Journal of Andrology, Vol. 15, No. 3, May/June 1994

Proacrosin Gene Expression in Rat Spermatogenic Cells

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ABSTRACT: Mammalian proacrosin gene expression was considered to be exclusively postmeiotic until recent studies detected the presence of proacrosin mRNA in mouse pachytene spermatocytes. To determine if rat proacrosin gene expression was initiated during meiosis, a 314-bp proacrosin cDNA fragment was amplified from rat round spermatid RNA, using proacrosin-specific primers, for use as a probe. Sequence analysis of the round spermatid 314-bp cDNA fragment confirmed >99% identity with the rat proacrosin cDNA sequence. This 314-bp fragment was subsequently used for Northern blot analysis of RNA isolated from testicular germ cells. A 1.6-kb transcript was detected in pachytene spermatocytes, round spermatids, and a mixed population of condensing spermatids/residual bodies, with the highest level of expression in round spermatids. Northern blot analysis of testicular RNA during development revealed the earliest timepoint of expression to be at 24 days of age, further demonstrating the association of proacrosin mRNA with spermatocytes. These data demonstrate diploid expression of the rat proacrosin gene, in agreement with mouse proacrosin gene expression but in contrast to the apparent haploid expression of proacrosin described for the bull and the boar. These studies provide evidence that, in the rat, the process of acrosome biogenesis begins during meiosis.

Key words: Testis, sperm development, meiosis, acrosome, germ cell.

J Androl 1994;15:244–249

The acrosome is an organelle that is unique not only in its sperm-specific localization, but also as a model system for organelle biogenesis. Although the morphogenesis of the mammalian acrosome has been well documented, the concomitant biogenesis of acrosomal proteins and their routing to the acrosome is less well defined. Until recently, in many mammalian species, the expression of acrosome-specific antigens had not been detected earlier than the round spermatid stage of spermiogenesis. This coordinated expression of acrosomal proteins and morphologic formation of the mature acrosome had been described not only for the protease acrosin (Florke et al, 1983; Phi-Van et al, 1983; Kallajoki et al, 1986; Arboleda and Gerton, 1988) but also for other acrosomal proteins (for a review, see Peterson et al, 1992).

Earlier studies relied in large part on immunochemical techniques to detect the presence of proacrosin protein. More recently, molecular biology techniques have allowed the earliest timepoint of proacrosin mRNA expression to be determined; the molecular cloning of proacrosin cDNAs from several mammalian species has provided the probes necessary for investigating mRNA expression. Northern blot analysis of RNA from prepubertal bull testis and *in situ* hybridization studies of transcript localization in boar testis suggested a postmeiotic expression pattern for proacrosin (Adham et al, 1989). Although initial Northern blot analyses of prepubertal mouse testes could not detect proacrosin mRNA earlier than day 20 of development (Kashiwabara et al, 1990a; Klemm et al, 1990), indicating postmeiotic expression, later studies by the same investigators using enriched spermatocyte and spermatid germ cell populations on Northern blots indicated proacrosin mRNA was expressed in pachytene spermatocytes (Kashiwabara et al, 1990b; Kremling et al, 1991). Additionally, the biosynthesis of human proacrosin has been reported during the diploid phase of spermatogenesis (Escalier et al, 1991).

It is unclear whether or not the timeframe for initiation of proacrosin gene expression is species-specific. The present study was undertaken to determine definitively the expression pattern of rat proacrosin mRNA, via analyses of RNA from enriched germ cell populations and developmental testis preparations. Our results suggest diploid expression of the rat proacrosin gene.

Supported by grants from the Minnesota Medical Foundation, WHO Basic Research Project 90009 (L.W.H.), and NIH Fellowship GM13906 (L.S.R.).

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Received for publication August 23, 1993; accepted for publication September 14, 1993.

