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ACROSIN BIOSYNTHESIS IN MEIOTIC AND POSTMEIOTIC SPERMATOGENIC CELLS

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It has been widely accepted that mammalian sperm acrosin is first synthesized only in the postmeiotic stages of spermatogenic cells. In this study, we carried out Northern blot analysis of RNAs prepared from purified populations of mouse spermatogenic cells. The acrosin mRNA was obviously found in meiotic pachytene spermatocytes, and the mRNA content markedly increased in postmeiotic round spermatids. Also, the acrosin mRNA in pachytene spermatocytes was functionally associated with polysomes. These results provide evidence that acrosin biosynthesis is already started in meiotic cells and continues through the early stages of spermiogenesis.

Spermatogenesis, differentiation of germ cells, involves morphological changes in cellular organelles and synthesis of germ-cell-specific proteins during the meiotic and haploid phases (1, 2). Acrosome is one of the sperm-specific organelles that are formed during the haploid phase of spermatogenesis, spermiogenesis (3). It contains a variety of hydrolytic enzymes which are required for the sperm-egg interaction. Although morphological features of acrosome formation and development during spermiogenesis have been extensively studied, the biochemical aspects of acrosome formation, including synthesis, processing, and targeting of the acrosome components, have not been characterized well.

Acrosin is a serine protease that is localized in the sperm acrosome as an enzymatically inactive zymogen, proacrosin, and is released as a consequence of the acrosome reaction (4, 5). It is reasonable to consider that acrosomal proteins, including acrosin, are synthesized during spermiogenesis, since acrosome formation is initiated immediately after the completion of meiosis (3). In fact, immunochemical studies on acrosin biosynthesis have shown that acrosins from various mammalian species begin to be synthesized in early, round spermatids (6-9). Recently, the cDNA clones for human (10, 11), porcine (12, 13), and mouse (14, 15) acrosins have been isolated. Hybridization analysis apparently demonstrated that the acrosin

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gene is first expressed only in the postmeiotic stages of spermatogenesis (13-15). Thus, it seems that the question concerning acrosin biosynthesis has been all settled.

In this study, we have re-examined expression of the mouse acrosin gene using purified populations of testicular cell types. The acrosin gene is expressed in both pachytene spermatocytes and round spermatids, and translation of the acrosin mRNA also occurs in the same stages of spermatogenesis.

MATERIALS AND METHODS

Purification of spermatogenic cells. Prepuberal (17-day-old) and sexually mature (60-day-old or more) ddY mice were obtained from Japan SLC Inc. (Shizuoka, Japan). Decapsulated testicles were placed in bicarbonate-buffered RPMI 1640 (Gibco) and incubated in the presence of 1 mg/ml collagenase with agitation from 5% CO_2 bubbling at 34°C for 15 min (16). The dispersed seminiferous tubules were washed three times with ice-cold bicarbonate-buffered RPMI 1640 and incubated in the same buffer containing 250 μg/ml trypsin and 5 μg/ml DNase I for 15 min, as described above. The cell aggregates were shared gently by repeated pipetting, filtered through 74-μm nylon mesh, and then centrifuged at 1,500 rpm for 10 min. The pellet was resuspended in bicabonate-buffered RPMI 1640 containing 0.5% BSA and 1 μg/ml DNase I and filtered again through the nylon mesh. The cell suspension was fractionated by a STA-PUT unit gravity sedimentation system (Johns Scientific, Canada), using a 2-4% BSA (w/v) gradient. Fractions (12 ml) were collected and assayed by phase-contrast microscopy, and then the identical cell populations were pooled. The purities of pachytene spermatocytes and round spermatids exceeded 90% in all experiments.

Isolation of RNA and Northern blot analysis. Total RNA was prepared from purified populations of spermatogenic cells by the guanidium isothiocyanate method followed by centrifugation on a 5.7 M CsCl cushion (17). The RNA samples were denatured with glyoxal (18), electrophoresed on 1.2% agarose gels, and then transferred onto GeneScreen Plus membranes. The blots were probed by the ^{32}P-labeled cDNA fragments of mouse acrosin and beta-actin. Stringencies employed for hybridization and washing were those described previously (12).

Preparation of polysome fractions. The purified spermatogenic cells were homogenized in 1 ml of HK buffer (20 mM Hepes/NaOH, pH 7.5, 100 mM KCl) containing 10 mM $MgCl_2$, 0.1% diethylpyrocarbonate, and 0.5% Triton X-100 at 4°C with a Teflon-glass homogenizer. The homogenate was centrifuged at 12,000g for 10 min. The half of the supernatant was placed on a 10-40% sucrose gradient (9 ml), which had a 0.45-ml cushion of 60% sucrose, in HK buffer containing 1.5 mM $MgCl_2$, and centrifuged at 40,500 rpm for 2 hr at 4°C in a Beckman SW-41 rotor (19). To the remaining supernatant EDTA was added to give a final concentration of 100 mM. The mixture was placed on the sucrose gradient and centrifuged, as described above, except that 1.5 mM $MgCl_2$ in the gradient and cushion was replaced by 10 mM EDTA. After centrifugation, fractions were collected from the bottom of the centrifuge tube. RNA was precipitated with ethanol, using yeast tRNA as a carrier. The pellet was dissolved in 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 0.5% SDS, treated with phenol/chloroform solution, and then precipitated with ethanol. The precipitate was dissolved in water and subjected to Northern blot analysis, as described above.

