Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 6086–6090, October 1980
Genetics

Quantitation of mRNAs during mouse spermatogenesis: Protamine-like histone and phosphoglycerate kinase-2 mRNAs increase after meiosis

(haploid gene expression/gamete equivalence/spermatozoa/gene transcription)

ROBERT P. ERICKSON, JAMES M. KRAMER, JUDITH RITTENHOUSE, AND ANN SALKELD

Department of Human Genetics, University of Michigan School of Medicine, Ann Arbor, Michigan 48109

Communicated by James V. Neel, July 24, 1980

ABSTRACT **Total RNA, prepared from immature or mature mouse testes or from spermatogenic cells separated on the basis of sedimentation velocity, was translated *in vitro*. Mouse protamine-like histone could be identified as an *in vitro* translational product when [^{3}H]arginine was used as the label. The mRNA for protamine-like histone was detected only after meiosis; the appearance of a peak of radioactivity comigrating with protamine-like histone occurred only when RNA from mature testes or late spermatid cell fractions was translated. Phosphoglycerate kinase-2 (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) was identified as an *in vitro* translational product by affinity chromatography followed by two-dimensional gel electrophoresis or by specific immunoprecipitation when [^{35}S]methionine was the label. The mRNA for phosphoglycerate kinase-2 was detected only in mature testes or late spermatid cell fractions. These translational assays for protamine-like histone and phosphoglycerate kinase-2 mRNAs suggest that these messages are transcribed after meiosis.**

It is usually considered that sperm function depends little or not at all on the genotype of the sperm but mainly or only on the diploid genotype of the male, and that all four products of meiosis form functional sperm (1). Thus, it has been generally assumed that postmeiotic (i.e., haploid) gene transcription does not occur. There are two lines of argument for the alternative view in mammals. First, although Mendelian transmission ratios require gamete equivalence, this could be accomplished in other ways than eliminating postmeiotic gene transcription. The large intercellular bridges connecting all spermatids allow a sharing of gene products and would contribute greatly to gametic equivalence (2). Second, the appearance of sperm specific proteins after meiosis requires new transcription or the use of stored mRNA. There is little evidence for the use of stored mRNA in mammalian embryogenesis, in contrast to the situation in lower vertebrates (3, 4). The situation remains to be explored for mammalian gametogenesis.

Evidence for postmeiotic RNA synthesis was first provided by Monesi (5), who demonstrated that early mouse spermatids exposed to short pulses of [^{3}H]uridine showed a small peak of incorporation when examined by autoradiography. Quantitative autoradiographic studies showed that the rate of RNA synthesis per cell decreased only 75% during meiosis in the ram testis; i.e., the RNA synthesis/DNA ratio was essentially unchanged (6). The newly synthesized RNA from separated, postmeiotic spermatogenic cells has been characterized by size and by the percentage of the RNA transcripts that contain poly(A) tracts (7, 8). By these criteria, synthesis of putative mRNA remains at about the same level in early postmeiotic as compared to late premeiotic cells.

Firm proof for postmeiotic transcription (and processing) of mRNA will require hybridization studies with cloned cDNAs or cloned structural genes for specific proteins involved in spermatogenesis. However, functional assays for mRNA activity could provide strong support for the notion of postmeiotic mRNA synthesis. We performed functional assays for the mRNAs for protamine-like histone (PLH) and phosphoglycerate kinase-2 (PGK-2; ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3). PLHs are small basic proteins abundant in spermatozoa (9, 10) whose synthesis occurs late during spermatogenesis (11, 12). In mice, PGK-2 is found only in the sperm and testes; its first detection during development, whether studied by starch gel electrophoresis (13) as an antigen (14) or by radiopulse synthesis studies (unpublished data), corresponds to a postmeiotic synthesis. We have studied the relative amounts of mRNAs for these two proteins by purifying RNA from testes at various stages of development or from separated spermatogenic cells and translating it *in vitro*.

