High accumulation of components of the RNA polymerase II transcription machinery in rodent spermatids

SCHMIDT, Edward Eric, SCHIBLER, Ulrich

Abstract
Levels of mRNA and protein encoded by the TATA-binding protein (tbp) gene are shown to increase dramatically during late spermatogenesis in rodents, culminating in a highly testis-enriched expression pattern. Whereas adult spleen and liver contained roughly 0.7 and 2.3 molecules of TBP mRNA per haploid genome-equivalent, respectively, adult testis contained 80-200 molecules of TBP mRNA per haploid genome-equivalent. Comparison of nuclear and cytoplasmic levels of TBP mRNA in liver and testis suggested that nuclear events (transcription or processing) contribute roughly 12-fold, and cytoplasmic events (mRNA stability) roughly 6-fold, to testis-specific overaccumulation. Levels of nuclear TBP protein in testis cells were, on average, 8- and 11-fold higher than those in liver and spleen cells, respectively. Overexpression of TBP mRNA in testis began about 20 days after birth and reached a plateau around day 40, corresponding to the developmental emergence of haploid cells. Besides TBP, two other components of the general RNA polymerase II machinery, TFIIB and RNA polymerase II, were also overexpressed in testis. By [...]
High accumulation of components of the RNA polymerase II transcription machinery in rodent spermatids

Edward E. Schmidt and Ueli Schibler
Department of Molecular Biology, University of Geneva, Sciences II, 30 Quai Ernest-Ansermet, CH-1211 Geneva-4, Switzerland

SUMMARY

Levels of mRNA and protein encoded by the TATA-binding protein (tbp) gene are shown to increase dramatically during late spermatogenesis in rodents, culminating in a highly testis-enriched expression pattern. Whereas adult spleen and liver contained roughly 0.7 and 2.3 molecules of TBP mRNA per haploid genome-equivalent, respectively, adult testis contained 80-200 molecules of TBP mRNA per haploid genome-equivalent. Comparison of nuclear and cytoplasmic levels of TBP mRNA in liver and testis suggested that nuclear events (transcription or processing) contribute roughly 12-fold, and cytoplasmic events (mRNA stability) roughly 6-fold, to testis-specific overaccumulation. Levels of nuclear TBP protein in testis cells were, on average, 8- and 11-fold higher than those in liver and spleen cells, respectively. Overexpression of TBP mRNA in testis began about 20 days after birth and reached a plateau around day 40, corresponding to the developmental emergence of haploid cells. Besides TBP, two other components of the general RNA polymerase II machinery, TFIIB and RNA polymerase II, were also overexpressed in testis. By immunostaining, it was found that TBP and RNA polymerase II were particularly rich in round spermatid nuclei. Our results suggest a molecular explanation for how early spermatids are able to accumulate all of the mRNA necessary for the final week of spermiogenesis.

Key words: RNA polymerase II, spermiogenesis, TATA-binding protein, transcription, rat

INTRODUCTION

Spermatogenesis is the process through which diploid stem cells (spermatogonia) differentiate into mature haploid spermatooza. Major postembryonic steps in mammalian spermatogenesis begin with mitotic proliferation of the spermatogonia. Some of the resultant daughter cells remain as stem cells; the rest differentiate into spermatocytes and undergo meiosis. Each spermatocyte yields four haploid spermatids. During spermiogenesis (the haploid stages of spermatogenesis), the acrosome and flagella are synthesized, histones are replaced by protamines leading to chromatin condensation, and finally, the condensed late spermatid is released from the bulk of its cytoplasm (reviewed in Bellvé et al., 1977; Kleene et al., 1983; Leblond and Clermont, 1952; Meistrich, 1989). The net result of this process is a gross morphological, biochemical, structural and genetic transformation that provides one of the most dramatic examples of cell differentiation and specialization.

