Localization of Protamine 1 mRNA in Different Stages of the Cycle of the Rat Seminiferous Epithelium

Pekka Mali,* Minna Sandberg,‡ Eero Vuorio,‡ Pamela C. Yelick,§ Norman B. Hecht,§ and Martti Parvinen*

Departments of *Anatomy and ‡Medical Biochemistry, University of Turku, SF-20520 Turku, Finland; and §Department of Biology, Tufts University, Medford, Massachusetts 02155

Abstract. A mouse protamine 1 cDNA probe was used to study P1 protamine gene expression during the cycle of the seminiferous epithelium in the rat. In situ hybridization experiments showed that transcription of the P1 protamine mRNA starts in the middle of step 7 of spermiogenesis during substage VIIc. The mRNA levels stay high in steps 7-14 spermatids but decrease during steps 15-16 and are virtually undetectable in steps 17-19 spermatids. Northern blot analyses of RNAs isolated from microdissected pools of seminiferous tubules show high P1 protamine mRNA concentrations during stages VIIc-XIV-III of the cycle and lower levels during stages IV-VIIb. Owing to a post-transcriptional shortening of the poly(A) tail by 130 bases, a decrease in the size of protamine 1 mRNA from ~580 to 450 nucleotides was observed in stages XIII-XIV suggesting an initiation of protamine 1 synthesis in step 13-14 spermatids. In stages II-VI (steps 16-18 spermatids), only the smaller size protamine 1 mRNA was detectable. The expression of protamine 1 mRNAs has been localized in the very last phase of the haploid gene activity. Although the in situ hybridization suggests a disappearance of protamine 1 mRNA after step 16 of spermiogenesis, Northern blot analysis shows that low levels of mRNA are present during the period of final condensation of the chromatin, reflecting the association of protamine with DNA.

IT has been known for a long period of time that the DNA-associated proteins of mature sperm cells may be different from histones found in somatic cells (Miescher, 1897). In mammals, small basic proteins called protamines, rich in arginine residues replace histones and transition proteins in spermatids. Their major function is to neutralize the charge of DNA and to aid in its compaction (Bellvé, 1979; Hecht, 1986, 1988a, b; Poccia, 1986). The mRNAs of two variants of mouse protamine have been found to be synthesized during the haploid phase in round nucleated spermatids, and to be translationally regulated (Kleene et al., 1983, 1984; Hecht and Penschow, 1987), i.e., translation in elongating spermatids occurs ~1 w after mRNA synthesis starts (Kleene et al., 1983, 1984; Balhorn et al., 1984). The exact stages of spermiogenesis where the transcription and translation of protamine mRNA occurs, are not known. Since rat testis is known to express the protamine 1 gene (Calvin, 1976; Kistler et al., 1976; Bower et al., 1987), we used nucleic acid hybridizations to localize protamine 1 mRNA expression in situ in histological sections, and Northern analysis of RNA isolated from specific stages of the cycle of the seminiferous epithelium.