MATERIALS AND METHODS

Pooled vas deferens and epididymal spermatozoa were prepared by a slicing and screening technique in 139 mM NaCl/34 mM fructose/0.5 mM Na_2HPO_4/1.3 mM NaH_2PO_4/7 mM KCl/1.4 mM Mg_2SO_4 as described (15). PLH was extracted from spermatozoa with 8 M urea/5% (vol/vol) 2-mercaptoethanol according to Goldberg *et al.* (16). PLH and salmon protamine (sulfate salt, grade I, Sigma) were electrophoresed on 15% (wt/vol) polyacrylamide gels containing 6.25 M urea, 0.357 M acetic acid, and 0.06 M KOH. The upper chamber buffer was 0.33 M alanine adjusted to pH 5.0 with acetic acid; the lower chamber buffer was 0.14 M KOH adjusted to pH 4.45 with acetic acid. Electrophoresis was for 5–8 hr at a constant amperage of 2–3 mA/gel.

Amino acid analyses were performed on Amido Schwartz-stained slices cut from the acid/urea acrylamide gels (17). Tyrosine was completely, and histidine partially, oxidized by gel-trapped air (despite special attempts to degas the slices) or gel components during the 20-hr hydrolysis and their values were estimated (see *Results*). Amino acid analyses were performed with a Beckman model 120C analyzer.

The procedures for cell dispersal and elutriation were generally those of Grabske *et al.* (18). Testes from eight random-bred CD-1 mice, 2–6 months old, were decapsulated, chopped into 1- to 2-mm pieces in phosphate-buffered saline, and incubated for 20 min at 31°C with constant shaking in 40 ml of phosphate-buffered saline with 0.1% glucose, 0.23 mg of trypsin per ml (GIBCO), and 10 μg of DNase per ml (Worthington). Fetal calf serum was added to a concentration of 8%, and the

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Abbreviations: PLH, protamine-like histone; PGK-2, phosphoglycerate kinase-2.

suspension was strained through an 80-gauge nylon mesh and cooled to 0–4°C on ice. The cells were separated in a JE-6 Elutriator rotor in a Beckman J-21/C centrifuge at 0–4°C. The elutriation buffer was phosphate-buffered saline with 0.5% bovine serum albumin and 5 mM 2-naphthol-6,8-disulfonic acid dipotassium salt (Eastman) buffered to pH 7.2. For each separation, 1–3 × 10^9 cells in 40 ml were added to the mixing chamber and elutriated in five fractions. Fraction 1 (rotor speed of 5000 rpm, buffer flow of 11.2 ml/min) was mainly cell debris and sperm. Fraction 2 (2020 rpm, 8.4 ml/min) was mostly late spermatids and some erythrocytes. Fraction 3 (2020 rpm, 16.5 ml/min), fraction 4 (2020 rpm, 24.2 ml/min), and fraction 5 (1500 rpm, 17.4 ml/min) appeared to be, respectively, early spermatids, an equal mix of spermatocytes and multinucleated spermatids, and predominantly spermatocytes (17). Cell viability (≥90%) and cell uniformity were monitored by supravital staining with trypan blue and microscopic examination.

Total RNA from whole mouse testes was prepared by guanidinium thiocyanate extraction (19). Testes from immature mice were taken at 17–19 days because the first appearance of postmeiotic cells is at 20 days. RNA from separated testicular cells was isolated by a modification of the micromethod of Braude and Pelham (20). Yeast tRNA (Sigma) was used as carrier. The volume of phenol used was 200 μl/10^8 spermatids or 2.5 × 10^7 spermatocytes. Phase separation was by centrifugation in Eppendorf 1.5-ml tubes. After lyophilization, the ethanol precipitate was dissolved in 1 μl of water per 8 μl of recovered aqueous phase and stored at −60°C for use in translation.

Translation was performed with lysates prepared from rabbits that had been made anemic by daily phenylhydrazine injections (21). The reticulocyte lysate was digested with micrococcal nuclease to destroy endogenous message and it was used for translational assay of added mRNA essentially as described (22). Incubation was at 37°C for 45 min. Purified rabbit globin mRNA was purchased from Miles. Overall incorporation of radiolabeled amino acids ([3,4,5-^{3}H]leucine, specific activity 117 Ci/mmol, New England Nuclear; [2,3-^{3}H]arginine, specific activity 23 Ci/mmol, New England Nuclear; and [^{35}S]methionine, specific activity 900–1350 Ci/mmol, Amersham) into protein was estimated by spotting 5 μl of each incubated mixture onto approximately 0.5-cm^2 Whatman no. 2 filter paper (1 Ci = 3.7 × 10^{10} becquerels). These were boiled batchwise in 5% (wt/vol) trichloroacetic acid for 5 min, cooled, washed several times with cold acetone, and oven-dried. Radioactivity was measured in scintillation vials in 10 ml of Instagel scintillation fluid. In some cases, translation mixtures were treated with RNase after this incubation: 6 μl of pancreatic RNase (2 mg/ml; Sigma, type I-A) in 20 mM unlabeled arginine and 0.1 M EDTA was added, and the mixture was incubated a further 20 min at 37°C.