Mature spermatooza exhibit highly compacted chromatin and are transcriptionally inactive (Monesi, 1964). In mammals, chromatin condensation involves the sequential replacement of somatic histones by transition proteins and protamines (Balhorn et al., 1984; Bellvé et al., 1975; Bellvé, 1979; Grimes et al., 1977; Kistler et al., 1973; Mayer et al., 1981; Meistrich, 1989). Chromatin condensation is one of the final steps in spermatogenesis. Many of the proteins involved in condensation are synthesized from mRNAs which were, themselves, synthesized several days earlier and stored in a translationally inactive state (Balhorn et al., 1984; Braun et al., 1989; Kleene et al., 1984). For example, transcription of the protamine 1 and 2 genes and the transition protein 1 and 2 genes is detected only in early spermatids, at which time these mRNAs accumulate to very high levels. Translation of these mRNAs occurs several days later (Hecht et al., 1986; Heidaran et al., 1987; Kleene and Flynn, 1987; Kleene et al., 1990; Kleene, 1993). The synthesis of these abundant mRNAs in early spermiogenesis might place stringent demands on the RNA polymerase II (pol II) machinery.

We have been interested in the mechanisms regulating overall rates of transcription in metazoan cells. Previous investigations revealed a correlation between cell size and overall transcription rates in various tissues (Schmidt and Schibler, 1995). Because overall transcription rates by all three RNA polymerases appear to be cell size-dependent, we began investigating whether components of the general RNA polymerase II machinery, TFIIB and RNA polymerase II, were overexpressed in testis. By immunostaining, it was found that TBP and RNA polymerase II were particularly rich in round spermatid nuclei. Our results suggest a molecular explanation for how early spermatids are able to accumulate all of the mRNA necessary for the final week of spermiogenesis.
stages. Further analysis suggested that RNA polymerase II was also highly abundant in the nuclei of round spermatids. Our findings suggest that overexpression of the pol II transcription apparatus might be a general property of this stage of spermatogenesis.

MATERIALS AND METHODS

Animals, sample preparations and immunostaining
Male laboratory rats (Lewis) or mice (MORO) were used in all experiments. RNA and nuclear extracts were prepared from tissues and cultured cells as described by Schmidt and Schibler (1995) and Schmidt et al. (1991). For immunostaining, small pieces of decapsulated rat testes were fixed for 2 hours at room temperature in 5% glutaraldehyde/PBS overlaid with heptane. After washing and dehydrating, tissues were embedded in Paraplast supplemented with 0.8% dimethylsulfoxide. Sections attached to 3-aminopropylsilane-coated slides were dewaxed in xylene followed by 100, 95, and 80% ethanol. Slides were treated for 20 minutes in 80% methanol, 3% hydrogen peroxide, hydrated, and postfixed in 4% paraformaldehyde/PBS. Sections were preincubated with PBS containing 0.1% Triton X-100, 1% nonfat dry milk (PTM) supplemented with 1% normal goat serum for 1 hour followed by first antibody (1:20) in PTM for 1 hour. Sections were washed five times in PTM and incubated in second antibody (goat-anti-rabbit, or anti-mouse, horseradish peroxidase-conjugated, 1:100 dilutions) in PTM for 1 hour and washed as above. Control sections were incubated with no antibody, with only second antibody-conjugates, or with non-immune rabbit serum followed by anti-rabbit peroxidase conjugate. Staining was with diaminobenzidine.

Rabbit-anti-human TBP antiserum (raised against the whole molecule) was a generous gift from A. Hoffmann and R. Roeder; mouse-anti-human TBP monoclonal antibody (undefined epitope) was purchased from Santa Cruz Biotechnology. The mouse-anti-human pol II antibody (CTD epitope) was a generous gift from C. Kedinger.

RNase protection assays
RNase protection assays were as described by Schmidt and Merrill (1989) and Schmidt and Schibler (1995). Signals were quantitated by liquid scintillation of excised gel bands. Plasmid 6His-pET IId contained the human TBP cDNA, which was a gift from A. Hoffmann and R. Roeder; mouse-anti-human TBP monoclonal antibody (undefined epitope) was purchased from Santa Cruz Biotechnology. The mouse-anti-human pol II antibody (CTD epitope) was a generous gift from C. Kedinger.

RESULTS
Average cell size differences in many rodent tissues correlate to differences in overall transcriptional activities (Schmidt and Schibler, 1995). Therefore, we began investigating whether components of the basal transcription machinery may limit transcription rates in small-celled tissues. The amount of mRNA encoding the TATA-binding protein, TBP, was measured in total RNA samples, normalized to equal DNA-equivalents of tissue, from rat liver, lung, kidney, spleen, brain, testis, heart and thymus. RNA:DNA ratios in these tissues vary over a 16-fold range, with liver having a ratio of 4.7 and thymus a ratio of 0.3 (Schmidt and Schibler, 1995). The results (Fig. 1A) showed that the organs with the smallest average cell size (lung, spleen and thymus) generally exhibited fewer TBP mRNA molecules per DNA than organs with larger average cell size (liver, kidney and heart; Schmidt and Schibler, 1995). Surprisingly, however, testis contained levels of TBP mRNA which were approximately two orders of magnitude higher than those in the other tissues.