Materials and Methods

Preparation of Testicular Germ Cell Populations

A mixed testicular germ cell suspension was isolated from the seminiferous epithelium essentially by the method of Romrell et al (1976), as modified by O'Brien (1987). This mixed popu-

lation was fractionated by unit gravity velocity sedimentation in a 2–4% bovine serum albumin gradient using a CelSep® sedimentation chamber (originally obtained from Wescor, Inc.; now available from Brinkmann, Westbury, New York) as described by Wolgemuth et al (1985). After settling for 95 minutes, the chamber was drained and 100 fractions were collected for examination by light microscopy using Nomarski optics. Fractions were pooled, based on cell morphology, to produce enriched populations. Pachytene spermatocytes (80–85% pure) and round spermatids (>90% pure) were highly enriched, whereas condensing spermatids were largely contaminated with residual bodies.

RNA Isolation

Tissue specimens were collected fresh from Sprague-Dawley rats and immediately frozen by submersion in liquid nitrogen, then stored at −70°C until use. Total RNA was isolated by the acid guanidinium–thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). For isolation of total RNA from germ cells and Clone 9 liver cells, cell pellets were processed essentially as previously described (Chomczynski and Sacchi, 1987), with the exception that 4 μg glycogen (molecular biology grade, Boehringer Mannheim) was added to the initial aqueous phase to facilitate RNA precipitation.

Primer Design

Oligonucleotide sequences derived from published rat proacrosin cDNA data (Klemm et al, 1991) representing conserved regions of DNA were analyzed as potential primers using PCR-Primer Selection software (Epicenter Software). The primer sequences were optimized to prevent primer–dimer and primer–primer self-annealing. As a second selection criterion, the potential primer sequences were checked against the nucleotide data banks using Intelligenetics, Inc. software (Brutlag et al, 1990) to avoid the use of a primer that could anneal to many cDNAs. Primers were synthesized and HPLC-purified at the Microchemical Facility, Institute of Human Genetics, University of Min-Minnesota. The forward primer used in this study was 5′-GACTGGTTTTTGGAGC-3′ and the reverse primer was 5′-AATGAGATCCACTCGG-3′.

Polymerase Chain Reaction (PCR) Amplification

For RNA-PCR, 1 μg of total RNA isolated as described above was reverse transcribed to cDNA using 2.5 U Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus) for 1 hour at 37°C. Following this step, the PCR reagents were added directly to the reverse transcription reaction tube and the PCR amplification carried out as described below.

For PCR, the target DNA in the reaction mixture was amplified with 2.5 U AmpliTaq® DNA polymerase (Perkin-Elmer Cetus) and 50 pmol of each of the appropriate forward and reverse primers. The reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler, using a cycle of 1 minute at 94°C (melt), 2 minutes at 55°C (anneal), and 3 minutes at 72°C (extension). After an initial melt of 3 minutes at 94°C, 35 cycles were used to amplify the DNA. PCR reaction products were analyzed by agarose gel electrophoresis, with DNA fragments visualized by ethidium bromide staining.

Probe Preparation

For preparation of digoxigenin-labeled DNA fragments to be used as nonradioactive probes, DNA fragments were purified from agarose gel slices using the Magic® PCR Preps DNA Purification System (Promega) and subsequently labeled by performing a PCR amplification in the presence of digoxigenin-labeled deoxyuridine triphosphate (Boehringer Mannheim), using a 65% dTTP/35% digoxigenin-11-dUTP ratio in the nucleotide mixture.

Alternatively, digoxigenin-labeled cDNA probes were synthesized by PCR amplification of PCR fragments subcloned into expression vectors. For proacrosin, PCR-amplified DNA prepared using the primers described above was subcloned into pCRII vector (TA Cloning Kit, Invitrogen). Digoxigenin-labeled proacrosin cDNA fragments could then be produced by amplification of insert DNA in purified plasmid preparations (purified by Magic® MiniPreps DNA Purification System). Proacrosin-specific primers corresponding to the ends of the insert or a primer set specific for the SP6 promoter and T7 promoter sites flanking the insert were used to prime the PCR amplification. Digoxigenin-labeled protamine 1 probe was also prepared by PCR amplification. Full-length mouse protamine 1 cDNA (Kleene et al, 1985; Yelick et al, 1987) subcloned into pGEM-2 (obtained from ATCC/NIH Repository of Human and Mouse Probes and Libraries) was digoxigenin-labeled by PCR amplification of purified plasmid DNA using primers specific for the SP6 and T7 promoter sites that flank the multiple cloning site. For all digoxigenin-labeled probes, the labeled cDNA fragments were separated from unincorporated digoxigenin-nucleotide and other reaction mix components with the Magic® PCR Preps DNA Purification System.

