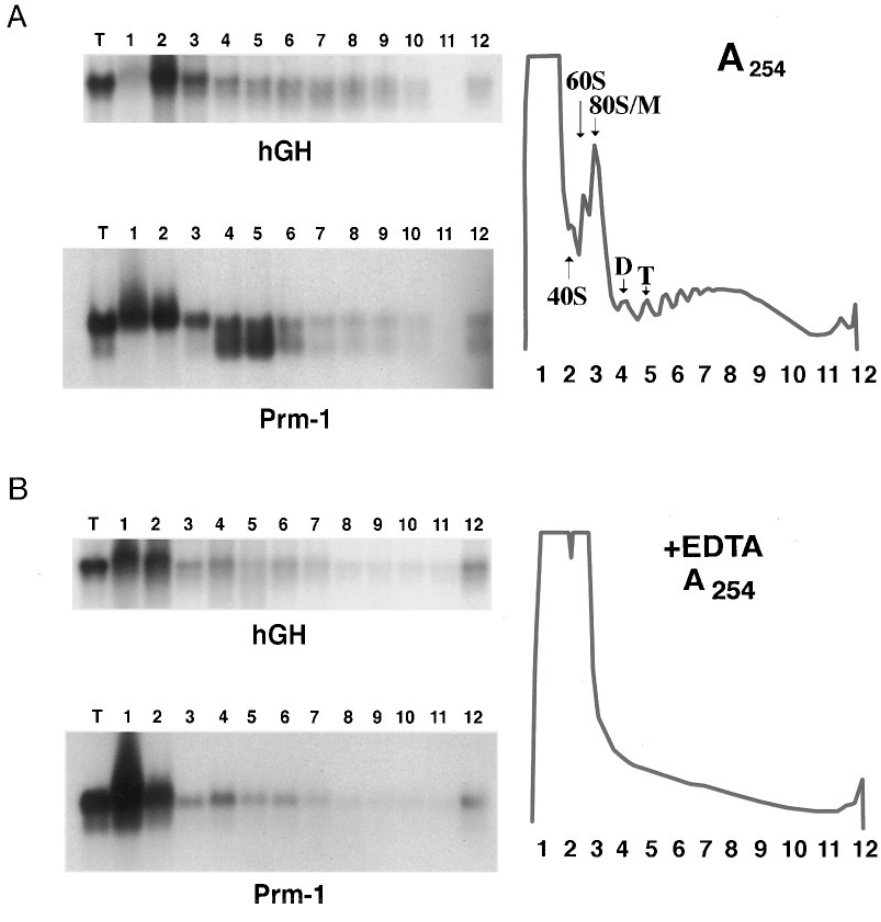

FIG. 5. Distribution of transgenic and endogenous *Prm-1* mRNAs in sucrose gradients. Testes from sexually mature transgenic mice were used to prepare a postmitochondrial supernatant that was allowed to sediment on a sucrose gradient, as described under Materials and Methods. The gradient was passed through a flow cell where the absorbance at 254 nm was recorded, and 12 fractions were collected. Prominent peaks are labeled on the A_{254} graph in (A). Single ribosomal subunits are labeled 40S and 60S, monosomes labeled 80S/M, and disomes and trisomes labeled with the letters D and T, respectively. Sample number 1 is the top of the gradient. A Northern blot was prepared from total RNA isolated from each fraction and hybridized with probes specific for *hGH* and *Prm-1* coding sequences (A). As a control for polysome association, RNA was analyzed from lysates prepared and allowed to sediment in the presence of EDTA which dissociates polysomes (B).

seen in animals expressing either transgenic mRNA B or D (Fig. 1) is most probably due to a low level of leaky hGH synthesis. With both sites present in the full-length *Prm-1* 3′ UTR translation is completely repressed. This added stringency may be beneficial to the process of male gametogenesis given the detrimental consequences of premature expression of Prm-1 protein on the developing spermatid (Lee *et al.*, 1995). Additionally, these elements may participate in establishing an mRNP conformation that is recognized by the factors responsible for proper temporal translational activation.