One difference between adult testis and most other tissues is that testis contains a high proportion of dividing cells. We therefore investigated whether TBP mRNA might generally be expressed at higher levels in proliferative as opposed to nondividing cells. This question was approached in two systems. First, TBP mRNA levels in adult testis were compared with those in several proliferating tissue culture cell lines (Fig. 1B). TBP mRNA levels in the cultured cell lines were much lower than those in testis. Second, accumulation of TBP mRNA was compared in RNA samples harvested from liver during postembryonic development. In newborn rat liver, almost all cells are proliferative; between 7 and 28 days after birth, the mitotic index of liver decreases, reaching a basal level of roughly 0.01% (reviewed by Leffert et al., 1988). Concomitantly, average RNA:DNA ratios for liver cells increase about 3-fold (Schmidt and Schibler, 1995); cellular TBP mRNA levels increased slightly as well (Fig. 1C). In conclusion, high overexpression of TBP mRNA...
did not correlate with cellular proliferation, but rather, was a specific property of testis.

To estimate cellular numbers of TBP mRNA molecules, we compared cellular RNA samples to a standard curve containing known amounts of synthetic TBP pseudo-mRNA by RNase protection (Gross et al., 1987; Fig. 2). The results showed that two human cell lines, HeLa and HepG2, contained roughly 6 molecules of TBP mRNA per cell. Mouse L cells and rat C2Rev7 hepatoma cells contained about 5 molecules of TBP mRNA per cell. Rat liver and spleen contained an average of 5 and 1.5 molecules of TBP mRNA per diploid genome-equivalent, respectively. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.

Several tests were performed to determine whether our data were truly TBP mRNA-specific. First, two distinct probes to the 5’ nonconserved region of rodent TBP mRNA, one spanning sequences from 36 to 135 (Fig. 3A) and one spanning sequences from 136 to 289 of the mouse cDNA (not shown), showed levels of TBP mRNA in testis and spleen which matched those detected using the probe for the 3’ conserved region. In contrast, the testis sample exhibited 350 TBP mRNA molecules per diploid genome-equivalent. Quantitation of TBP mRNA levels from numerous individual animals indicated that adult rat testis contains between 160 and 400 molecules of TBP mRNA per diploid genome-equivalent.
whereas whole testis contained 50- to 80-fold more TBP mRNA per genome-equivalent than whole liver (Fig. 3D). Because TBP mRNA represented a much higher proportion of total than of nuclear RNA in testis, whereas in liver TBP mRNA was somewhat more abundant in nuclear than in total RNA preparations, the results suggest that cytoplasmic TBP mRNA was more stable in testis than in liver. Thus, overaccumulation of TBP mRNA resulted from both testis-specific nuclear (transcription or processing, about 12-fold) and cytoplasmic (mRNA stability, about 6-fold) determinants (see Discussion).

Western blots indicated that, consistent with the high levels of TBP mRNA, TBP protein levels in testis nuclei were much higher than those in nuclei from other tissue types (Fig. 4). Densitometric quantitation of signal intensities suggested that the magnitude of TBP protein overexpression in testis was less than the magnitude of TBP mRNA overexpression (compare Figs 1A and 4). Thus, testis gave a TBP-specific band intensity which was 7.9- and 11.4-fold above the TBP-specific band intensity for an equal DNA-equivalent of nuclei from liver and spleen, respectively. It might be noteworthy that uniquely for testis, much of the input DNA will be from transcriptionally inactive late spermatids. As a result, the proteins associated with transcriptionally active nuclei will be diluted by this additional DNA, causing the testis lane to be somewhat underloaded for analysis of components of the transcription

