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Differential Gene Expression during Mouse Spermatogenesis[1]

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ABSTRACT

The temporal expression of genes corresponding to eighteen cDNA clones isolated from testis mRNA was analyzed. Two sets of RNA samples were surveyed by means of Northern blot analysis; one set contained poly[A$^+$] RNA from the seminiferous epithelium of mice at different developmental ages and the other contained total cellular RNA from purified germ cells. The purified germ cells represented several morphological stages of spermatogenesis, from spermatogonia to condensing spermatids (steps 12–16) and residual bodies. In this study, four distinct phases of gene expression were observed during spermatogenesis. The clones that were characterized provide molecular markers for stage-specific transcription during germ cell differentiation.

INTRODUCTION

Morphological and biochemical observations suggest that germ cell differentiation is accompanied by selective gene expression. However, the molecular mechanisms regulating the cell-type specificity of gene expression during mammalian spermatogenesis have yet to be resolved. Germ cells undergoing spermatogenesis pass through a series of morphological transitions, beginning with spermatogonial proliferation and renewal, followed by meiosis, and finally terminal differentiation during spermiogenesis. In mice, spermatogenesis begins at postnatal Day 5 or 6, when the primitive type A spermatogonia begin to proliferate (Bellvé et al., 1977). These progenitor cells undergo a series of mitoses to give rise to the types A_{1-4}, intermediate, and eventually type B spermatogonia (Bellvé, 1979). On Day 9, the type B spermatogonia pass through a mitotic division to yield preleptotene spermatocytes, which undergo a final round of DNA replication before entering meiotic prophase. By Day 10, leptotene spermatocytes appear and quickly differentiate to form zygotene spermatocytes in which homologous chromosomes undergo pairing. By Day 14, pachytene spermatocytes form, and subsequently genetic recombination and extensive RNA synthesis occurs. Around Day 18 of development, the first meiotic division occurs to form secondary spermatocytes. These cells rapidly divide to produce haploid round spermatids at the onset of spermiogenesis. The morphological changes in germ cells during spermatogenesis occur concomitantly with alterations in the complement of constituent proteins, and these presumably reflect differences in the mRNA populations coding for stage-specific proteins (Boitani et al., 1980; Millette and Moulding, 1981; Kramer and Erickson, 1982; Gold et al., 1983; Stern et al., 1983).

The appearance of stage-specific mRNAs during spermatogenesis has been demonstrated. Experiments involving in vitro translation of mRNA from isolated germ cells have suggested that transcription of the phosphoglycerate kinase-2 (PGK-2) and lactate dehydrogenase (LDH-C) genes first occurs in pachytene

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spermatocytes and continues in round spermatids (Erickson et al., 1980; Weiben, 1981; Gold et al., 1983). Screens of testis cDNA libraries (Kleene et al., 1983; Dudley et al., 1984) have identified cDNA clones that are transcribed specifically by round spermatids, such as the protamines (Kleene et al., 1985; Yelick et al., 1987). Several multigene families express germ cell-specific isotypes, including α-tubulin (Villasante et al., 1986; Hecht et al., 1988), phosphoglycerate kinase (Kramer and Erickson, 1981), lactate dehydrogenase, (Goldberg and Hawtrey, 1968), and β-actin (Waters et al., 1985). In addition, various oncogenes (Ponzetto and Wolgemuth, 1985; Shakleford and Varmus, 1987) and t-complex polypeptide-1 (TCP-1) (Willison et al., 1986) are expressed in somatic tissues and germ cells. Collectively, these observations suggest that precise controls exist for determining stage-specific gene expression during spermatogenesis.

In the present study, developmentally regulated patterns of gene expressions have been explored by using a series of cloned cDNAs corresponding to transcripts that are expressed at various stages of mouse spermatogenesis. This report presents the isolation and initial characterization of several cDNA clones studied by Northern analysis of RNA obtained from prepubertal seminiferous epithelium and purified germ cells. The data suggest the following: 1) stage-specific patterns of transcription occur coincidently with the appearance and accumulation of distinct germ cell types within the seminiferous epithelium; 2) transcription of a variety of genes occurs exclusively within haploid spermatids; 3) several transcripts, including LDH-C and PGK-2, are detected initially during meiotic prophase and continue to accumulate in round spermatids; and 4) specific transcripts accumulate in spermatogonia and meiotic germ cells but not in spermatids.

MATERIALS AND METHODS

Isolation of the Seminiferous Epithelium and Spermatogenic Cells

Seminiferous cords and tubules were prepared from Swiss Webster mice (Charles River Breeding Labs, Wilmington, MA) by collagenase treatment, and monodisperse suspensions of spermatogenic cells were obtained from these isolated seminiferous tubules by trypsin digestion (Romrell et al., 1976; Bellvé et al., 1977). Types A and B spermatogonia were isolated from the testes of Day 8 prepubertal mice. Preleptotene spermatocytes, leptotene/zygotene spermatocytes, and pachytene spermatocytes (P_{17}) were isolated from seminiferous epithelia of Day 17 mice (Bellvé et al., 1977). Pachytene spermatocytes (P_{60}), round spermatids (steps 1–8), elongating spermatids (steps 12–16), and residual bodies were isolated from the testes of ≥60-day-old mice. The germ cells were separated by velocity sedimentation at unit gravity on 2–4% bovine serum albumin (BSA) gradients; their purity, as determined by cytological examination, was >92% and their viability, was by dye exclusion, >98%. TM_3, leydig culture cells were obtained from the American Tissue Culture Collection (Rockville, MD). TM_4 Sertoli culture cells were a gift from J. Mather (Genentech, San Francisco, CA).