Materials and Methods

Histological Procedures

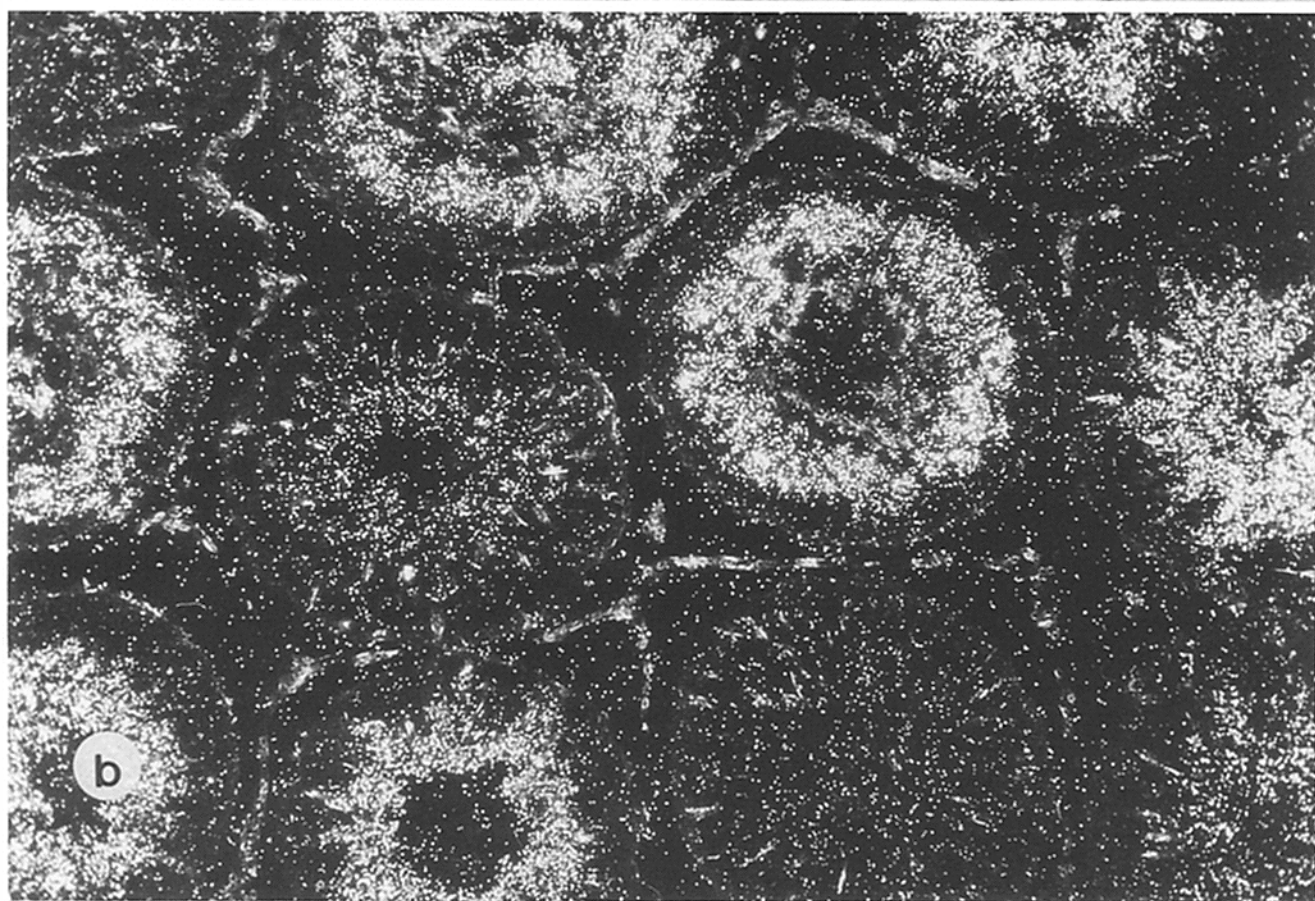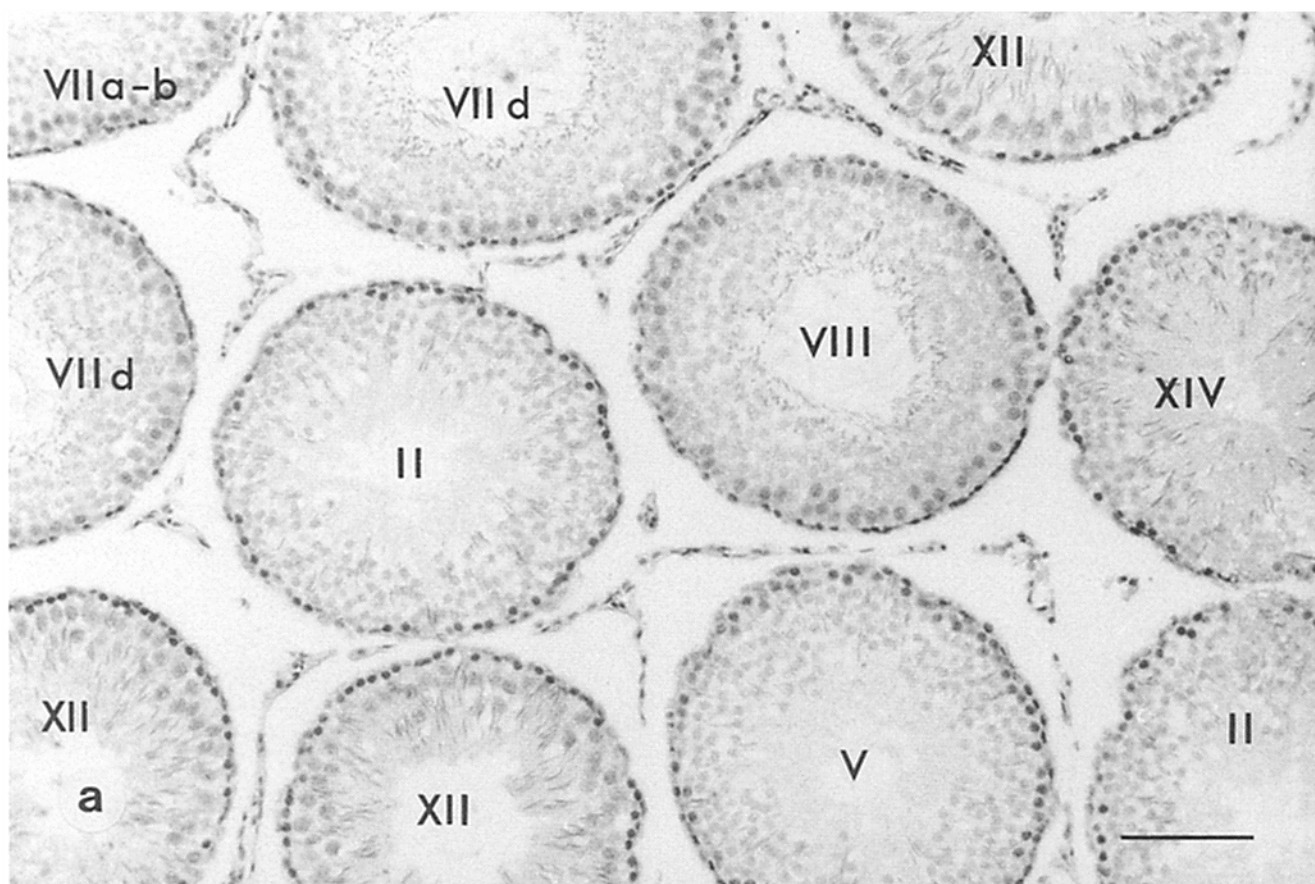Testes of adult (3-mo-old) Sprague-Dawley rats were fixed in 10% buffered formaline at room temperature for 24 h, dehydrated in ethanol, and cleared in xylene and embedded in paraffin. Sections (5 µm in thickness) were cut on microscope slides treated with Denhardt's solution (Denhardt, 1966) and acetylated as described by Brahic and Haase (1978). To ensure a firm attachment of the sections, 1% Elmer's glue together with heating at 60°C overnight were used (Sandberg and Vuorio, 1987). Deparaffinization was performed using a series of washes in xylene, absolute ethanol, and 70% ethanol solutions.