RESULTS

To examine expression of the mouse acrosin gene during spermatogenesis, spermatogenic cell suspensions were separated by unit gravity sedimentation into populations of pachytene spermatocytes, round spermatids, and a mixture (residual body fraction) of elongating spermatids and residual bodies. The pachytene spermatocytes obtained by this technique are usually contaminated with multinucleate spermatids. Since the seminiferous cell suspension from 17-day-old mice contains predominantly meiotic germ cells (20), we purified the pachytene spermatocytes from these mice. On the other hand, mouse testis contains two kinds of actin mRNAs that are 2.1 and 1.5 kb in length (21, 22). The 2.1-kb actin mRNA is found throughout spermatogenesis, whereas the 1.5-kb mRNA is first detected in round spermatids (21, 22). We used the mouse beta-actin cDNA as a hybridization probe to verify the purity of isolated pachytene spermatocytes. The population of pachytene spermatocytes from 17-day-old mice contains no 1.5-kb actin mRNA (Fig. 1-B, lane 1). This result clearly demonstrates that this cell population is not contaminated with round spermatids. The preparation of pachytene spermatocytes from sexually mature mice is still contaminated with round spermatids (Fig. 1-B, lane 2). When total RNA samples from purified cell populations were probed by a RsaI/RsaI fragment of the mouse acrosin cDNA (15), a 1.8-kb acrosin mRNA was obviously found in the pachytene spermatocytes from 17-day-old mice (Fig. 1-A, lane 1). The level of the acrosin mRNA content increased in the

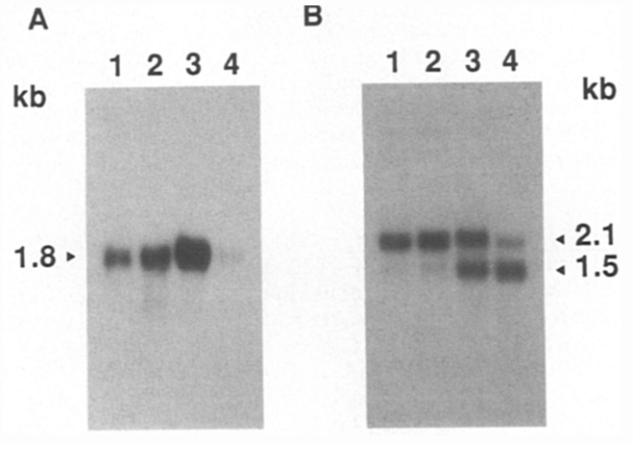

Fig. 1. **Northern blot analysis of acrosin mRNA in purified meiotic and postmeiotic spermatogenic cells.** Total RNAs (5 μg each) of pachytene spermatocytes from prepuberal (17-day-old) mice (lane 1), and pachytene spermatocytes (lane 2), round spermatids (lane 3), and a mixture of elongating spermatids and residual bodies (lane 4) from adult (60-day-old or more) mice were denatured with glyoxal (18), electrophoresed on 1.2% agarose gels, and transfered onto GeneScreen Plus membranes. The blots were probed by (A) a 435-bp RsaI/RsaI fragment of mouse acrosin cDNA (15) or (B) a 421-bp AluI/AluI fragment of mouse beta-actin cDNA (29).

round spermatids, and remarkably decreased in the residual body fraction (Fig. 1-A, lanes 3 and 4). Thus, the acrosin gene is already expressed in meiotic, pachytene spermatocytes. It is most unlikely that the presence of acrosin mRNA in the residual body fraction (Fig. 1-A, lane 4) is attributed to actual expression of the acrosin gene, since these cells are transcriptionally inactive (23-25). This may be due to the contaminating round spermatids in the residual body fraction and/or the remaining acrosin mRNA which had been transcribed during the earlier stages of spermatogenesis.