Fig. 2. Quantitation of TBP mRNA levels. The alignment of rodent (r) and human (h) tbp sequences in the 3′ probe region is indicated at the top, with X denoting mismatches and the translational stop codons underlined. RNase-resistant hybrids between probe and rodent (r/h) or human (h/h) TBP mRNAs are denoted by heavy lines. The indicated molar amounts of synthetic TBP pseudo-mRNA or the indicated weights of total RNA from tissues or cell lines were analyzed by RNase protection with the 3′ TBP probe as in Fig. 1. The cell lines used were: HeLa, a human cervical carcinoma; HepG2, a human hepatoma; tk L, a mouse fibroblast; and C2Rev7, a rat hepatoma. Bands were excised and the associated radioactivity was assayed by liquid scintillation counting. Rodent TBP mRNA signals were corrected for the difference in length between the rodent and pseudo-mRNA protected fragments. Calculated molar quantities are listed below the autoradiogram. Dashes correspond to lanes in which the signal was greater than the highest point in the standard curve and therefore could not be reliably estimated. Molar values were converted into molecules per cell by multiplying by the RNA:DNA ratio and assuming 6.6 pg DNA per diploid genome-equivalent. Abbreviations as in Fig. 1; P denotes a roughly 1:100 dilution of nondigested probe.
Fig. 3. Physical characterization of testis TBP mRNA. (A) 5' and 3' sequences of TBP mRNA are overexpressed in testis. At the top is a schematic of the 1640 bp mouse TBP cDNA with the protein-coding region hatched, the conserved protein coding region underlined, and probe positions indicated by 3'-5' oriented arrows. Hybridizations were as in Figs 1 and 2 using 25 μg DNA-equivalents of testis (T) and spleen (S) RNA supplemented to 100 μg with yeast RNA. Size markers (M) from top were 221/220, 154 and 74 bases. (B) Sedimentation analysis of TBP mRNA. Total RNA purified from the indicated tissues was sedimented in parallel through sucrose gradients under conditions which empirically optimize separation of RNAs between roughly 100 and 3000 bases. Stained denaturing gels were used to determine which fractions contained tRNA (85 bases) and 18S (1950 bases), or 28S (4712 bases) rRNAs. The position of TBP mRNA was determined by RNase protection using the 3' probe and RNA purified from each fraction. (C) Comparison of TBP mRNA levels in total and poly(A)+ RNA preparations. RNase protections using the 3' probe contained the indicated amounts of RNA supplemented to 100 μg with yeast RNA. Poly(A)-enrichment yielded 5% mass recovery; methylene-blue staining of the A+ RNA after denaturing gel electrophoresis and transfer to Nytran membranes showed little or no residual rRNA (not shown). (D) Nuclear/cytoplasmic distributions of TBP mRNA in testis and liver. RNase protections were as in preceding Figs using the 3' probe and an equal weight of nuclear RNA, or equal DNA-equivalents of total RNA, supplemented to 100 μg with yeast RNA. The marker lane (M) contains only the 74/75-base doublet.
machinery relative to the other tissues. In conclusion, in testis as compared to other tissues, levels of TBP mRNA and protein per genome-equivalent were roughly two and one orders of magnitude higher, respectively (see Discussion).

We next measured whether TBP overexpression was a property that arose during sexual maturation. Juvenile rat testis exhibited low levels of TBP mRNA (Fig. 5A). Overaccumulation of TBP mRNA began between 18 and 28 days after birth. Average per-cell levels of TBP mRNA increased until about 40 days after birth, at which time levels were 50-fold above those observed in juvenile testis. A similar analysis was performed in mice using higher resolution time points to more accurately characterize the transition (Fig. 5B,C). The increase was most rapid between 20 and 30 days of age. The time-course of testis maturation has been characterized in mouse (Bellvé et al., 1977; Nebel et al., 1961). At precise ages, specific new cell types arise in the spermatogenic pathway. The developmental accumulation pattern of TBP mRNA roughly correlates with the beginning of spermiogenesis and the appearance of the first haploid spermatids. As the onset of the TBP mRNA increase was seen as early as 17 days in mice (Fig 5B), up-regulation of the \( tbp \) gene may already begin in late spermatocytes.