Preparation of the cDNA Probe

The mouse protamine cDNA clone pMP1, isolated from a mouse testis cDNA library (Kleene et al., 1983, 1985; Yelick et al., 1987), was used as the hybridization probe. For in situ hybridization, the 437-bp insert was released from its vector by digestion with the restriction enzyme Sal I, and isolated after electrophoresis on 1% agarose gel by binding to a DEAE membrane (Schleicher & Schuell, Inc., Keene, NH). It was then nick-translated to a specific activity of ca. 5×10^8 cpm/µg using [³⁵S]dATP (Amersham, U.K.). The lengths of the nick-translated fragments varied between 100 and 200 bp as determined by electrophoresis on 2% alkaline agarose gels. For Northern blots, plasmids pMP1, pRGAPDH-13 (a constitutively expressed mRNA for rat glyceraldehyde-3-phosphate dehydrogenase, Fort et al., 1985) and pI-19 (containing a cloned gene coding for 28 S mouse ribosomal RNA; Arnheim, 1979) were labeled with [³²P]dCTP and used as hybridization probes.

In Situ Hybridization

The prehybridization and hybridization procedures were performed essentially as described earlier (Brahic and Haase, 1978; Moench et al., 1985; Syrjänen et al., 1986; for detailed information see Sandberg and Vuorio, 1987), using 0.1 µg/ml of ³⁵S-labeled pMP1 probe. Control slides were hybridized with nick-translated BglI generated fragments of bacteriophage lambda DNA. Autoradiography was performed by dipping the slides in Ko-

dak NTB-2 emulsion at 40°C. The slides were exposed for 7-14 d in desiccant-containing boxes at 4°C, developed and stained with hematoxylin. The grain densities above different cell types were determined from dark field photomicrographs from 32 tubular cross sections above areas varying from 3,000 to 30,000 μm^2 that were measured by a morphometer (MOP 3, Reichert-Jung, Austria). The exact stages of the cycle were identified from adjacent periodic acid Schiff-hematoxylin-stained sections using the criteria of Leblond and Clermont (1952). For statistical significance, the logarithmic values of grain counts were assayed by analysis of variance together with Student-Newman-Keuls multiple range test using a BMDP statistical program (Los Angeles, CA).