Isolation of RNA

Cytoplasmic RNA was isolated according to Chirgwin et al. (1979). Seminiferous cords and tubules, purified germ cells, and somatic cells were lysed in 0.5% NP-40, 1.5 mM $MgCl_2$, 14 mM NaCl, 10 mM vanadyl complexes (Bethesda Research Labs, Bethesda, MD) in 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5). The nuclei and membranes were removed by centrifugation at 4000 rpm for 15 min. Guanidine thiocyanate was added to 6 M and then CsCl to 0.9 M final concentrations. The homogenate was layered over a 2-ml cushion of 5.7 M CsCl and centrifuged in an SW 50.1 rotor, at 35,000 rpm for 18–24 h at 20°C. After the supernatant was removed, the pelleted RNA was resuspended in 0.25% sodium dodecyl sulfate (SDS) and precipitated with 3 M sodium acetate and absolute ethanol (vol:vol, 0.1:2) at –20°C. RNA for Northern blot analysis was isolated by LiCl precipitation (Cathala et al., 1983). The samples were homogenized briefly in 5 M guanidine thiocyanate, 10 mM ethylene diamine tetraacetate (EDTA), 8% β-mercaptoethanol (βME), 50 mM Tris-HCl (pH 7.5). The RNA was precipitated at 4°C by addition of LiCl to 4.0 M final concentration, centrifuged, and then redissolved in 0.1% SDS, 1 mM EDTA in 10 mM Tris-HCl (pH 7.5). The resuspended RNA was extracted with phenol/chloroform, (vol:vol, 1:1) and precipitated by addition of 3 M sodium acetate and ethanol (vol:vol, 0.1:2) at –20°C.

Isolation of Poly[A⁺] RNA

Poly[A⁺] RNA was isolated by affinity chromatography over oligo-dT cellulose (Bantle et al., 1976). Total RNA was dissolved in 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5); this procedure was followed by

the addition of 9 volumes of dimethylsulfoxide (DMSO) and an equal volume of 1.0 M LiCl, 5 mM EDTA, 2% SDS in 10 mM Tris-HCl (pH 7.5). The RNA was heated to 55°C for 5 min and then diluted tenfold with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M LiCl, 0.5% SDS (Binding Buffer) before loading into the oligo-dT cellulose column. The flow-through was recycled twice and the column finally was washed with at least five bed-volumes of Binding Buffer. The bound poly[A^+] RNA was eluted with 0.5% SDS in 10 mM Tris-HCl (pH 7.5) and precipitated with 3 M sodium acetate and ethanol (vol:vol, 0.1:2.0) at –20°C.

Construction of cDNA Libraries

First-strand and second-strand syntheses were carried out with 1–10 μg poly[A^+]-selected RNA (Maniatis et al., 1982). Synthetic *Eco*RI linkers were added to the S1 (New England Biolabs, Beverly, MA) -treated, methylated, double-stranded DNA. After the *Eco*RI digestion, free *Eco*RI linkers were separated from double-stranded DNA by repeated (three times) precipitations with 2.0 M ammonium acetate and isopropanol (vol: vol, 0.1:0.8), followed by column chromatography through Sephadex G-100 or Sepharose 4B. The double-stranded cDNA was cloned into *Eco*RI-digested λgt10 vector (Huynh et al., 1985). Fractionation on denaturing 1.2% agarose gels indicated that for testes the double-stranded DNA ranged from 4000 to 500 bps, whereas for pachytene spermatocytes it ranged from 2500 to 500 bps. cDNA inserts were isolated and characterized by using *Bgl*II and *Hind*III restriction sites, which closely flank the *Eco*RI cloning site in λgt10.

Screening of cDNA Libraries and Isolation of Germ Cell-Specific Recombinant Clones

Total testis libraries were constructed by using 10 μg poly[A^+] RNA. These libraries contained 5×10^6 plaque-forming units (pfu) of which 80% represented recombinants. The pachytene spermatocyte library was constructed by using 1 μg poly[A^+] RNA and contained 10^6 pfu with 30% recombinants. From each of these libraries, 200,000 plaques (20,000 plaques per 150-cm plate) were screened on nitrocellulose (Benton and Davis, 1977). Single-stranded, ^{32}P-labeled, cDNA probes were prepared from poly[A^+] RNA of 1) liver, 2) pachytene spermatocytes, and 3) round spermatids (steps 1–8) to specific activities of $\geq 10^8$ cpm/μg DNA by a reverse-transcriptase reaction. Hybridization to triplicate plaque lifts were done at 42°C in 50% formamide, fivefold concentration of SSPE buffer (1.1 M NaCl, 5 mM EDTA, 50 mM NaH_2PO_4 (pH 7.4), single concentration of Denhardt's buffer (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS, sheared calf thymus DNA (100 μg/ml), and poly dA/dT (50 μg/ml). The filters were washed three times for 15 min in 0.1% SDS, single concentration of SSC (0.15 M NaCl 0.015 M $Na_3C_6H_5O_7$) at 22°C, followed by three more stringent washes at 15 min each in 0.1% SDS and one-tenth (0.1) concentration of SSC at 60°C.