Northern Blotting

RNA samples were electrophoresed in agarose gels containing 6% formaldehyde (Rave et al, 1979). After a brief incubation in 10× saline–sodium citrate (SSC), the gel was placed next to a sheet of Magnagraph nylon membrane (Micron Separations Inc.) in a setup for capillary transfer in 10× SSC. Following transfer, the membrane was baked at 80°C for 30–60 minutes, then UV-crosslinked at a setting of 200 μjoules (×100) in a Stratalinker® 2400 (Stratagene). Blots were prehybridized in 50% formamide, 5× Denhardt's solution (Denhardt, 1966), 0.1 mg/ml salmon sperm DNA, 0.1% sodium dodecyl sulfate (SDS), and 5× SSC for 4–6 hours at 42°C. Membranes were then hybridized overnight at 42°C in 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% Sarkosyl, and 0.02% SDS containing 10–20 ng/ml of dioxigenin-labeled probe.

Following probe incubation, washes were performed with 2× SSC and 0.1% SDS at room temperature, followed by washes at 68°C with 0.1× SSC and 0.1% SDS. Membranes were then blocked with 1% blocking reagent in 0.1 M maleic acid, pH 7.5, and 0.15 M NaCl. After blocking, blots were incubated with alkaline phosphatase-conjugated (1:10,000 dilution; Boehringer Mannheim) sheep anti-digoxigenin F_{ab} fragment. Excess antibody was washed away and blots equilibrated in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$. LumiPhos® substrate was diluted (1:50) in this pH 9.5 buffer and incubated with the membrane for 5 minutes. Membranes were then re-

moved from the substrate solution and sealed in a heat-sealable bag while still damp. Membranes were exposed to Kodak X-OMat film to prepare a "lumigram" after a short incubation at 37°C to initiate the alkaline phosphatase reaction.

Sequencing

For sequencing of PCR-amplified DNA, the double-stranded DNA Cycle Sequencing System (GIBCO BRL Life Technologies, Inc.) was used. Sequencing was performed on approximately 50 fmol of purified DNA obtained from agarose gel slices as described above (sequencing protocol: 20 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 70°C, followed by 10 cycles of 30 seconds at 95°C and 60 seconds at 70°C).

To perform additional sequencing, PCR-amplified DNA subcloned into pCRII vector was sequenced by the chain termination method (Tabor and Richardson, 1989) using M13 primers and Sequenase® (US Biochemical Corporation). Radiolabeled nucleotides (^{35}S-dATP and γ^{32}P-dATP) for sequencing were purchased from DuPont-New England Nuclear.

Sequencing reaction products were electrophoresed on denaturing gels of 7 M urea, 1.2× Tris-borate-ethylenediaminetetraacetic acid (EDTA), 5% Long Ranger acrylamide (J.T. Baker). Following electrophoresis, gels were dried under vacuum at 80°C, without a prior fixation step, then placed against Kodak X-OMat AR film. Sequences were read from the developed films and analyzed using Intelligenetics, Inc. software.

Results

Rat proacrosin-specific sequences, as indicated in Figure 1, were used as primers for RNA-PCR amplification from rat round spermatid RNA. A single product of the expected size of 314 bp was observed on ethidium bromide-stained agarose gels (data not shown). This 314-bp DNA fragment was excised from the agarose gel, purified, and sequenced by double-stranded cycle sequencing. Additional PCR product was removed directly from the reaction tube, subcloned into pCRII vector, and sequenced using Sequenase®. The sequence presented in Figure 1 represents a consensus sequence compiled by comparing four separate sequencing runs using cycle sequencing and three separate runs of Sequenase® sequencing. The cDNA fragment was found to have sequence identity of >99% with rat proacrosin cDNA (Klemm et al, 1991), confirming that the PCR reaction product is a proacrosin cDNA fragment.

Based on the previous data (Klemm et al, 1991), the expected cDNA fragment length was 314 nucleotides. However, the consensus sequence for the PCR product represented only 312 nucleotides, because of two deletions observed upon comparison to the previously published sequence (Fig. 1). The deletions represent the only areas of nonidentity. For consistency, the proacrosin cDNA fragment utilized in this study will be referred to as pcr314, based on the initial expectation of its size.