For assay of *in vitro* synthesis of PLH, 50-μl translation mixtures were allowed to incorporate [^{3}H]arginine. After the incubation, 500 μg of carrier salmon protamine and approximately 150 μg of mouse sperm PLH were added, followed by 25 μl of 200 mM sodium acetate (pH 4.0) to adjust the pH to between 5 and 6. These mixtures were applied to small Whatman CM32 carboxymethyl-cellulose columns (in pasteur pipettes). Free arginine was removed and PLH was eluted as described (23). Lyophilized eluates were dissolved in 60 μl of 8 M urea containing 5% mercaptoethanol, and 50 μl were applied to the acid/urea polyacrylamide tube gels which were electrophoresed as described, frozen, cut into 2-mm slices, and incubated overnight at 37°C with 1 ml of NCS solubilizer to which 10 ml of toluene-based scintillation fluid was then added. Radioactivity was then measured.

For assay of *in vitro* synthesis of PGK-1 and PGK-2 with RNA from immature and mature testes, translation reaction mixtures were combined with carrier testes homogenate and adsorbed to a 25-μl ATP-hexane-agarose column (P. L. Biochemicals) as described (24). Approximately 80% of the material retained by the column is PGK, and recoveries for PGK are 85–95%. The column bed was resuspended in lysis buffer and run on a two-dimensional gel system similar to that of O'Farrell (25) except that a 3:1:3 mixture of pH 7–9/5–7/3.5–10 ampholines (LKB) was used to extend the basic end of isoelectric focusing gels. Gels were fixed and stained and the spots representing PGK-1 and PGK-2 were excised and digested with NCS solubilizer for liquid scintillation spectrometry. Identity of PGK-1 and PGK-2 has been confirmed by immunoprecipitation (26), tissue specificity, and developmental appearance in the testis (13). For assay of *in vitro* synthesis of PGK-2 with RNA from separated cells, aliquots of reticulocyte translation mixtures containing 10^5 acid-precipitable cpm were combined with 2 μl of antiserum against PGK-2 (14) in a total volume of 500 μl containing 0.1% $NaDodSO_4$, 1% Triton X-100, 0.2% sodium deoxycholate, 10 mM L-methionine, and 1 mg of bovine serum albumin per ml in borate-buffered saline. After 30 min of incubation at room temperature, sufficient sheep antiserum against rabbit gamma globulin was added to precipitate all of the primary antibody. The mixtures were incubated a further 30 min at room temperature and then overnight at 4°C. The precipitation mixture was layered over 150 μl of 1 M sucrose/1% Triton X-100 and centrifuged for 5 min with a Beckman Microfuge B. The precipitate was washed three times with 0.5% Nonidet P-40, 2 mM L-methionine, and 1 mg of bovine serum albumin per ml in borate-buffered saline and finally in borate-buffered saline alone. Washed precipitates were dissolved in $NaDodSO_4$ buffer [2% $NaDodSO_4$/10% (vol/vol) glycerol/5% 2-mercaptoethanol/6 M urea/62.5 mM Tris·HCl, pH 6.8].

The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with the buffer system of Laemmli (27) with a 3% stacking gel and 8% separating gel. All samples were heated at 100°C for 3 min prior to electrophoresis. Gels were fixed in 10% acetic acid, prepared for fluorography (28), dried, and exposed to Kodak XR-5 film at −80°C. [*methyl*-^{14}C]Ovalbumin and bovine serum [*methyl*-^{14}C]albumin (New England Nuclear) were used as markers.

RESULTS

Acid/urea gel electrophoresis of proteins soluble in 8 M urea/5% 2-mercaptoethanol prepared from mouse spermatozoa gave a major Amido Schwartz-stained band with an R_F of 0.7. Extracts prepared in a similar manner from liver and expected to contain histones did not demonstrate protein bands with R_F values greater than 0.5. Confirmation that the protein of high R_F was PLH was provided by amino acid analyses of the stained band. The amino acid composition of this protein closely approximated published values (29) for mouse PLH (Table 1). Thus, the unique basicity and size of PLH provided a basis for detecting its synthesis after *in vitro* translation.