Southern, Northern, and Dot-Blot Assays

DNA was isolated from individual plaque-purified recombinant clones by double digestion with either *Bam*HI and *Bgl*II or *Hind*III and *Bgl*II, electrophoresed through 0.7% agarose gels in Tris-borate buffer and blotted onto Genescreen filters (Southern, 1975). Dot blots were constructed by first denaturing phage DNA with 0.2 M NaOH followed by neutralization with 1.0 M ammonium acetate. Three tenfold-serial dilutions of each sample were then applied to Genescreen membrane filters (NEN Research Products, Boston, MA) by using a dot-blot manifold apparatus. Two Northern blots (Thomas, 1980) were constructed by using poly[A^+] RNA (6 μg) from the seminiferous epithelium of prepubertal mice aged 6–60 days. A third Northern blot was made of the same RNA but omitted Day 6, 8, 12, and 16. A fourth Northern blot was made of total RNA (30–40 μg) from seminiferous epithelium of prepubertal mice and purified germ cells from prepubertal and adult male mice. The RNA was denatured in 50% formamide buffer at 55°C for 15 min and loaded onto 1.4% agarose gels containing 2.2 M formaldehyde. The samples were electrophoresed at 27°C until the bromophenol blue dye was two-thirds towards the bottom of the gel. After blotting onto Genescreen for 24 h, the filters were UV-treated for 4 min and baked at 80°C for 30 min.

Hybridization Conditions and Probes

All hybridization assays were carried out at 42°C in 50% formamide, 0.1% SDS, fivefold concentration of SSPE, single concentration of Denhardt's, and sheared calf thymus DNA (100 μg/ml). Three washings were undertaken at 15-min intervals initially in 0.1% SDS, and single concentration of SSC at 22°C. If the backgrounds were excessive, the filters were washed three

times at 15 min each with 0.1% SDS in 0.1 concentration of SSC at 60°C, before filters were exposed to Kodak film with intensifier screens at –70°C (Maniatis et al., 1982).

The ^{32}P-labeled, double-stranded, hybridization probes were prepared by nick translation of *Eco*RI or *Bgl*II-*Hind*III restriction fragments (containing the cDNA insert flanked by 1.1 kb of λgt10 DNA). The DNA restriction fragments used for nick translation were recovered from agarose gels by $NACIO_4$ precipitation, followed by elution with 0.1 concentration of TE buffer (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) from GF/C filters (Whatman, Maidstone, England). The specific activity of all double-stranded probes was $\geq 10^9$ cpm/μg DNA. The sensitivity of detection of the LDH-C nick-translated probe on Northern blots was 160 fg mRNA/μg total RNA. Single-stranded cDNA hybridization probes were prepared from poly[A$^+$]RNA templates by using reverse transcriptase and [^{32}P]dNTPs. The specific activity of the cDNA probes was $\geq 10^8$ cpm/μg DNA.

RESULTS

Differential Screening of Testis cDNA Libraries: Isolation and Characterization of Spermatogenesis-Specific cDNA Clones

Two cDNA libraries were constructed from poly[A$^+$] RNA isolated from either adult testes or purified adult pachytene spermatocytes (see *Materials and Methods*). These testis libraries were differentially screened to identify clones that are expressed in germ cells and absent or expressed at reduced levels in somatic tissues. To this end, the cDNA libraries were screened in triplicate with three probes, each prepared by incorporation of [^{32}P]dNTPs into cDNAs, with reverse transcriptase and poly[A$^+$] RNA as template. The cDNA probes were made from RNA isolated from a) liver, b) pachytene spermatocytes, and c) round spermatids (steps 1–8). Recombinant clones that hybridized strongly to the liver probes were not examined further, with the exception of a few clones that served as controls in later experiments.

Two hundred plaque-purified recombinant clones were selected on the dual criteria of strong hybridization to adult pachytene spermatocyte cDNA probes and negligible hybridization to the liver cDNA probes. To verify that mRNAs complementary to these 200 clones were abundant in germ cells, DNA was prepared from each clone and subjected to dot-blot and/or Southern analyses. The blots were rescreened by using the same three cDNA probes described for the initial plaque hybridization. These assays permitted classification of the clones according to whether they hybridized to 1) pachytene spermatocyte cDNA probe, 2) round spermatid cDNA probe, or 3) both pachytene spermatocyte and spermatid cDNA probes. Of the 200 clones rescreened at high stringency, 60 clones showed relatively weak hybridization with liver cDNA probe and were not analyzed further. Forty-eight clones showed strong hybridization with the spermatid cDNA probe and did not hybridize with the adult pachytene spermatocyte cDNA probe. Ninety-two clones hybridized to the pachytene cDNA probe and hybridized weakly, or not at all, to the spermatid cDNA probe. These 92 clones were rescreened with a cDNA probe prepared from poly[A$^+$] RNA that was isolated from Day 15 prepubertal seminiferous epithelium to identify clones that were expressed in early spermatocytes. Ten of the clones that hybridized to the Day 15 cDNA probe were further analyzed by Northern blot of poly[A$^+$] RNA, but only two (C3D and P6A), detected discrete transcripts that were expressed in early germ cells and not in spermatids. The 200 cDNA clones were also screened by dot-blot analysis for internal redundancy. Hybridization probes were prepared from twenty clones, including