Given that the *Prm-1* mRNA is found homogeneously throughout the cytoplasm (Morales *et al.*, 1991) and is stored as a 20S mRNP (Kleene, 1993), it has been modeled that RNA–protein interactions in the 3′ UTR facilitate temporal repression. Our working hypothesis is that *trans*-acting factors interact with respective *cis* elements in the 3′ UTR and by some unknown mechanism inhibit translation initiation. It is known that the *Prm-1* mRNA does not undergo compartmentalized storage (Morales *et al.*, 1991) in a manner similar to *Vg-1* for *Xenopus laevis* or a number of maternal mRNAs such as *bicoid* and *nanos* in *Drosophila* (reviewed by St Johnston, 1995), nor is there any evidence for the existence of *Prm-1* 3′ UTR antisense RNAs (Lee and Braun, unpublished observations) found for *lin-14* mRNA in *C. elegans* (Lee *et al.*, 1993). Such results argue against these types of mechanisms playing a role in *Prm-1* mRNA regulation.

There are several possible ways in which interactions at the 3′ end of the mRNA may prevent efficient translation

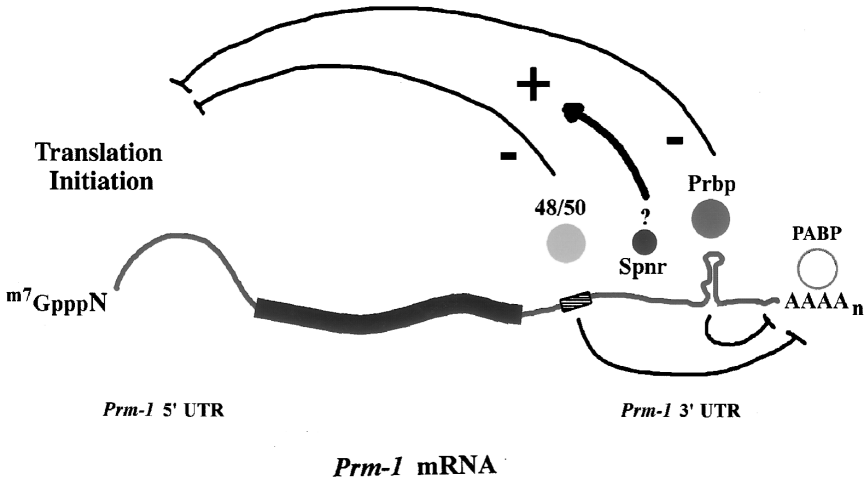

FIG. 6. Model. The RNA binding proteins Prbp (Lee *et al.*, 1996), and the 48- and 50-kDa RNA binding proteins previously described (Fajardo *et al.*, 1994), interact with *cis* elements in the *Prm-1* 3′ UTR to repress translation initiation. Possible mechanisms of inhibition include preventing poly(A) binding protein (PABP) function or interaction with the 5′ cap to block binding of the 40S ribosomal complex. Translational activation requires sequences present in the 3′ most 62 nt of the *Prm-1* 3′ UTR and may be dependent on the binding of the microtubule associated RNA binding protein, Spnr (Schumacher *et al.*, 1995; Schumacher *et al.*, submitted), for publication or an as yet unidentified protein.