RNA prepared from intact testes by guanidinium thiocyanate extraction and several reprecipitations gave ultraviolet spectra expected for nucleic acids and were quantitated on the basis of A_{260}. RNA prepared from the small quantities of separated cells was heavily contaminated with protein; we have compared fractions on the basis of the number of cells from which they were prepared. Either kind of testicular RNA stimulated *in vitro* incorporation of [^{3}H]leucine and [^{3}H]arginine (Table 2). Testicular RNA and RNA from purified late spermatids stimulated the incorporation of arginine to a greater extent than

Table 1. Analysis of amino acid composition of mouse PLH from disc-gel slices

Amino acid	mol % Observed*	Expected†
Ala	1.5 (1.4)	1.2
Arg	54.2 (51.6)	52.6
Asp	0	0.5
Cys	14.4 (13.7)	10.5
Glu	0.5 (0.5)	1.0
Gly	3.9 (3.7)	3.5
His	9.8 (12.7)	12.7
Ile	1.1 (1.0)	1.7
Leu	1.0 (1.0)	1.3
Lys	4.5 (4.3)	5.2
Met	0	0
Phe	0	0
Pro	0	0
Ser	7.8 (7.4)	8.1
Thr	1.2 (1.1)	1.1
Tyr	0 (2.2)	2.2
Val	0	0
Trp	—	0

* Experimental value (corrected value in parentheses) in which the correction is based on the published amino acid composition and assumes 23% loss of histidine and 100% loss of tyrosine (i.e., uses the expected values of these two amino acids).
† Ref. 29.

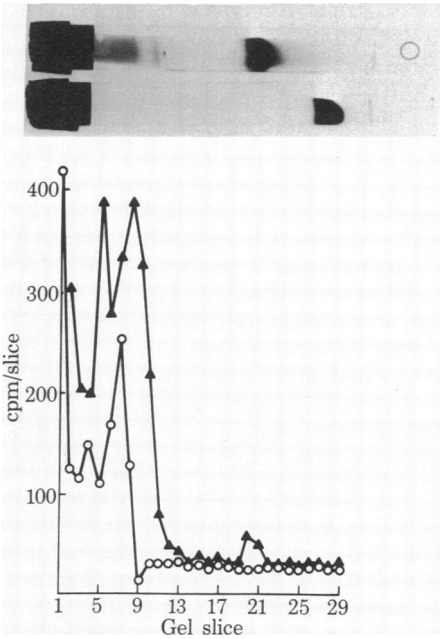

FIG. 1. (*Upper*) Electrophoretograms of acid/urea acrylamide gels stained with Amido-Schwartz. The electrophoretic mobility of purified PLH (upper gel) is compared to that of salmon protamine (lower gel). (*Lower*) Gels were sliced and radioactivity was measured. [^{3}H]Arginine incorporation stimulated by RNA isolated from late spermatids (▲) is compared to endogenous (no added RNA) [^{3}H]-arginine incorporation (○). This pair of *in vitro* translational mixtures had been treated with RNase after the reaction.

leucine, and about the expected proportion of arginine (3 residues) compared to leucine (17 residues) was found in the β-globin controls. The relative efficiency with arginine suggested that basic protein synthesis was being stimulated by the testicular RNA.