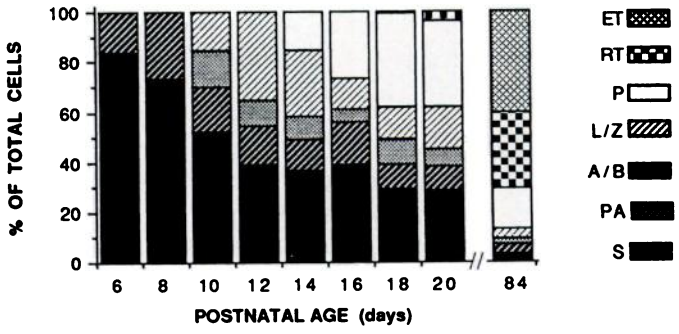
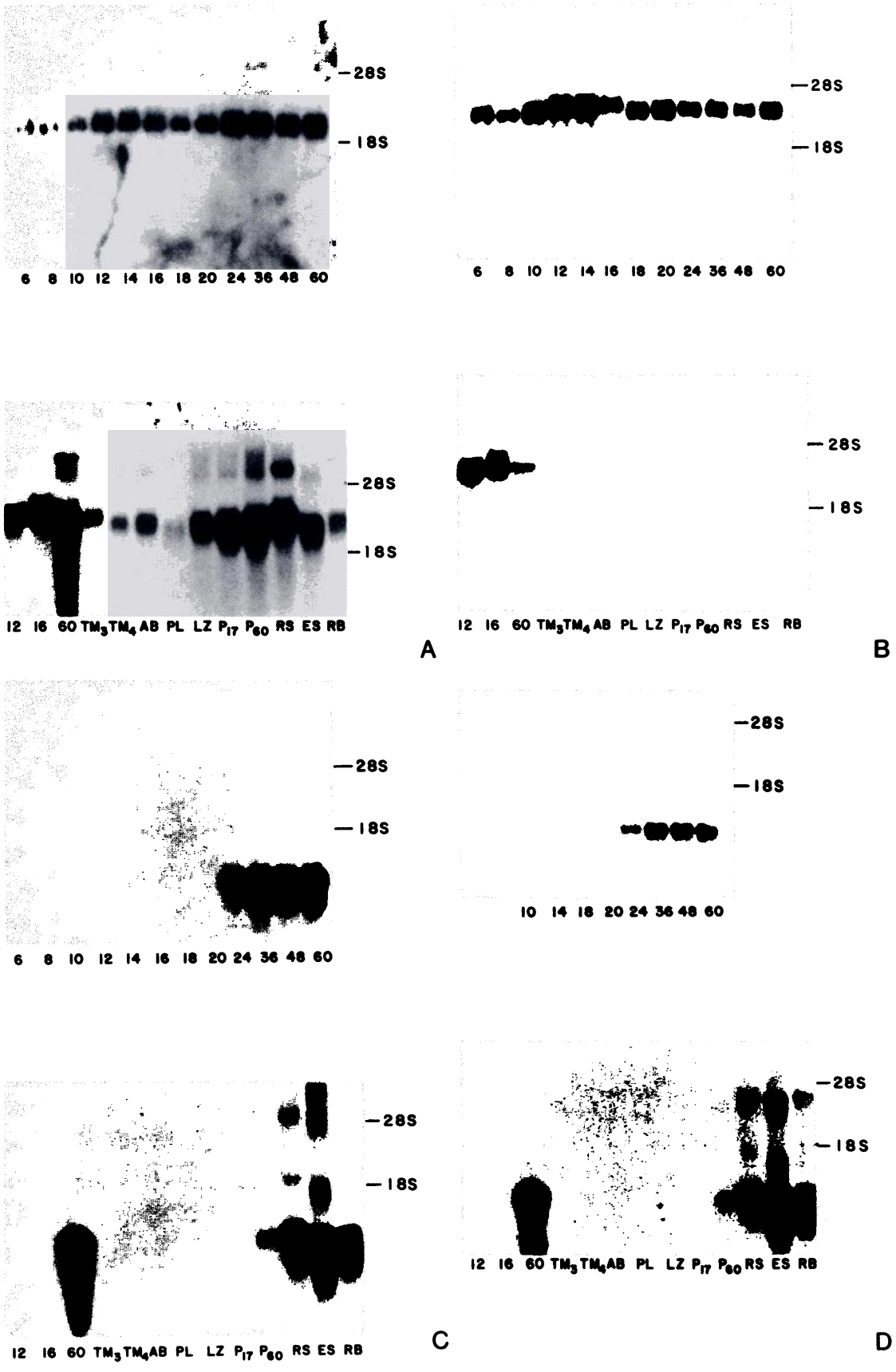
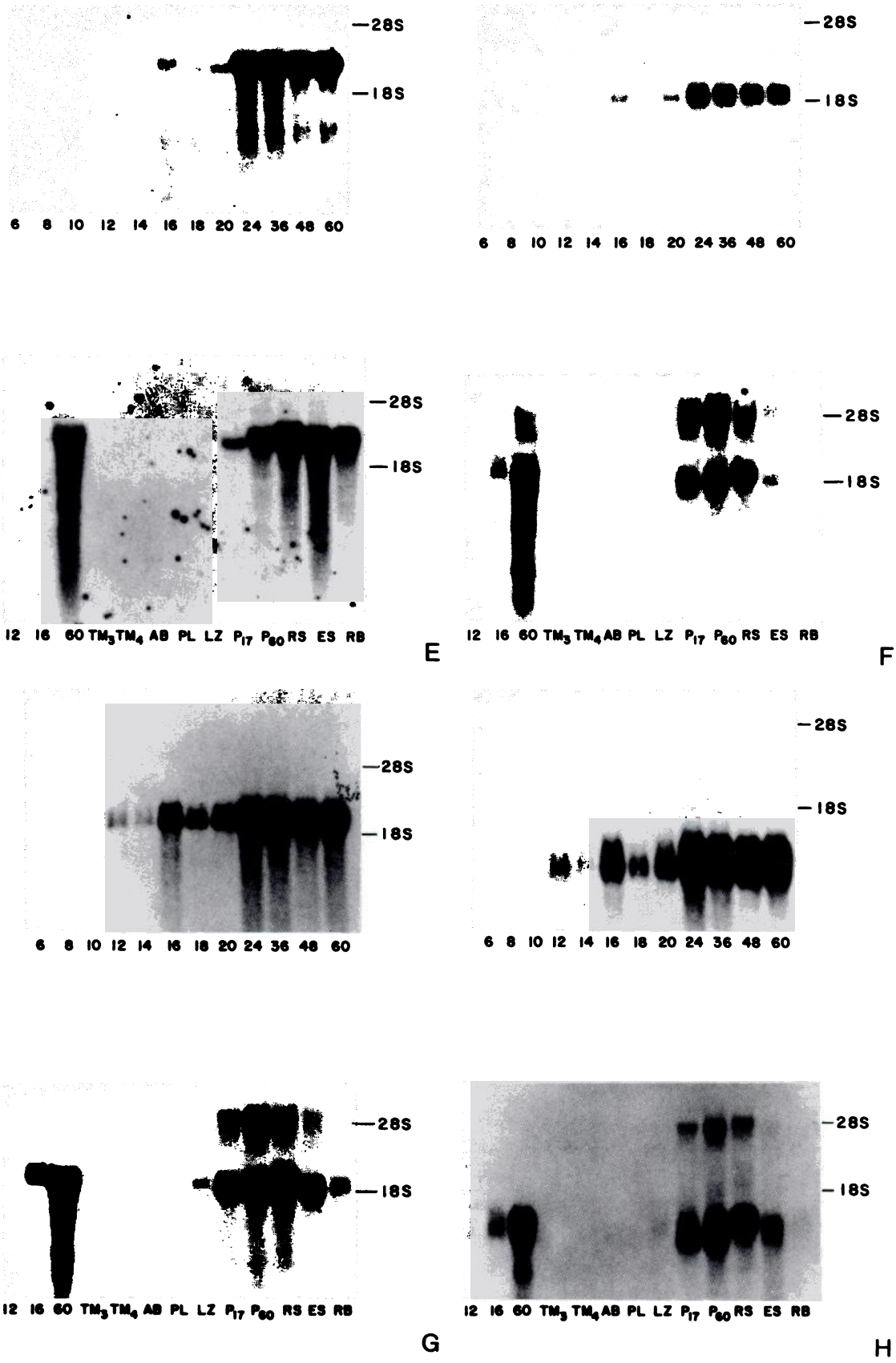