initiation events at the 5′ end of the message. RNA binding proteins could bind specific sites located in the 3′ UTR and either directly interact with the 5′ terminal cap element (^{m7}-GpppN) to prevent translation initiation or they could prevent binding of poly(A) binding protein to the poly(A) tail. Recently, studies in *Saccharomyces cerevisiae* suggest that the 3′ end of the mRNA, namely, the poly(A) tail and the poly(A) tail binding protein (Pab1p), actively cooperates with the 5′ end to efficiently stimulate translation initiation (Tarun and Sachs, 1995). Alternatively, an RNA binding site located in the 3′ UTR could serve as a nucleation site for RNA binding proteins like Prbp and the Y-box proteins, which then could mask the entire mRNA (Lee *et al.*, 1996; Wolffe, 1994). We have previously described the general translational silencing properties of Prbp (Lee *et al.*, 1996). Prbp lacks complete specificity for *Prm-1* containing RNAs *in vitro*, is highly expressed in round spermatids, inhibits translation at high molar excesses in a cell-free translation system, and binds to an *in vivo* functional region of the *Prm-1* 3′ UTR, where it appears to initiate masking of the mRNA by oligomerization (Lee, Fajardo, and Braun, unpublished observations). The Y-box family of proteins also repress translation *in vitro* at high protein/mRNA molar ratios, with multiple protein molecules coating a single mRNA, shielding it from the translation machinery (Darnbrough and Ford, 1981; Evdokimova *et al.*, 1995; Marello *et al.*, 1992; Matsumoto *et al.*, 1996). These types of molecular interactions are not necessarily mutually exclusive. For some classes of mRNAs both masking and 5′ cap-dependent mechanisms may cooperate to achieve optimal regulation.

Translational activation requires that the mRNA be mobilized from repressed mRNPs onto polysomes in elongated spermatids. By some unknown mechanism the repression apparatus must be modified to release the mRNA for translation. The finding that newly synthesized Prm-1 protein is found only in the nuclear fraction of sonication-resistant nuclei suggests that the protein is immediately deposited into the nucleus upon synthesis (Green *et al.*, 1994; Lee *et al.*, 1995). One interpretation of this observation is that Prm-1 protein synthesis occurs at or near the nuclear pore. To facilitate this restricted subcellular synthesis the *Prm-1* mRNA may be localized to the nuclear periphery perhaps through interaction with the cytoskeleton. Developmentally regulated protein production near the site of action has been established for a number of mRNAs such as those involved in pattern formation during embryogenesis of *Drosophila*, and in addition there is correlative evidence for the involvement of the cytoskeleton in translational control processes (reviewed by Hesketh, 1994). The recent evidence that mRNPs and polysomes can associate with cytoskeletal elements supports the notion that this is a mechanism for targeted protein synthesis. In elongating spermatids a specialized microtubule array called the manchette forms and may function in nuclear shaping (Fawcett *et al.*, 1971; Russell *et al.*, 1991). It has also been suggested this structure plays a role in overall sperm differentiation by serving as a ''track'' that is utilized for the movement of organelles, vesicles, and mRNPs (Fawcett *et al.*, 1971; MacKinnon and Abraham, 1972; Schumacher *et al.*, 1995). In the molecular screen that yielded *Prbp*, a gene called *Spnr* (spermatid perinuclear RNA binding protein) was also cloned and characterized (Schumacher *et al.*, 1995). *Spnr* encodes an RNA binding protein that is highly expressed in elongating haploid germ cells, is localized to the manchette structure, and

appears to be a microtubule-associated protein (MAP) (Schumacher *et al.*, submitted for publication). Given that Spnr was cloned based on its ability to bind the *Prm-1* 3′ UTR, it may function as a MAP that links the *Prm-1* mRNA to the manchette and thereby plays a role in the putative subcellular localization of protamine mRNA molecules that are destined to be activated for translation at the nuclear periphery. In this capacity, the transgenic mRNA used in this study may lack *Prm-1* 3′ UTR sequences required for Spnr interaction and therefore fails to be properly activated. Since the elongating spermatid is a highly polarized cell where many mRNAs are translationally regulated, it is plausible that certain mRNAs are localized to specific subcellular regions where translation ensues and the protein product is immediately used. The protamine mRNAs might be targeted by such a regulatory mechanism.

MATERIALS AND METHODS

Transgenic Constructs

DNA manipulations were performed using standard procedures. Deletion variants of the *Prm-1* 3′ UTR were fused to a heterologous reporter to evaluate translational control function *in vivo* as previously described (Braun *et al.*, 1989). This reporter cassette contains 4.1 kb of mouse *Prm-1* 5′-untranscribed sequence up to the transcriptional start site, chimeric 5′ UTRs of 159 bp (91 bp of *Prm-1* 5′ UTR, 7 bp of linker DNA, and 61 bp of *hGH* 5′ UTR), and the complete *hGH*-coding sequence and introns (DeNoto *et al.*, 1981).