Microdissection of Seminiferous Tubules, RNA Isolation and Northern Blots

Pooled segments of seminiferous tubules (50 cm each, ~50 mg wet weight) from stages I, II-III, IV-V, VI, VIIa-b, VIIc-d, VIII, IX-XI, XII and XIII-XIV (Leblond and Clermont, 1952) were collected by transillumination-assisted microdissection (Parvinen and Vanha-Perttula, 1972; Parvinen and Ruokonen, 1982) and stored at −70°C. The RNA was isolated by the guanidine isothiocyanate method as described by Chirgwin et al., (1979). The total RNA yields for each group of pooled tubules ranged from 125 to 140 µg.

In some cases, RNase H digestion of total RNA annealed with oligo(dT) was used for selective digestion of poly(A) segments before Northern analysis as described by Heidaran and Kistler (1987). Total RNA (10 µg) from pooled stages IV-V, VIII, and XIII-XIV-I was first incubated with oligo-(dT)$_{12-18}$ (1 µg, Gibco, Grand Island, NY; Bethesda Research Laboratories [BRL], Bethesda, MD) in 25 µl of distilled water for 3 min at 65°C. Then, RNase H buffer was added to give final concentrations of 100 mM KCl, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 50 mg/ml sucrose and the solution was kept on ice for 15 min, followed by addition of RNase H (3 U, Gibco-BRL). The samples were incubated at 37°C for 45 min, extracted with phenol/choroform and precipitated with ethanol.

The RNA fractionations were performed on 0.75-2% agarose gels after denaturation with glyoxal and DMSO or formaldehyde (Thomas, 1980). One set of samples was stained with ethidium bromide to visualize the rRNAs, and the other set was transferred by blotting to Pall Biodyne membrane. The prehybridizations, hybridizations with nick-translated ([^{32}P]-dCTP-labeled) plasmid DNAs, washes and autoradiography were performed as described earlier (Thomas, 1980). Densitometric analyses of multiple exposures of the X-ray films were performed by LKB 2222-020 Ultroscan densitometer (Bromma, Sweden) to quantify the protamine 1 mRNAs, using pRGAPDH-13 and pI-19 clones as references for both localization and quantitation.

Results

The in situ hybridizations showed marked differences between individual seminiferous tubules in the level of mRNA detected by the radioactive protamine 1 probe (Fig. 1). The epithelial stages from IV to early VII (to substage VIIb, Perey et al., 1961) contained less labeled probe than other stages. In a well defined zone in mid-stage VII, radioactivity was detected in a sharply defined cell layer in the seminiferous epithelium occupied by round spermatids at step 7 of spermiogenesis. The label was first seen during substage VIIc, a cell association characterized by large residual bodies at the level of the nuclei of step 19 spermatids. Along with the penetration of the step 12 spermatids towards the basal part of the seminiferous epithelium, the labeled area during stages XII-XIV-I of the cycle was found in the uppermost layer of the seminiferous epithelium, a region that is mainly occupied by steps 12-15 spermatids (Fig. 1). The radioactiv-
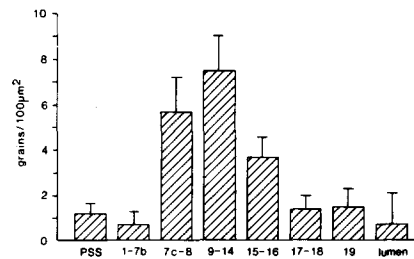
Figure 2. Quantitative grain counts detecting protamine 1 mRNA over the pachytene and diakinetic primary spermatocytes (PSS, from stages IX-XII, $n = 12$), steps 1-7b spermatids (stages I-VIIb, $n = 17$), steps 7c-8 spermatids (stages VIIb-VIII, $n = 6$), steps 9-14 spermatids (stages IX-XIV, $n = 12$), steps 15-16 spermatids (stages I-III, $n = 6$), steps 17-18 spermatids (stages IV-VI, $n = 8$), steps 19 spermatids (stages VII and VIII of the cycle, $n = 7$), and over the lumen of the seminiferous tubules on stages VIIc-XIV of the cycle ($n = 14$). The values over steps 7c-16 spermatids are significantly ($p < 0.01$) above the background grain density, seen over all other cell types. The statistical significances between other groups are: 1-7b and lumen vs. PSS and 17-19, $p < 0.05$; 7c-8 vs. 15-16, $p < 0.05$ and 9-14 vs. 15-16, $p < 0.01$.

ity then rapidly decreased during step 16 of spermiogenesis at stages II and III of the cycle. The grain densities above different cell types at defined stages of the cycle are presented in Fig. 2.