FIG. 1. Temporal appearance of germ cells during development of the mouse seminiferous epithelium. Data are expressed as a percentage of total cells in the seminiferous epithelium at each of the designated ages of development, with birth occurring on Day 0. Cell types include Sertoli cells *(S)*; primitive type A spermatogonia *(PA)*; type A and type B spermatogonia *(A/B)*; leptotene and zygotene spermatocytes *(L/Z)*; pachytene spermatocytes *(P)*; round spermatids, steps 1–8 *(RT)*; elongating spermatids, steps 12–16 *(ET)*. (After Bellvé et al., 1977a; reproduced by permission of the Editor, Journal of Cell Biology).





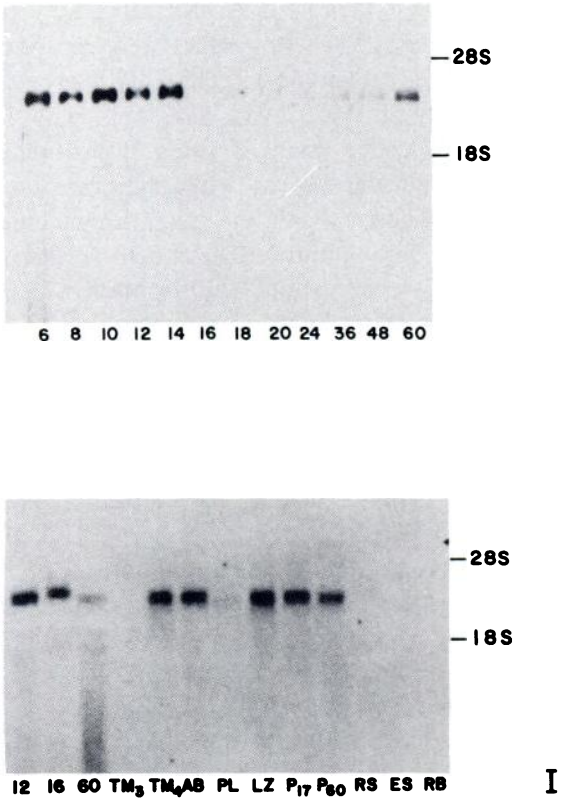


FIG. 2. *Top panels, A–I.* Poly[A+] RNA (6 μg/lane) was isolated from the seminiferous epithelium at the designated ages (Day 6–60) for Northern analysis. Two identical Northern blots were used in panels A–C and E–I. A third Northern blot *(panel D)* omitted RNA from Day 6, 8, 12, and 16 prepubertal testis. *Bottom panel, A–I.* Total RNA (30–40 μg/lane) was isolated from the seminiferous epithelium at Day 12 (12), Day 16 (16) and Day 60(60); TM_4 Sertoli cells (TM_4); from TM_3 Leydig cells (TM_3); from types A and B spermatogonia *(AB)*; preleptotene spermatocytes *(PL)*; leptotene and zygotene spermatocytes *(LZ)*; Day 17 prepuberal pachytene spermatocytes (P_{17}); Day 60 adult pachytene spermatocytes (P_{60}); round spermatids, steps 1–8 *(RS)*; elongating spermatids, steps 12–16 *(ES)*; and residual bodies *(RB)*. Note that *Lane PL* was underloaded. The hybridization probes used were as follows: in *A)* E32B (mRNA 1100 nucleotides); *B)* rat SGP-2 (mouse mRNA 1500 nucleotide); *C)* D13 (mouse protamine-1) (mRNA 450–550 nucleotides); *D)* D28 (mRNA 600 nucleotides); *E)* mouse PGK-2 (mRNA 1600 nucleotides); *F)* P3C (mRNA 1000 nucleotides); *G)* mouse LDH-C (mRNA 1400 nucleotides); *H)* D20B (mRNA 700 nucleotides); *I)* C3D (mRNA 3500 nucleotides).