Microinjection

Transgenic mice were generated by microinjecting ~2 pl of DNA solution into pronuclei of fertilized eggs derived from (C57BL/6 × C3H)F_1 females mated with identical hybrid males (Brinster *et al.*, 1985). Pseudopregnant C57BL/6 foster females were used for oviduct implantation of eggs that survived microinjection. Two lines of transgenic mice (11246 and 11303) were derived carrying transgene D described in Fig. 1. Additionally, three founder males that harbored transgene D, and were known expressors of the transgene, were evaluated for *Prm-1*-like translational control.

Northern Analysis

Total RNA was isolated from dissected mouse testes as described previously (Braun *et al.*, 1989; Cathala *et al.*, 1983). RNA samples (15 μg) were electrophoresed in 2.0% agarose–formaldehyde gels, transferred to nylon membrane (Hybond N, Amersham Life Science), and hybridized 12–15 hr with radioactive [α-^{32}P]DNA probes prepared by random oligonucleotide-primed synthesis (Feinberg and Vogelstein, 1984). The nylon membrane was subsequently washed at a final stringency of 0.1× SSC and 0.5% SDS at 55°C and autoradiographed.

Protein Extracts and Western Blotting

Testes were dissected and homogenized in 0.8 ml of buffer containing 10 m*M* Hepes, pH 7.3, 50 m*M* NaCl, 10 m*M* EDTA, supplemented with protease inhibitors (0.5 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml apoprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 1 mM benzamidine, and 1 μg/ml Na-t-BOC-deacetylleupeptin). The proteins were solubilized in 1% SDS on ice for 15 min, sonicated until they were no longer viscous, and microfuged for 5 min. Protein concentration was determined by the enhanced alkaline copper protein assay (Lowry, 1951) and equivalent amounts of proteins were mixed with Laemmli buffer (Laemmli, 1970), boiled, and electrophoresed in SDS–15% polyacrylamide gels. The proteins were electroblotted onto nitrocellulose membrane (Towbin *et al.*, 1979) for 4 hr at 200 mA at 4°C. The membrane was blocked at room temperature for 1 hr in 5% nonfat dry milk and phosphate-buffered saline (BPBS) and then incubated overnight at 4°C with primary antibody (anti-hGH). After being washed once in BPBS supplemented with 0.05% Tween 20 and twice in BPBS for 20 min, the membrane was incubated with a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad) for 3 hr at room temperature. After washing as above, the HRP enzyme was detected using a modified enhanced chemiluminescence (ECL) protocol for Western blots described previously by Schneppenheim and Rautenberg (1987). ECL reagent (50 ml) was made immediately before using by dissolving 40 mg of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and 10 mg of 4-iodophenol in 1.0 ml of DMSO. Following the addition of 10 ml of 0.1 *M* Tris–Cl, pH 8.5, 5.0 ml of 5 *M* NaCl, 17 ml of H_2O, and 125 ml H_2O_2 the membrane was incubated for 1–2 min, drained, and exposed to X-ray film (Kodak XAR 5).

Immunocytochemistry

Testes were dissected from sexually mature males, fixed in 60% ethanol, 30% chloroform, and 10% glacial acetic acid, embedded in paraffin, and cut into 5-μm sections. Sections were deparaffinized with xylene and rehydrated using standard procedures. Tissue sections were treated with primary antibody (rabbit anti-hGH) as previously described (Braun *et al.*, 1989). The rabbit anti-hGH antibody was obtained from the National Hormone and Pituitary Program (Baltimore, Maryland). Biotinylated goat anti-rabbit IgG and streptavidin conjugated to horseradish peroxidase were used as recommended by the manufacturer (Zymed Laboratories, Inc.). Peroxidase activity was visualized with the chromogen aminoethyl carbazole. Sections were counterstained with hematoxylin. Negative controls using both primary and secondary antibodies were performed on testes isolated from nontransgenic animals.