No significant variations occurred in the total RNA content between stages I, II-III, IV-V, VI, VIIab, VIIcd, VIII, IX-XI, XII and XIII-XIV of the cycle, as evaluated by the yield of total RNA and analysis of rRNA fractions after electrophoresis and fluorescent staining with ethidium bromide. Hybridization with the labeled protamine 1 cDNA-probe showed marked differences in the concentration and size of the corresponding protamine 1 mRNA between different stages of the cycle of the seminiferous epithelium. Stages II-III, IV-V and VI showed a low level of hybridization of the probe to the smaller protamine 1 mRNA, the nadir being at stage VI of the cycle (Fig. 3). The amount of protamine 1 mRNA increased during substages VIIc and VIId to a constant level that covered stages VIII-XIV of the cycle. Only the larger mRNA species was observed up to stage XII. The pools of stages XIII-XIV and particularly of stage I tubule segments showed the maximal hybridization intensity of labeled cDNA probe while the levels obtained with the reference probes (GAPDH mRNA and 28 S rRNA) remained relatively unaltered. In addition to the larger protamine 1 mRNA species, increasing amounts of the smaller size mRNA were observed in the sample isolated from stage I (Fig. 3). The densitometric analysis showed about a 10-fold difference between the minimal (stage VI) and maximal (stages VIII-XIV) expression of protamine 1 mRNA during the cycle of the rat seminiferous epithelium (Fig. 4). This is in accordance with the grain counts from the in situ hybridization figures. The Northern blot analysis of selected RNA samples was also performed after removal of poly(A) tails by

Figure 1. Normal (a) and dark-field (b) photomicrographs of a hematoxylin-stained paraffin section of rat testis to show the in situ hybridization of the protamine 1 cDNA probe. Stages VIId, VIII, XII, and XIV have strong hybridization signals over the steps 7-14 spermatids, whereas stage V has a background level of grain density. An intermediate radioactivity is seen above a stage II tubule. Bar, 100 µm.
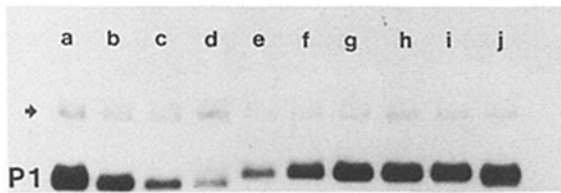
Figure 3. Northern blot analysis of protamine 1 mRNA isolated from pooled seminiferous tubules from stages I (*a*), II–III (*b*), IV–V (*c*), VI (*d*), VIIab (*e*), VIIcd (*f*), VIII (*g*), IX–XI (*h*), XII (*i*) and XIII–XIV (*j*) of the cycle. High mRNA levels are found in stages VIIcd–XIV–I of the cycle, and lower values in stages II–VIIab. The electrophoretic mobility of the protamine 1 mRNA (*P1*) is slightly faster (450 nucleotides) in stages II–VIIab than in stages VIIcd–XIV (580 nucleotides). Stages XIII–XIV and I seem to have both size classes of protamine 1 mRNA. For a hybridization controls for mobility and intensity, the GAPDH mRNA (1,269 nucleotides, *arrow*) and 28 S rRNA (not shown) were analyzed.

RNase H. Both the larger (580 base, from stages VIII and XIII–XIV) and the smaller (450 base, from stages IV–V) form of protamine 1 mRNA were reduced to an identical length of ∼420 bases (Fig. 5).

Figure 5. Northern blot analysis of protamine 1 mRNA before (lanes *1*, *2*, and *3*) and after (lanes *4*, *5*, and *6*) RNase H digestion of poly(A) tracts. In stages XIII–XIV and I (lanes *1* and *4*) and VIII (*3* and *6*) the length of the removed chain is ∼160 nucleotides, but it shortens to ∼30 nucleotides in stages IV–V (lanes *2* and *5*) after translation. The size markers were from a combined Hin fI–Eco RI digest of pBR322.