The observed TBP abundance prompted us to ask whether other components of the basal RNA polymerase II transcriptional machinery were also overexpressed in adult testis. Western blots for the large subunit of RNA polymerase II (pol II) in nuclei from testis, spleen, lung, brain and kidney indicated that average nuclear pol II protein levels were higher in testis than in the somatic tissues (Fig. 6A). Comparison with the dilution curve revealed that the signals for pol II in testis were between 3- (spleen and lung) and 1.5-fold (kidney and brain) higher than in somatic tissues. Although these magnitudes are modest, it should be reiterated that the results represent the biochemical average for all nuclei in each sample. A subset of testis nuclei are transcriptionally inactive (see above). Moreover, if pol II overexpression were limited to only a fraction of the remaining nuclei, the levels in these nuclei could be significantly higher (see below).

A third essential component of the basal pol II transcription machinery, TFIIB, was also investigated. Unfortunately, using a commercial antibody (Santa Cruz Biotechnology), we were

![Fig. 4. Overaccumulation of TBP protein in testis.](image)

![Fig. 5. TBP mRNA levels during sexual maturation in rat testis. Total nucleic acid and pure RNA was harvested from rodent testes. For rat (A) all samples were prepared in parallel. Total nucleic acid was used to determine the RNA:DNA ratios for each sample. Each sample represents an individual animal. The RNA:DNA ratios for the rat samples, in order as they appear in the autoradiogram from left to right, were 1.23, 1.26, 1.35, 1.02, 0.51, 0.79, 1.24, 1.14, 1.60, 1.82, 1.96, 2.42, 1.30, 2.45, 2.42, 2.58. The amount of RNA corresponding to 20 \( \mu \)g of DNA was supplemented to 100 \( \mu \)g with yeast RNA and hybridized to the 3' TBP probe. The age and weight of each rat, and the combined weight of both testes, are listed below the autoradiogram as indicators of the stage of sexual development. For mouse (B), the 17-30 day samples were prepared from littermates harvested sequentially. Each sample represents an individual animal except the 14-day mouse sample (B), for which testes from 4 brothers were pooled. Bands were excised from the gels and the associated radioactivity was determined by liquid scintillation. Values for the mouse samples are presented in C.](image)
unable to detect TFIIB on western blots. By RNase protection, samples from eight different adult tissues showed that, with the exception of testis, all tissues contained similar numbers of TFIIB mRNA molecules (Fig. 6B). In contrast, testis contained, on average, 6- to 11-fold more TFIIB mRNA molecules per unit DNA than the other tissues. Comparison with a standard curve (Fig. 6C) indicated that testis cells contained, on average, 46 TFIIB mRNA molecules; somatic tissue types contained, on average, roughly 6 TFIIB mRNA molecules per diploid genome-equivalent. During testis maturation in rat, TFIIB mRNA levels increased from somatic cell levels in juvenile rats to adult levels (Fig. 6D) with a time course that resembled that of TBP mRNA (Fig. 5A).

Testis contains cells of numerous types and developmental stages. To visualize which cells overaccumulated TBP and pol II, immunohistochemistry was used. DNA counterstaining allowed estimation of cell ploidy from the micrographs. Immunostaining with the pol II antibody showed that overexpression of the antigen was restricted to a specific subset of nuclei in the sectioned tubules (Fig. 7C-L). Thus, for example, Fig. 7G shows 3 tubules at different spermatogenic stages with distinct pol II distributions. The uppermost tubule exhibits strong pol II staining in most haploid cells and almost none in the diploid and tetraploid peripheral cells; the lowermost tubule exhibits diminished staining in the haploid spermatids, and increased staining in a subset of peripheral cells. In the middle tubule, little staining persists in haploid cells. Higher magnification micrographs (Fig. 7I-L) confirmed that staining was nuclear and predominantly in haploid cells, leading us to conclude that it is primarily the round spermatid nuclei which are overaccumulating pol II (note that the staining interferes strongly with Hoechst’s fluorescence). We remain uncertain of the identity of the peripheral cells which stained predominantly in tubules lacking early spermatids (e.g. Fig. 7E).

Epitopes detected with the TBP antibodies were also predominant in round spermatids (Fig. 7M-P). TBP epitopes were below detectable levels in most of the diploid and tetraploid cells at the periphery of the tubules, including the subset of peripheral nuclei that stained for pol II. Both a commercial mouse monoclonal antibody (Fig. 7M-P) and a rabbit polyclonal antibody (not shown) gave similar localization of antigen in tubules, suggesting that the detected epitopes truly represented TBP. The bulk of TBP antigen was enriched in the nuclei of the round spermatids (note that the staining interferes strongly with Hoechst’s fluorescence, thereby allowing precise localization of antigen). However, perhaps in contrast to pol II, TBP epitopes appeared to persist into later stages at which point they became more punctate in nuclei (lower tubule in Fig. 7M) and perhaps partially extranuclear (upper tubule in Fig.