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  1GACTGGTTTTTGGAGCCCATGAAATTGAATACGGAAGAAACAAGCCAGTG
323.................................................

 51AAAGAGCCCCAGCAGGAAAGATACGTGCAGAAAATTGTCATCCACGAGAA
373.................................................

101ATACAACGCTGTGACCGAGGGGAACGACATTGCCCTCTTGAAAGTCACTC
423.................................................

151CTCCTGTTACATGTGGGGACTTCGTTGGGCCTGGCTGCCTACCTCATTTT
473.................................................

201AAGTCTGGTCCTCCCAGAATCCCCCACACCTGCTACGTGACTGGGTGGGG
523.................................................

251ATACATAAAAGATAACG-CCCCAGACCATCACCTGT-CTGATGGAGGCCC
573.....C.....C.....

299GAGTGGATCTCATT
623..............
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FIG. 1. Sequence analysis of 314-bp PCR product. A proacrosin-specific forward primer, marked here by underlining, represents nucleotides 323–338 of rat proacrosin cDNA (Klemm et al., 1991). A proacrosin-specific reverse primer, the inverse complement of nucleotides 621–636 (marked by bold underlining) of rat proacrosin cDNA (Klemm et al., 1991) was utilized in conjunction with the forward primer for RNA-PCR amplification of rat round spermatid RNA; the resulting 314-bp product was sequenced. The consensus sequence for the cDNA fragment from several sequencing runs (top line) is compared to the previously published sequence (bottom line). Periods represent base identity; a dash indicates a deletion.

The pcr314 fragment was labeled with digoxigenin and used for analysis of proacrosin gene expression in spermatogenic cells. RNA preparations from pachytene spermatocytes, round spermatids, condensing spermatids, and Clone 9 liver cells were separated by formaldehyde–agarose gel electrophoresis, transferred to nylon, and probed with digoxigenin-labeled pcr314 and protamine 1 cDNA (Fig. 2). The expected proacrosin 1.6-kb transcript was detected as early as the pachytene spermatocyte stage of spermatogenesis, with highest levels of expression seen in round spermatids. No transcripts were detected in a somatic cell line, Clone 9 liver cells.

Protamine 1 transcripts of sizes 500 and 600 bp are restricted in expression to haploid cells (Hecht, 1988; Klemm et al, 1989); the amount of protamine mRNA signal observed in the pachytene spermatocyte RNA lane serves as an indicator of the level of contamination with spermatids. Based on the relative protamine 1 signals detected in lanes PS and RS (Fig. 2), spermatids represent a minor component of the pachytene spermatocyte preparation. Therefore, a comparison of the proacrosin transcript signals observed in the pachytene spermatocyte RNA (lane PS) and round spermatid RNA (lane RS) demonstrates that proacrosin is truly expressed in pachytene spermatocytes, as the transcript level is much higher than that expected due solely to spermatid contamination.

As an additional assay for determining the onset of proacrosin gene expression, testis RNA samples representing a developmental continuum from postnatal day 17 to adulthood were examined by Northern blot analysis, using digoxigenin-labeled pcr314 as a probe. As demon-