Discussion

Although a meiotic expression of protamine mRNA in salmonoid fish has been described (Iatrou et al., 1978), its postmeiotic transcription has been suggested in the mouse (Erickson et al., 1980). In prepuberal mice, protamine mRNAs are first detected in testes from 22-d–old animals, coincident with the appearance of steps 5–8 spermatids (Hecht et al., 1986). This observation suggests that protamine mRNA is synthesized during the last half of the active transcription period of the haploid genome. The present analysis demonstrates that in the rat the protamine 1 mRNA transcription initiates during a very short period of time in mid-step 7 of spermiogenesis. In the rat, stage VII has a duration of 2.3 d (Clermont and Harvey, 1965). The time from the beginning of stage VIII to the beginning of stage II when the highest levels of protamine 1 mRNA are present in steps 7–16 spermatids is 6.7 d. This is close to the estimated value
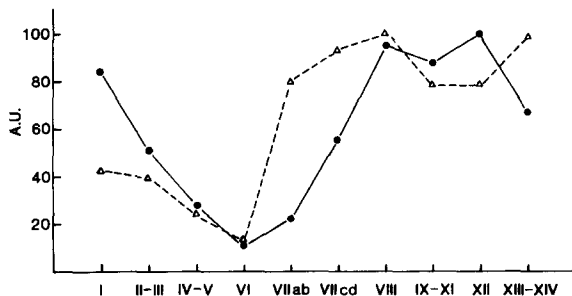

Figure 4. Densitometric analysis (arbitrary units, highest value = 100) of the protamine 1 mRNA in relation to GAPDH mRNA (*dashed line*) and to 28 S rRNA (*solid line*). The activation of the gene transcription occurs during stage VII of the cycle, and smallest amount of protamine 1 mRNA is found at stage VI, the difference between minimal and maximal values being 10-fold.
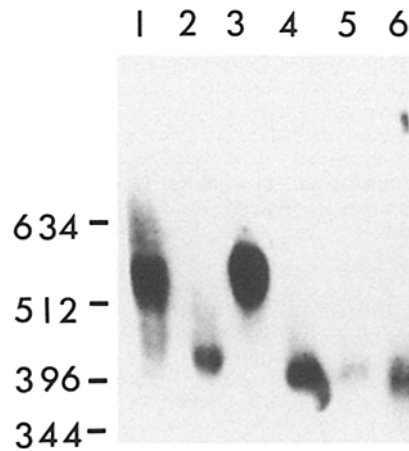
in the mouse from the appearance of the mRNA to the initiation of protamine synthesis (Hecht, 1988*b*). In the rat, protamines are present in steps 16–19 spermatids (Grimes et al., 1977; Meistrich et al., 1978) suggesting an initiation of their synthesis during step 15. The final condensation of the chromatin during this step of spermiogenesis is further supported by DNA flow cytometric observations (Toppari et al., 1985). Each of the stages I–III of the cycle have characteristic locations of the hypohaploid peak induced by steps 15–16 spermatids. This has been interpreted to reflect the second nucleoprotein transition and gradual chromatin condensation in these steps that is also concomitant with a reduced phosphotungstic acid stainability (Courtens and Loir, 1981). The beaded ultrastructure of the chromatin in spread preparations is replaced by a smooth type that facilitates the packaging of the male gamete genome (Kierszenbaum and Tres, 1975, 1978). Although this phenomenon is not accurately correlated with particular stages of spermiogenesis, it is apparently related to the changes in nucleoprotein composition of late spermatids.

The present results suggest that protamine 1 mRNA undergoes posttranscriptional processing before it is translated in spermatids. In stages VIII–XIV, the size of the mRNA recognized by the protamine 1 cDNA probe, is ∼130 bases larger than in the other stages. This is in agreement with earlier observations. In both mouse and rat, the polysomal form of protamine 1 mRNA is ∼130 nucleotides shorter than the stored ribonucleoprotein protamine 1 mRNA (Kleene et al., 1984; Bower et al., 1987). Based upon this size difference, we can conclude from our Northern blot analysis that the protamine 1 protein is first synthesized in steps 13–14 spermatids in stages XIII–XIV. Although the 450 nucleotide polysomal form of protamine 1 is detectable in tubules between stages XIII–VI, we cannot determine precisely when protamine synthesis terminates because the protamine 1 mRNA has been found in the residual body fraction of dissociated mouse testicular cells (Hecht, N. B., unpublished

observation). The level of this mRNA is, however, below the detection limit in the present in situ hybridization analysis. Previous kinetic-labeling studies of rat testicular nuclear proteins have suggested that rat protamine synthesis occurs subsequent to steps 14–15 and declines to a low level by step 18 (Grimes et al., 1977).

The superior resolution provided by Northern blot analysis of RNA isolated from dissected staged seminiferous tubules reveals that the 580-nucleotide ribonucleoprotein particle form of protamine 1 mRNA is not present in all stages of the seminiferous epithelium. It is first detectable in stage VIIab tubules (step 7 spermatids) and diminishes to a level below detection in stages IV-V (step 17 spermatids). The RNase H digestion suggests that a small poly(A) fragment remains in this RNA during and after translation.