**Fig. 6.** Tissue-specific expression of RNA polymerase II and TFIIB. (A) Whole nuclei (1.0 corresponds to 25 μg DNA-equivalents) from the indicated tissues were sonicated in SDS/loading dyes, separated on polyacrylamide gels, blotted, and visualized with the anti-pol II monoclonal antibody. Signals were quantitated by comparison to dilutions of testis nuclei. (B) TFIIB mRNA levels in rat tissues. Total RNA (20 μg DNA-equivalents) were used in an RNase protection assay with the TFIIB probe. Bands were excised and assayed by liquid scintillation counting. The TFIIB-specific values were, 294, 193, 194, 169, 188, 1810, 263, and 203 cpm for L, U, K, S, B, T, H, and Y, respectively. (C) Absolute TFIIB mRNA levels in testis. TFIIB probe was hybridized to the indicated molar quantities of TBP pseudo-mRNA or total rat testis RNA. All samples were supplemented to 100 μg with yeast RNA. Calculated molar values are listed below the autoradiogram. (D) TFIIB mRNA accumulation during rat testis maturation. Samples and assay conditions were exactly as in Fig. 5A except that the TFIIB-specific probe was used.
Commercial TFIIB antibody gave only weak signals in immunostaining, and thus precluded rigorous immunolocalization of this protein.

Most nuclei that stained for pol II and TBP were large and exhibited a relatively low DNA content by Hoechst’s staining. As these nuclei were roughly the same size as, or larger than, the diploid nuclei around the periphery of the tubule, which did not stain strongly, we conclude that the concentrations of antigens in these nuclei were indeed higher than those in most diploid cells. Because these cells appear haploid, the amount of TBP protein per genome-equivalent was likely 2-fold higher yet. In addition, as no immunostaining was seen in condensed sperm nuclei, which maintain the same DNA content as the early spermatids concentrated into a much smaller volume, we conclude that most or all epitopes were either lost from the nuclei prior to condensation or became masked (see Discussion).

Fig. 7. Immunolocalization of pol II and TBP. Adult rat testis sections (5 μm) were immunostained using monoclonal anti-pol II antibody (C-L) or monoclonal anti-TBP antibody (M-P) and goat-anti-mouse peroxidase or using only goat-anti-mouse peroxidase as controls (A,B). Sections were counterstained for DNA using Hoechst’s, and visualized with white light (A,C,G,K,M,O), Hoechst’s fluorescence (panels B,D,F,H,J,L,N,P), or both (E,I). Extranuclear fluorescence (e.g. in center of tubules) is Hoechst’s stain-independent, and represents endogenous fluorescence which was accentuated by glutaraldehyde fixation. Because the staining product accumulates at the antibody-accessible surfaces, at high magnification, immunostaining and DNA fluorescence are in slightly different focal planes giving rise to the ‘faded-out’ appearance of immunostaining in O as compared to P. Scale bars represent 200 μm (A-F); 100 μm (G,H,M,N); 32 μm (I,J); 20 μm (K,L,O,P).
tion). In conclusion, our results suggest that TBP and pol II are overexpressed in the early haploid stages of rodent spermiogenesis. In addition, pol II overexpression occurred in a subset of peripheral cells whereas TBP expression persisted into somewhat later stages.

**DISCUSSION**

Accumulation of TBP and TFIIB in somatic cell types

Our investigation of tbp expression patterns was initiated in an attempt to examine whether TBP might play a role in determining cell size-specific transcription rates. Our data suggest that TBP levels do vary somewhat between somatic tissue types as a function of cell size and increase concomitant with the increase in RNA:DNA ratios during liver maturation. Presently, however, we remain uncertain of whether these modest differences contribute to determination of cell size-dependent transcription rates (see Schmidt and Schibler, 1995).