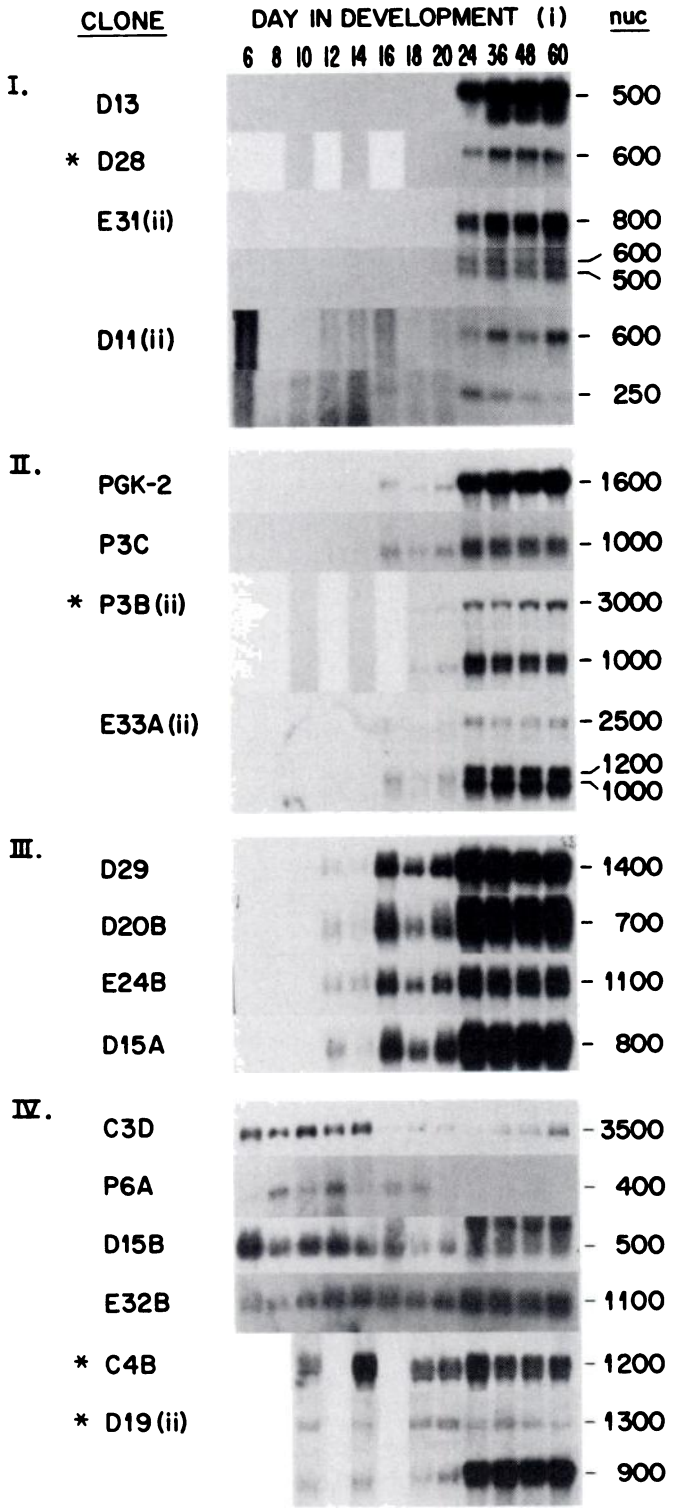
protamine-1 and LDH/C, and each was represented only once among the two hundred. Germ cell expression of a few of the more interesting clones is presented below. Expression of the clones assayed in Figures 2C–2H was undetectable by Northern analysis of poly[A+]RNA isolated from various somatic tissues.

Temporal Gene Expression of Selected cDNA Clones

After birth, development of the seminiferous epithelium is characterized by a defined accumulation of germ cells at progressively more advanced stages of differentiation (Fig. 1) (Bellvé et al., 1977). Thus, by undertaking Northern analyses on poly[A+] mRNAs isolated from the seminiferous epithelium at different ages, it was possible to deduce the cell type first expressing the complementary mRNA (Fig. 2A–I, top). To confirm and extend the resulting data, these developmental blots are accompanied by a second series of Northern blots prepared from total RNA isolated from the respective populations of purified spermatogenic cells (Figs. 2A–I, bottom). These included types A and B spermatogonia, preleptotene, leptotene/zygotene and pachytene spermatocytes (Days 17 and 60), round spermatids (steps 1–8), elongated spermatids (steps 12–16), and residual bodies. For these analyses, all hybridization probes were radiolabeled by nick-translation of restriction fragments containing cDNA inserts. From the results of these hybridization experiments, four distinct phases of gene expression were observed to occur during mouse spermatogenesis.