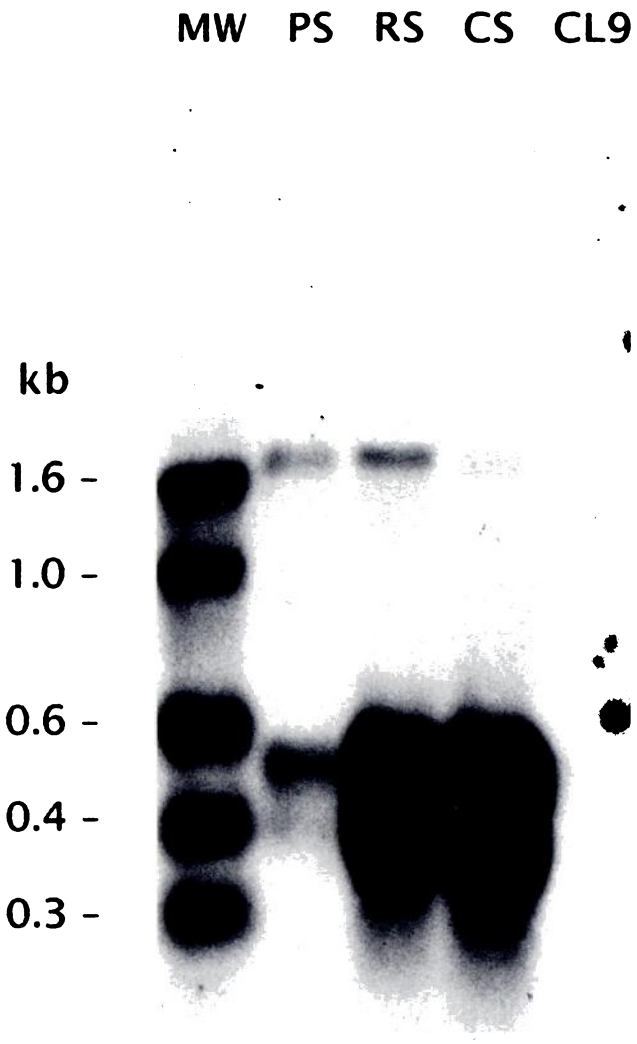

FIG. 2. Northern blot analysis of germ cell RNA. Fifteen μg of total RNA isolated from pachytene spermatocytes (PS), round spermatids (RS), condensing spermatids (CS), and Clone 9 liver cells (CL9; Weinstein et al, 1975) was electrophoresed, blotted onto nylon, and probed with digoxigenin-labeled pcr314 and protamine 1 cDNA. Numbers to the left represent the size of RNA molecular weight standards (MW, digoxigenin-labeled RNA Molecular Weight Marker III, Boehringer Mannheim).

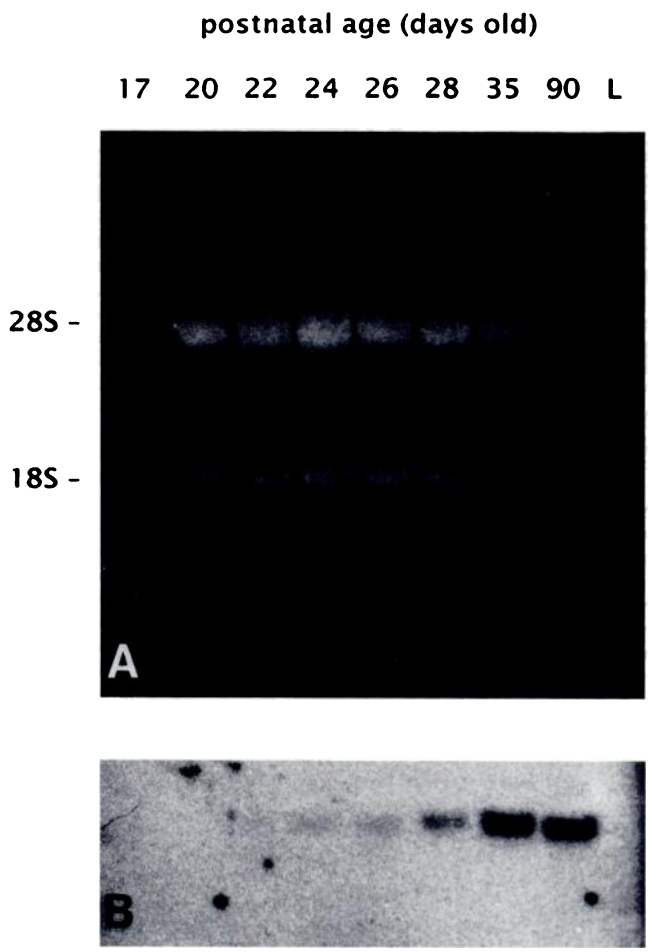

FIG. 3. Northern blot analysis of rat testis RNA. Rat tissue was processed to isolate total RNA as described in the Materials and Methods. For samples prepared from day-17 to day-35 animals, tissue from three individual animals was collected and combined prior to RNA isolation to minimize animal-to-animal variation. Fifteen μg of total RNA was electrophoresed in each lane and blotted onto nylon. Panel **A**, ethidium bromide-stained gel. Panel **B**, membrane probed with digoxigenin-labeled pcr314 and processed to produce a lumigram. Numbers to the left in Panel **A** denote the 28S and 18S rRNA species. Lanes are labeled with the postnatal age of the animals used as source of tissue. The liver sample (L) was obtained from an adult male rat.