In Situ Hybridizations

Testes were dissected from adult animals, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were deparaffinized with xylene and rehydrated using standard procedures. The sections were washed once in phosphate-buffered saline, pH 7.4 (PBS), for 5 min, washed once in 0.1 *M* triethanolamine (TEA) for 3 min, treated with 0.25% acetic anhydride in TEA for 5 min, washed again in PBS for 5 min, and prehybridized in hybridization solution (50% formamide, 5× SSC, 100 μg/ml sonicated salmon sperm DNA, 300 μg/ml yeast RNA, 100 μg/ml heparin (Sigma H2149), 0.1% Tween 20, and 10% dextran sulfate) for 4–5 hr at 50°C. Single-stranded ^{33}P-containing sense and antisense riboprobes were made *in vitro* with linear templates and either SP6 or T7 RNA polymerase (Melton *et al.*, 1984). The mouse *Prm-1* riboprobe was 250 bp in length and contained a por-

tion of exon 1, the intron, and a portion of exon 2 as previously described by Braun *et al.* (1989). The *hGH* riboprobe was 170 bp in length and contained hGH exon 5 (Braun *et al.*, 1989). Reaction yield and full-length integrity were assessed by gel electrophoresis, and probes were used at a concentration of ~5 μg/ml. Sections were hybridized with radioactive probes for 12–15 hr at 50°C, washed in 4× SSC and 10 m*M* DTT for 15 min at room temperature, incubated for 30 min at 37°C in 10 m*M* Tris, pH 8.0, 0.5 *M* NaCl, 10 m*M* DTT, and 20 μg/ml RNase A, and washed sequentially in 2× SSC, 50% formamide, and 10 m*M* DTT for 30 min at 55°C, 1× SSC, 50% formamide, and 10 m*M* DTT for 30 min at 55°C, 0.1× SSC for 30 min at 37°C. Slides were dehydrated with ethanol using standard procedures, dried for 12–15 hr, dipped in Kodak NTB-3 emulsion, stored at 4°C, developed after 4–21 days, and counterstained with hematoxylin. Transgenic mRNA accumulation and persistence was qualitatively compared to that of the endogenous *Prm-1* mRNA.

Polysome Analysis

RNA was isolated and purified from polysomes fractionated by sucrose gradient centrifugation as described previously (Kleene, 1993; Mach *et al.*, 1986). Briefly, testis was dissected from a sexually mature mouse carrying transgene D (Fig. 1) and homogenized in 1 ml of lysis buffer (100 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 20 m*M* Hepes, pH 7.6). The nuclei and mitochondria were collected by centrifugation for 2 min at 12,000*g*, and the supernatant was layered over a 11.0-ml linear 15–50% (w/w) sucrose gradient in lysis buffer. After centrifugation at 36,000 rpm in a Beckman SW 40 rotor for 110 min, the gradients were fractionated into 12 1-ml fractions using an Isco Density Gradient Fractionator (Model 185), while monitoring ultraviolet absorbance at 254 nm. SDS was added to 1% and the fractions were treated with proteinase K at 0.2 mg/ml for 90 min at 37°C. NaCl was added to 0.2 *M*, the samples were extracted with phenol:chloroform (1:1 v/v), and the nucleic acid was precipitated with ethanol. Polysome profiles for the transgenic mRNA and the endogenous *Prm-1* mRNA were evaluated using Northern blot analysis as described earlier. As a control to verify mRNA association with polysomes, equivalent supernatants were prepared and centrifuged in sucrose gradients in buffer in which the $MgCl_2$ was replaced by 20 m*M* EDTA. The presence of EDTA causes mRNA and ribosomes to dissociate; hence, polysomal mRNAs sediment more slowly than single ribosomes (Penman *et al.*, 1968; Kleene, 1989). Polysome distribution of chimeric and endogenous mRNAs was assessed in at least two separate experiments.

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