The hybridization data presented here is based upon our ability to detect rat protamine 1 mRNA with a mouse protamine 1 cDNA. Although the protamine 1 genes of the rat and mouse are not likely to be identical in sequence, the first 15 amino acids are the same (Kistler et al., 1976; Kleene et al., 1985).

Although the in situ hybridization and the Northern blot data presented here are complementary and in agreement, differences in the ability to detect low levels of protamine 1 mRNA are apparent. For instance, the Northern blot procedure detects protamine 1 mRNA in all stages of the seminiferous epithelial cycle. The differences in size of the protamine mRNAs in stages VI and VII suggest little or no cross contamination between stages where low levels of protamine 1 mRNA are seen. In contrast, strong hybridization signals are detected by both techniques in stages VIIc–III of the cycle. The fact that protamine 1 mRNA levels related to GAPDH mRNA and 28 S rRNA are not identical suggests a cyclicity in their levels at different stages of the seminiferous epithelium.

The regulation of protamine mRNA synthesis in spermatids remains to be investigated. Its synthesis occurs during those steps of spermiogenesis that have the least ability to differentiate in vitro (Toppari and Parvinen, 1985), and also are the most sensitive cells for the effects of hypophysectomy (Clermont and Morgentaler, 1955). It is therefore possible that the steps of spermiogenesis involving nucleoprotein transitions, are critical for optimal culture conditions, hormonal stimulation and interaction with the Sertoli cells.

References

Arnheim, N. 1979. Characterization of mouse ribosomal gene fragments purified by molecular cloning. Gene. 7:83–96.

Balhorn, R., S. Weston, C. Thomas, and A. J. Wyrobek. 1984. DNA packaging in mouse spermatids. Synthesis of protamine variants and four transition proteins. Exp. Cell. Res. 150:298–308.

Bellvé, A. R. 1979. The molecular biology of mammalian spermatogenesis. Oxford Rev. Reprod. Biol. 1:159–261.

Bower, P. A., P. C. Yelick, and N. B. Hecht. 1987. Both P1 and P2 protamine genes are expressed in mouse, hamster and rat. Biol. Reprod. 37:479–488.

Brahic, M., and A. T. Haase. 1978. Detection of viral sequences of low reitera-

tion frequency by in situ hybridization. Proc. Natl. Acad. Sci. USA. 75: 6125–6129.

Calvin, H. I. 1976. Comparative analysis of the nuclear basic proteins in rat, human, guinea pig, mouse and rabbit spermatozoa. Biochim. Biophys. Acta. 434:377–389.

Chirgwin, J. M., A. R. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18:5294–5299.

Clermont, Y., and S. C. Harvey. 1965. Duration of the cycle of the seminiferous epithelium of normal, hypophysectomized and hypophysectomized-hormone treated albino rats. Endocrinology. 76:80–89.

Clermont, Y., and H. Morgentaler. 1955. Quantitative study of spermatogenesis in the hypophysectomized rat. Endocrinology. 57:369–382.

Courtens, J.-L., and M. Loir. 1981. A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat and stallion spermatids. J. Ultrastruct. Res. 74:327–340.

Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641–646.

Erickson, R. P., J. M. Kramer, J. Rittenhouse, and A. Salkeld. 1980. Quantitation of mRNAs during mouse spermatogenesis: protamine-like histone and phosphoglycerate kinase-2 mRNAs increase after meiosis. Proc. Natl. Acad. Sci. USA. 77:6068–6090.

Fort, P., L. Marty, M. Piechaczyk, S. ElSabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate multigenic family. Nucl. Acids Res. 13:1431–1442.

Grimes, S. R., Jr., M. L. Meistrich, R. D. Platz, and L. S. Hnilica. 1977. Nuclear protein transitions in rat testis spermatids. Exp. Cell. Res. 110:31–39.

Hecht, N. B. 1986. Evidence for haploid-specific genes in the mammalian testis. In Proceedings of the IVth European Workshop on Molecular and Cellular Endocrinology of the Testis. M. Stefanini, M. Conti, R. Geremia, and E. Ziparo, editors. Elsevier, Amsterdam. 199–213.

Hecht, N. B. 1988a. Haploid gene expression and the regulation of post-meiotic structural genes. In Development and Function of the Reproductive Organs. Vol. II. M. Parvinen, I. Huhtaniemi, and L. J. Pelliniemi, editors. Serono Symposia Review No. 14. Ares-Serono Symposia. Rome. 325–334.

Hecht, N. B. 1988b. Mammalian protamines and their expression. In Basic chromosomal proteins: Structure, organization and regulation of the gene. G. Stein, J. Stein, and L. Hnilica, editors. CRC Press, Boca Raton, FL. In press.