strated in Figure 3 (Panel B), proacrosin mRNA is first detected at 24 days of age. Ethidium bromide staining of RNA prior to transfer demonstrated that the absence of proacrosin signal in the early stages of development was not due to degradation of the RNA in these samples (Fig. 3, Panel A). Hybridization of protamine 1 probe is not seen until postnatal day 35 of development (data not shown), in agreement with previous data (Klemm et al, 1989). However, it is likely that protamine 1 transcript would first appear in a day-29 or day-30 testis RNA sample, based on the initiation of protamine 1 mRNA synthesis in step 5 spermatids in other species (Morales et al, 1991).

Discussion

The results in Figure 2 indicate that, as in the mouse, rat proacrosin mRNA is first expressed in pachytene spermatocytes. This diploid expression pattern contrasts to the haploid expression described for boar and bull proacrosin (Adham et al, 1989). The examination of isolated germ cell populations that represent the different stages

of spermatogenesis has allowed detection of proacrosin mRNA in mouse pachytene spermatocytes (Kashiwabara et al, 1990b; Kremling et al, 1991).

In Figure 2, the highest level of proacrosin transcript appears in the population of purified round spermatids. This expression pattern has previously been described for the mouse proacrosin system (Kashiwabara et al, 1990b). In the same study, proacrosin mRNA was found to be polysome-associated in pachytene spermatocytes, suggesting protein translation occurred at this early stage of germ cell development. These results are in contrast to other reports, which demonstrate that proacrosin protein is first detected in the round spermatid stage of germ cell development (Flörke et al, 1983; Phi-Van et al, 1983; Kallajoki et al, 1986; Arboleda and Gerton, 1988). Translational control mechanisms have been described for several testis genes, including PGK-2 (Gold et al, 1983), transition proteins 1 and 2 (Kleene and Flynn, 1987; Yelick et al, 1989; Morales et al, 1991), and protamines 1 and 2 (Kleene et al, 1984; Hecht, 1989; Braun, 1990; Morales et al, 1991). These same mechanisms may be utilized if proacrosin is transcribed at the spermatocyte stage but not translated until days later, at the round spermatid stage.

In the developing rat testis, haploid cells first appear at postnatal day 25 (Zhengwei et al, 1990). Our results with respect to the developmental expression of rat proacrosin (Fig. 3) indicate that proacrosin mRNA is synthesized prior to day 25 and therefore is initially associated with spermatocytes. However, proacrosin protein synthesis may not occur until the postmeiotic stage of development.

The timeframe of proacrosin gene expression and protein synthesis relates to the study of acrosome biogenesis. Acrosomal proteins have been detected prior to spermiogenesis, including acrogranin (Anakwe and Gerton, 1990), guinea pig autoantigen AA1 (Hardy et al, 1988), and mouse and guinea pig 1D4 antigen (Gerton et al, 1988; O'Brien et al, 1988). Additional studies will be required to determine if the genes for all acrosomal proteins are coordinately expressed, how the biosynthesis of acrosomal proteins is regulated, and how various acrosomal proteins are trafficked to the developing organelle to create a mature acrosome. Transgenic mouse studies using fusion constructs of mouse protamine 1 gene and the human growth hormone structural gene demonstrated that the timepoint in spermatogenesis when these transgenes were translated altered trafficking of the gene product; synthesis in round spermatids resulted in acrosomal localization, whereas protein synthesis in condensing spermatids yielded cytoplasmic localization (Braun et al, 1989a,b). It is apparent that the temporal regulation of acrosomal gene expression and subsequent protein biosynthesis are of importance in the biogenesis of the acrosome in the developing germ cell.

Acknowledgments

We thank Jerry Sedgewick for his aid in preparation of the illustrations in this report.

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