The observation that somatic cells contained a steady-state level of only 1.5 to 6 TBP and TFIIB mRNA molecules per diploid genome-equivalent might provide some insight into the kinetics of how TBP and TFIIB proteins function. In rodents, both proteins are encoded by 316-codon open reading frames (Tamura et al., 1991; Tsuboi et al., 1992). Eucaryotic ribosomes elongate at an in vivo rate of approximately 10 amino acids per second (Hunt et al., 1969). Liver contains about 5 molecules of TBP mRNA per diploid genome-equivalent (Fig. 2), which are engaged by an average of roughly 5 ribosomes per mRNA (data not shown). Thus, we can estimate that liver cells synthesize and degrade roughly 2800 TBP protein molecules per hour.

In proliferating cells such as HeLa, the amounts of all cellular macromolecules must double each generation. As a result, even if the chemical halflife of a molecule were nearly infinite, such as for DNA, cells must synthesize a second cellular complement of the molecule each generation (Schmidt, 1990). HeLa cells contained only 3 molecules of TBP mRNA per haploid genome-equivalent (Fig. 2), which, if translation rates are similar in HeLa and liver, is expected to yield 1600 TBP protein molecules per hour per haploid genome (see above). During each cell cycle, the cell exhibits 2N (G1 phase), 4N (G2) and intermediate (S) DNA-contents. Assuming an average of 3N, HeLa cells synthesize TBP at an average rate of 4800 molecules per hour; in a typical 18 hour generation a cell can synthesize 8.6×10^4 molecules of TBP protein. However, depending on the stability of TBP protein, only a fraction of this amount will accumulate. For example, if the halflife of TBP were 1 hour, HeLa cells would contain 3600 molecules of TBP in early G1 phase and 7200 at M phase (i.e. 1800 per haploid genome); if the halflife were 10 hours, the cells would maintain 15,000 per haploid genome. As HeLa cells express an estimated 10,000 genes per haploid genome (reviewed by Lewin, 1974), our results suggest that TBP protein is not present in great excess over the number of active genes in a cell (from <1 TBP to, at most, 4.3 TBP per active promoter, depending on the halflife). This is compatible with models wherein promoters compete for a limited pool of TBP and TFIIB for assembly of an initiation complex (e.g., Choy and Green, 1993).

High accumulation of the pol II machinery in round spermatids

Several highly abundant testis-specific mRNAs have been characterized which are synthesized only during early spermiogenesis in rodents and are stored as RNP particles (reviewed by Kleene, 1993). These include the mRNAs for protamines 1 and 2, and transition proteins 1 and 2. The cognate proteins contribute to the transition from somatic chromatin to the condensed sperm chromatin (Kleene, 1993). Precocious accumulation of these proteins might be incompatible with transcription (reviewed by Meistrich, 1989). As a result, the early spermatids must synthesize and accumulate all of the RNA that will be required for the final week of sperm maturation.

The synthesis of storage mRNAs by early spermatids might require unusually high levels of RNA polymerase II activity. Kleene et al. (1983) demonstrated that isolated round spermatids (including both transcriptionally active early spermatids and later stages) contain roughly 6-fold higher ratios of poly(A):ribosomal RNA than either liver or immature testis. Of the polyadenylated RNA in whole testis, roughly 70% is found in nonpolysomal RNP particles (data not shown; and Gold et al., 1983; Stern et al., 1983; Tafuri et al., 1993). These observations are consistent with the accumulation and storage of a large mass of translationally inactive mRNA by early spermatids.

Our work suggests that TBP, TFIIB, and pol II are highly abundant in round spermatids. This may suggest a mechanistic explanation for how early spermatids are able to synthesize all of the mRNAs they will require for the final days of spermiogenesis in a relatively short burst. Thus, by overaccumulating the basal transcriptional machinery, spermatids might achieve an unprecedented rate of pol II transcription. Various tenets of this model remain to be tested, however. For example, as yet we have no direct assay for spermatid-specific pol II activity. Curiously, although examples of transcriptional regulation via promoter-specific transcription factors are numerous, this work suggesting that up-regulation of the RNA pol II machinery leads to increased spermatid transcription is among the first studies to indicate that transcription might be regulated in a cell type-specific manner by directly regulating levels of the basal transcription machinery.