Hecht, N. B., and J. D. Penschow. 1987. In situ localization of mRNAs coding for mouse testicular structural genes. Exp. Cell Res. 173:274–281.

Hecht, N. B., P. A. Bower, S. H. Waters, P. C. Yelick, and R. J. Distel. 1986. Evidence for haploid expression of mouse testicular genes. Exp. Cell Res. 164:183–190.

Heidaran, M. A., and W. S. Kistler. 1987. Transcriptional and translational control of the message for transition protein 1, a major chromosomal protein of mammalian spermatids. J. Biol. Chem. 262:13309–13315.

Iatrou, K., A. W. Spira, and G. H. Dixon. 1978. Protamine messenger RNA: evidence for early synthesis and accumulation in rainbow trout. Dev. Biol. 64:82–98.

Kierszenbaum, A. L., and L. L. Tres. 1975. Structural and transcriptional features of the mouse spermatid genome. J. Cell Biol. 65:258–270.

Kierszenbaum, A. L., and L. L. Tres. 1978. RNA transcription and chromatin structure during meiotic and postmeiotic stages of spermatogenesis. Federation Proc. 37:2512–2516.

Kistler, W. S., P. S. Keim, and R. L. Heinrikson. 1976. Partial amino acid sequence of the basic chromosomal protein of the rat spermatozoon. Biochim. Biophys. Acta. 427:752–757.

Kleene, K. C., R. J. Distel, and N. B. Hecht. 1983. cDNA clones encoding cytoplasmic poly(A)⁺ RNAs which first appear at detectable levels in haploid phases of spermatogenesis in the mouse. Dev. Biol. 98:455–464.

Kleene, K. C., R. J. Distel, and N. B. Hecht. 1984. Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. Dev. Biol. 105:71–79.

Kleene, K. C., R. J. Distel, and N. B. Hecht. 1985. Nucleotide sequence of a cDNA clone encoding mouse protamine 1. Biochemistry. 24:719–722.

Leblond, C. P., and Y. Clermont. 1952. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55:548–573.

Meistrich, M. L., W. A. Brock, S. R. Grimes, R. D. Platz and L. S. Hnilica. 1978. Nuclear protein transitions during spermatogenesis. Federation Proc. 37:2522–2525.

Miescher, F. 1897. Die Histochemischen und Physiologischen Arbeiten von F. Miescher. Vogel Vlg., Leipzig. 359–371.

Moench, T. R., J. E. Gendelman, J. E. Clements, O. Narayan, and D. E. Griffin. 1985. Efficiency of in situ hybridization as a function of probe size and fixation technique. J. Virol. Method. 11:119–130.

Parvinen, M., and A. Ruokonen. 1982. Endogenous steroids in rat seminiferous tubules. Comparison of different stages of the epithelial cycle isolated by transillumination-assisted microdissection. J. Androl. 3:211–220.

Parvinen, M., and T. Vanha-Perttula. 1972. Identification and enzyme quantitation of the stages of the seminiferous epithelial wave in the rat. Anat. Rec. 174:435–450.

Perey, B., Y. Clermont, and C. P. Leblond. 1961. The wave of the seminiferous epithelium in the rat. Am. J. Anat. 108:47–77.

Poccia, D. 1986. Remodeling of nucleoproteins during gametogenesis, fertilization and early development. *Int. Rev. Cytol.* 105:1-65.

Sandberg, M., and E. Vuorio. 1987. Localization of types I, II and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. *J. Cell. Biol.* 104:1077-1084.

Syrjänen, S. K., R. Syrjänen, R. Mäntyjärvi, S. Parkkinen, M. Väyrynen, S. Saarikoski, and O. Castren. 1986. Human papillomavirus (HPV) DNA sequences demonstrated by in situ DNA hybridization in serial paraffin-embedded cervical biopsies. *Arch. Gynecol.* 239:39-48.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201-

5205.

Toppari, J., and M. Parvinen. 1985. In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle: morphologic and immunolocalization analysis. *J. Androl.* 6:334-343.

Toppari, J., E. Eerola, and M. Parvinen. 1985. Flow cytometric DNA analysis of defined stages of rat seminiferous epithelial cycle during in vitro differentiation. *J. Androl.* 6:325-333.

Yelick, P. C., R. Balhorn, P. A. Johnson, M. Corzett, J. A. Mazrimas, K. C. Kleene, and N. B. Hecht. 1987. Mouse protamine 2 is synthesized as a precursor whereas mouse protamine 1 is not. *Mol. Cell. Biol.* 7:2173-2179.