The cDNA probe E32B hybridized to mRNA in all lanes on both the developmental and the germ cell Northern blots (Fig. 2A). Hybridization of E32B cDNA was most intense to RNA from testicular germ cells. However, this probe also hybridized to a Northern blot of RNA isolated from somatic tissues including kidney, intestine, spleen, pancreas, heart lung, and Leydig (TM_3) and Sertoli (TM_4) tissue culture cells. Although expression was not germ cell-specific, cDNA clone E32B provided an important control probe demonstrating the mRNA was intact in each lane (it is also clear that lane PL in the Northern blots of the purified cell types was underloaded). The possibility of contamination by Sertoli cell RNA in samples on the purified germ cell blots was assayed by hybridization with a cDNA probe for the rat sulfated glycoprotein-2 (SGP-2), which is expressed only by Sertoli cells within the seminiferous epithelium (Morales et al., 1987). All samples on the developmental Northern blots hybridized to the SGP-2 cDNA (Fig. 2B, top), because Sertoli cells comprise at least 30% of the total cell mass within the developing epithelium (Fig. 1). However, SGP-2 cDNA did not hybridize to RNA from any of the purified germ cells (Fig. 2B, bottom), verifying that these samples were not contaminated by Sertoli cell RNA. It is interesting that RNA from the cultured TM_4 Sertoli cells did not hybridize with SGP-2 (Fig. 2B), suggesting that, in culture, these cell did not maintain the ability to express this gene product.

cDNA clones D13 (Fig. 2C) and D28 (Fig. 2D) were expressed only during the haploid cell phase of sperma-



togenesis. D13 was identified as protamine-1 by oligonucleotide hybridization with probes derived from the sequence of the protamine-1 cDNA (Kleene et al., 1984; Peschon et al., 1987). The developmental Northern blots showed that each of these transcripts first appeared by postnatal Day 24 when spermatids (steps 1–8) become abundant within the seminiferous epithelium. The accompanying purified germ cell Northern blot substantiated this observation. Transcripts for D13 and D28 were detected only in round spermatids (steps 1–8), elongating spermatids (steps 12–16), and residual bodies, consistent with the premise that these genes are expressed only during spermiogenesis. These probes did hybridize to RNA isolated from adult pachytene spermatocytes (P_{60}) but not to Day 17 pachytene spermatocytes (P_{17}) (Figs. 2C and 2D, bottom). This difference, presumably, is due to contamination of the adult pachytene sample with round spermatids (~4%) (Romell et al., 1976). The appearance of smaller protamine-1 transcripts by Day 36 (Fig. 2C, top) has been shown previously to result from shortening of the poly[A^+] tail (Hecht et al., 1985). Several other cDNA clones also were expressed exclusively during spermiogenesis (Fig. 3). All of these clones first demonstrated hybridization to RNA from Day 24 prepubertal epithelium. However, individual clones displayed different patterns of mRNA accumulation at subsequent periods of testicular development.

A second phase of gene expression was demonstrated by the developmental and purified germ cell Northern blots hybridized with the cDNA probes for mouse PGK-2 (Fig. 2E) and P3C (Fig. 2F). Both transcripts were first detected at relatively low levels on the developmental Northern blot by Day 16, following the appearance of pachytene spermatocytes within the seminiferous epithelium. A dramatic increase in the abun-

FIG. 3. Pattern of mRNA accumulation within the developing mouse seminiferous epithelium. (i) Poly[A^+] RNA (6 μg/lane) was isolated from the seminiferous epithelium at the designated ages and probed with restriction fragments of the designated cDNA clones. Four phases of gene expression during spermatogenesis are designated as *(I)* cDNA clones that are first expressed in spermatids, *(II)* cDNA clones that are first expressed in pachytene spermatocytes, *(III)* cDNA clones that are first expressed in leptotene or zygotene spermatocytes, and *(IV)* cDNA clones that are expressed in spermatogonia. *(ii)* Identifies the cDNA probes that detect multiple transcripts; each set displayed identical expression kinetics. (*) Hybridization with these probes was to a Northern blot that did not include RNA from Days 6, 8, 12, and 16 (see also *Figure 2D, top panel*). All other probes were hybridized to the same filters shown in the *top panel* of Figure 2A–C, E–I. Transcript length is given in number of nucleotides to the *right* of each clone.

dance of these transcripts was evident by Day 24, coincident with the accumulation of round spermatids (steps 1–8). As confirmed by the spermatogenic cell Northern blot, this marked increase by Day 24 was due to an increase in relative abundance of PGK-2 and P3C mRNAs during spermiogenesis. PGK-2 and P3C appeared to be expressed equally well in round spermatids (steps 1–8). Although the abundance of PGK-2 mRNA remained approximately equivalent throughout spermatid differentiation, the levels of the P3C transcript decreased considerably in elongating spermatids (steps 12–16) and residual bodies (Figs. 2E and 2F, bottom).

Hybridization with the mouse LDH-C (Fig. 2G) and D20B probes (Fig. 2H) illustrated a third pattern of RNA accumulation. These transcripts first appeared on the developmental RNA blots at Day 12, just following the differentiation of leptotene and zygotene spermatocytes (Fig. 1). Northern blots of the spermatogenic cell RNAs confirmed that both of these transcripts were expressed first in leptotene/zygotene spermatocytes (Fig. 2G and 2H, bottom). Both transcripts were relatively abundant in pachytene spermatocytes and round spermatids (steps 1–8), but declined in elongating spermatids (steps 12–16), and were barely detectable in residual bodies.