Monesi (1964) reported that uridine incorporation by spermatogenic cells was highest in late pachytene spermatocytes, that is, the tetraploid cells just before the first reductive division. Lower uridine incorporation was detected in early spermatids and none in later stages. However, ploidy was not considered in the analysis by Monesi. Thus, the pachytene nuclei will have four times more copies of all genes than the early spermatids exposed to roughly the same area of photographic emulsion. This may account for a large part of the increased silver grain count over these nuclei. Spermatids appear to remain relatively small (e.g., see tight nuclei packing in Fig. 7), they do not exhibit dense cytoplasmic staining (Clermont, 1972; Leblond and Clermont, 1952), and they do not exhibit higher RNA:DNA ratios than pachytene spermatocytes (Kleene et al., 1983). Thus, it seems likely that ribosomes do not overaccumulate at this stage, only mRNA. The preexisting ribosomes may be stable enough to persist throughout spermiogenesis. If only pol II transcription were increased in the early spermatids, the study by Monesi might not have detected increased uridine incorporation.
Drosophila have a \( \text{tbp} \)-related gene, \( \text{trf} \), which is preferentially expressed in testis (Crowley et al., 1993). Vertebrate \( \text{trf} \) has not been reported, and it is unlikely that our data represent cross-detection of a \( \text{tbp} \)-related gene. Even in the conserved \( 3' \) region, Drosophila \( \text{tbp} \) and \( \text{trf} \) are less similar than Drosophila \( \text{tbp} \) is to human or yeast \( \text{tbp} \) (Crowley et al., 1993). Fig. 2 shows that under our conditions, a region of 3 out of 5 mismatches between our \( 3' \) RNase protection probe and an mRNA yields complete sensitivity to RNase digestion. Yeast RNA exhibits no protected fragments large enough to remain on gels, and thus an mRNA like \( \text{trf} \), which is even more divergent, would not likely be detected. Moreover, two probes to the \( 5' \) nonconserved region of mouse \( \text{tbp} \) quantitatively corroborated the results we obtained with the \( 3' \) conserved region probe. Thus, the signal we detect is almost certainly TBP mRNA.

The degree of TBP mRNA overexpression in testis, as compared to somatic tissues, was greater than the degree of TBP protein overexpression that we detected. Thus, in testis, either the average translational efficiency of TBP mRNA or the average stability of TBP protein is lower than in somatic tissues. Preliminary data suggest that much of the TBP mRNA in testis is nonpolysomal (not shown), and may therefore be translationally repressed. This sequestration of TBP mRNA in RNPs may also account for the greater stability of TBP mRNA in testis as compared to liver (Fig. 3D). It is conceivable that the nonpolysomal TBP mRNA is being stored for a later function, as has been shown for the mRNAs encoding protamines and transition proteins (see above). Interestingly, however, after the early spermatid stage, the sperm DNA (and its progeny) will not be transcribed again until activation of embryonic transcription. Thus, if the translationally blocked TBP mRNA were going to subsequently give rise to TBP protein which would function in transcription initiation, it would first have to weather a lengthy journey in the spermatoozoon. Recent reports have suggested that TBP might be able to affect gene activity in Xenopus zygotes (Prieouleau et al., 1994) and might play a regulatory role in mouse early embryonic development (Worrad et al., 1994). Thus, we were curious about whether the spermatoozoon might package TBP mRNA for transport and delivery into the zygote. However, we have been unable to extract either TBP mRNA or TBP protein from spermatoozoa isolated from epididymus (not shown). We therefore suspect that overaccumulation of TBP mRNA in testis is required for performing testis-specific, rather than post-spermatogenic, functions. Whether these functions extend beyond activation of pol II transcription in early spermatids remains to be determined.

We are grateful to A. de Agostini and R. Luthi at the Laboratoire d’Andrologie, Hôpital Cantonal, Université de Genève, for assistance in interpreting histological data. We thank J.-M. Matter for discussion and instruction in sample preparation, U. Laemmli and N. Roggli for providing equipment and expertise for figure preparation, R. Roeder and A. Hoffmann for the TBP cDNA and antibody, C. Kedinger for the anti-pol II antibody, T. Tamura for the mouse TBP cDNA, R. and J. Conaway for the TFII B cDNA, D. Lavery for commenting on the manuscript, and P. Bucin for animal care. This work was supported by the Swiss National Science Foundation and the State of Geneva.

REFERENCES


New York: Raven press.


(Accepted 6 May 1995)