Polysome and non-polysome fractions were prepared from purified populations of pachytene spermatocytes and round spermatids, and the distibution of acrosin mRNA was assessed by Northern blot analysis (Fig. 2). In the presence of Mg^{2+}, the 1.8-kb acrosin mRNA was detected in the polysome fraction in both cell types. The acrosin mRNAs detected in the polysome fraction were shifted to the non-polysome fraction in the presence of EDTA which is known to dissociate mRNA from the polysome (26). This result shows that acrosin mRNAs in both pachytene spermatocytes and round spermatids are functionally associated with polysomes. When beta-actin was used as

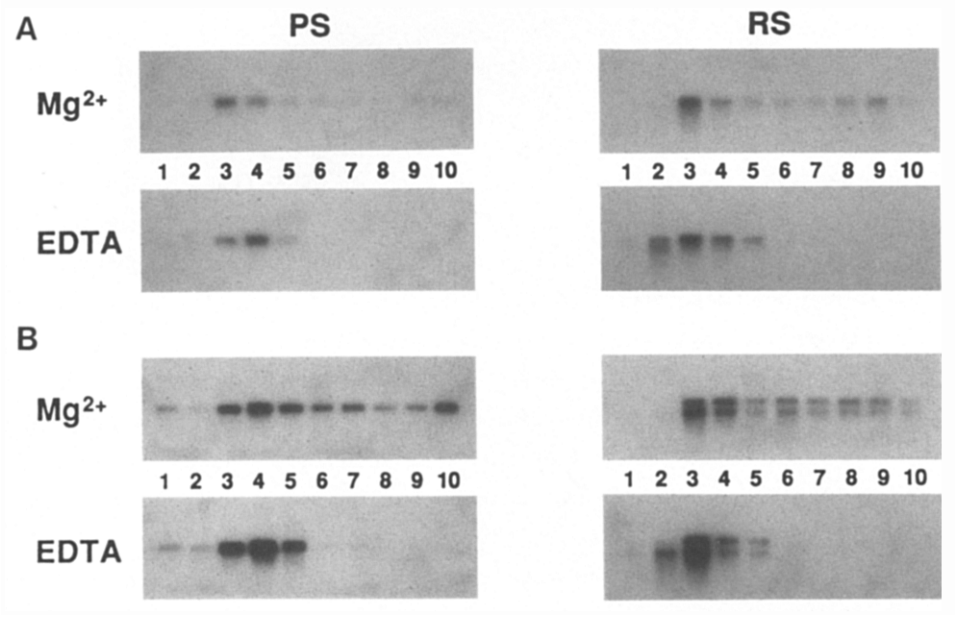

Fig. 2. **Distribution of acrosin mRNA in polysome and non-polysome fractions of pachytene spermatocytes and round spermatids.** Postmitochondrial supernatants of pachytene spermatocytes (1.3×10^7 cells) and round spermatids (7.5×10^7 cells) from prepuberal and adult mice, respectively, were sedimented in 10-40% sucrose gradients containing either $MgCl_2$ or EDTA (see MATERIALS AND METHODS). Fractions (1 ml) were collected, and total RNA prepared from each fraction was subjected to Northern blot analysis, using the acrosin (A) and beta-actin (B) cDNA fragments as probes, as described in Fig. 1. Fractions 1-5 and 6-10 correspond to polysome and non-polysome fractions, respectively. PS, pachytene spermatocytes; RS, round spermatids.

a probe, no 1.5-kb actin mRNA was detected in the polysome and non-polysome fractions of pachytene spermatocytes, confirming that the purified pachytene spermatocyte population was not contaminated with round spermatids.

DISCUSSION

We have already reported that using Northern blot analysis of testicular RNAs from prepuberal and adult mice of different ages, the acrosin mRNA was first detectable at the time when spermatogenic cells in the seminiferous epithelium begin to differentiate into round spermatids (15). The acrosin mRNA was mostly found in round spermatids by in situ hybridization analysis on histological sections of seminiferous tubules, and no significant signal was observed at detectable level in the spermatocytes (15). These results seem to conclude that acrosin gene expression first occurs in the early stages of round spermatids, as described by Engel and his co-workers (13, 14). In the present experiment, the acrosin gene is expressed in pachytene spermatocytes as well as in round spermatids (Fig. 1). The reason for this discrepancy may be due to the increased ratio of the acrosin mRNA in total RNA from purified population of pachytene spermatocytes. At any rate, this study provides evidence for acrosin gene expression in the pachytene spermatocytes. However, it is not certain at present that the acrosin gene is expressed during the spermatogenic stages of leptotene and zygotene spermatocytes.

Acrosin mRNAs are functionally associated with polysomes in pachytene spermatocytes (Fig. 2), indicating that acrosin mRNA translation occurs in these cells. This result is different from those of the previous immunological studies showing that acrosin is not found at detectable level in pachytene spermatocytes (6-9). However, several workers observed occasionally spermatocyte-like cells containing granules which immunoreacted with anti-acrosin antibodies (9). Other acrosomal proteins, such as autoantigen (27) and acrogranin (28), have been also reported to be detected in pachytene spermatocytes. Therefore, we conclude that biosynthesis of acrosin, probably including other acrosomal components, begins prior to the haploid phase of spermatogenesis.

Germ cell differentiation requires selective gene expression of the stage-specific proteins. In order to establish the biosynthetic mechanisms of organelles in germ cells, including acrosome, it is necessary to study transcriptional and translational controls of the germ-cell-specific genes.

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