Another phase of gene expression during spermatogenesis was revealed by Northern analysis with probes for cDNA clones E32B (Fig. 2A) and C3D (Fig. 2I). Both probes hybridized to all samples on the prepubertal Northern blots. However, the two differed in that the relative abundance of E32B increased through Day 24, whereas C3D was more abundant in the prepubertal seminiferous epithelium during Days 6–14 than at later stages of development (Days 16–60). Examination of the purified germ cell Northern blot shows that E32B was expressed in all germ cells (Fig. 2A, bottom); in contrast, C3D was not expressed at detectable levels in spermatids (Fig. 2I, bottom). Although the lack of expression in spermatids contributed to the hybridization pattern of C3D on the prepubertal Northern blot, it was not sufficient to explain the dramatic drop in hybridization intensity after Day 14, because spermatid RNA first accumulated in the Day 24 sample on the Northern blot of prepuberal RNA. Of all the cDNA clones that were analyzed, only C3D and P6A (Fig. 3) hybridized to mRNA of spermatogonia and meiotic germ cells but not to spermatids.

DISCUSSION

Screening by differential hybridization of two mouse testis cDNA libraries has enabled us to isolate two hundred individual cDNA clones corresponding to gene products that are expressed by spermatogenic cells in the developing mouse seminiferous epithelium. Previous efforts by Kleene et al. (1983) and Dudley et al. (1984), using similar techniques, led to the isolation of a few cDNA clones corresponding to transcripts expressed in the testis only by the haploid germ cells. Dudley et al. (1984) reported that all transcripts isolated were present at lower abundances in liver and spleen tissue. Included within this group of clones was TCP-1, which is encoded by a gene within the t-locus on mouse chromosome 17 (Willison et al., 1986). Other genes that are expressed in germ cell-specific patterns included the mouse protamines (Erickson et al., 1980; Hecht et al., 1985), α-tubulin (Distel et al., 1984), and β-actin (Waters et al., 1985). Subsequently, several other reports described haploid expression of the homeobox genes (Rubin et al., 1986; Wolgemuth et al., 1986) and the oncogenes c-abl (Ponzetto and Wolgenmuth, 1985; Oppi et al., 1987), int-1 (Shakleford and Varmus, 1987), c-mos (Propst and Woude, 1985) and erb A, the thyroid hormone receptor (Benbrook and Pfahl, 1987).

By using RNA isolated from the developing seminiferous epithelium and isolated spermatogenic cells, it has been possible to follow the accumulation of specific transcripts during spermatogenesis. Four transcriptional phases are apparent. These include 1) haploid gene expression, exemplified by cDNAs D13, D28, E31, and D11, which was first detected by Day 24 of prepubertal development (Fig. 3); 2) pachytene gene expression, revealed by cDNA clones PGK-2, P3C, P3B, and E33A, was first apparent by Day 16 of development, but the relative abundance of these transcripts increased dramatically by Day 24 with the development of round spermatids; 3) leptotene/zygotene gene expression, observed with cDNA clones D29 (LDH-C), D20B, E24B, and D15A, was first seen by Day 12, and these transcripts were also expressed during the subsequent differentiation of germ cells; and 4) gene expression in spermatogonia and spermatocytes. Transcripts corresponding to cDNA clones D15B, E32B, C4B, and D19 were expressed in various somatic tissues and in all germ cells assayed, whereas transcripts corresponding to cDNA clones C3D and P6A were not expressed in spermatids.

Several facts pertaining to the expression of specific transcripts during spermatogenesis are demonstrated by the present results. We observed that PGK-2 transcripts first are expressed within the prepubertal seminiferous

epithelium by Day 16, the time at which the mid to late pachytene spermatocytes have differentiated. This result corroborates previous research that demonstrated that PGK-2 gene expression is initiated in pachytene spermatocytes, as assayed by in vitro translation of poly[A+] mRNA (Erickson et al., 1980; Kramer and Erickson, 1981; Gold et al., 1983). LDH-C expression is detected on Northern blots by Day 12 of development, much earlier than was observed by a similar in vitro translation assay (Weiben, 1981). The Northern analyses also have provided information on the stability of germ cell-specific transcripts. For example, it appears that transcripts such as protamine-1 and PGK-2 are relatively stable, since both mRNAs are detectable within the anucleate residual body. In contrast, the transcript for LDH-C is barely detectable in the residual body. Since there is no transcription following chromatin condensation in elongating spermatids, the drop in LDH-C abundance must be due to selective RNA degradation (Weiben, 1981).

This study has focused on those clones for which the respective transcripts were detectable on the Northern blots with high specific activity, nick-translated probes. A significant number of cDNA probes just barely or failed to detect mRNA on the poly[A+] prepubertal Northern blots. Further characterization of these cDNA clones will require more sensitive techniques. The possibility exists that the patterns of germ cell gene expression may not be confined only to those described in this study. Additional cDNA libraries derived from poly[A+] mRNA isolated from germ cells at earlier stages of differentiation are being prepared by using unidirectional cloning vectors. With these techniques, it should be possible to isolate more cDNA clones that correspond to transcripts which are expressed exclusively in spermatogonia and/or early spermatocytes.

The specificity of gene expression revealed by each of the analyzed cDNA clones is likely to be regulated, at least in part, at the transcriptional level. Indeed, the promoters of three spermatogenic genes have been shown to direct germ cell-specific expression in transgenic mice. The mouse protamine-1 (Behringer et al., 1988), protamine-2 (Stewart et al., 1988) and the human PGK-2 promoter (M. Robinson, personal communication) all contain sequences within 900 bps of the transcription initiation site that regulate appropriate gene expression. Further studies may reveal protein transcription factors and the specific promoter sequences that these recognize.

In summary, this study emphasizes that all stages of germ cell differentiation are characterized by selective gene expression. The availability of these cDNA probes provides powerful tools for studying mechanisms regulating gene expression during spermatogonial proliferation and renewal, meiotic prophase, and spermiogenesis.

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