Acid/urea gel analysis of [^{3}H]arginine-labeled translational products stimulated by RNA prepared from late spermatids demonstrated a small peak of radioactivity comigrating with PLH (Fig. 1). Although this peak occurred at a level only about twice background, this region of the gels has always been devoid of peaks in a large number of controls. Such a peak was absent when exogenous RNA was not present. Similarly, when RNA was prepared from immature (up to 19-days-old) testes (which contain spermatogonia and spermatocytes but no spermatids), this peak of incorporation was not seen (Fig. 2*B*) whereas it was found with RNA from mature testes (Fig. 1). Because we were unable to obtain RNA preparations from immature testes that stimulated *in vitro* translation effectively, only about half as much radioactivity (over the background due to endogenous message) had been placed onto the gels and, thus, the lack of any detectable PLH peak was only a suggestive difference. However, analysis of *in vitro* translational products stimulated by RNA preparations from separated testicular cells confirmed the postmeiotic increase of PLH mRNA. RNA from spermatocytes and early spermatids did not stimulate the incorporation of [^{3}H]arginine into material comigrating with PLH, whereas RNA from late spermatids did (Fig. 2). The small peaks of [^{3}H]arginine incorporated at the position of stained PLH were reproduced in several translation mixtures stimulated by the RNA from mature testes and from the separated late spermatid cell fraction. No such peak was seen with RNAs from immature whole testes or from separated testicular cells fractions of early stages of spermatogenesis. The same result was found if the translational mixture was precipitated twice with ethanol before carrier was added and free [^{3}H]arginine removed. Assuming

Table 2. Stimulation of incorporation of [^{3}H]arginine and [^{3}H]leucine into polypeptides by various RNAs during *in vitro* translation

mRNA	Reticulocyte lysate batch	Amino acid incorporated minus endogenous incorporation* [^{3}H]Leucine (A)	[^{3}H]Arginine (B)	[^{3}H]Arginine/[^{3}H]leucine (column B/column A)
β-Globin	1	4964	1002	0.20
β-Globin	2	1637	500	0.31
Whole testis				
Immature	1	2.6	3.1	1.2
Mature	1	45.9	109	2.4
Mature	2	142	334	2.4
Separated cell fractions				
Spermatocyte	5	38.9	25.8	0.66
Early spermatid	5	11.6	10.9	0.93
Late spermatid	5	5.7	8.2	1.43

Volume of translation mixtures was 50 μl. A counting efficiency of 0.13 was determined by spotting isotope (diluted into acetone) onto similar sized filter pieces and measuring the radioactivity as described for translation assays.
* Values for β-globin and whole testis are in fmol/μg of RNA; values for separated cell fractions are in fmol/10^6 cells.

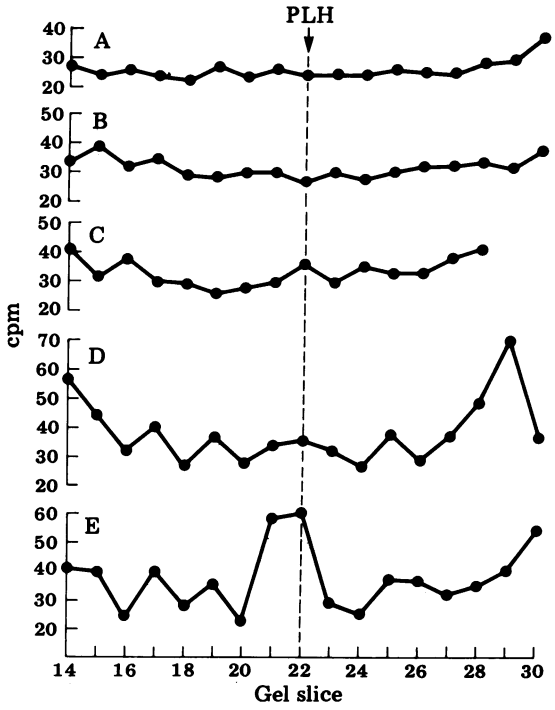

FIG. 2. Profiles of [^{3}H]arginine incorporation into basic proteins synthesized by the rabbit reticulocyte *in vitro* translational system. Translation mixtures (50 μl) were processed and run on acid/urea polyacrylamide gels. Gels were sliced and radioactivity was measured. Total cpm of [^{3}H]arginine incorporated into protein over endogenous synthesis for each RNA preparation were: (*B*) immature testis, 618 cpm; (*C*) spermatocyte, 2000 cpm; (*D*) early spermatid, 2430 cpm; and (*E*) late spermatid, 2000 cpm. (*A*) Endogenous synthesis.

that counting efficiencies were similar for acrylamide gel slices and Whatman paper, about 1.5% of the [^{3}H]arginine incorporated (over endogenous incorporation) by RNA from late spermatids was in PLH whereas at most 0.6% of the early spermatid product and $\leq$0.5% of the spermatocyte product were PLH. These yields of PLH occurred despite the opposite trend in recovery of mRNA (as measured by stimulation of *in vitro* translation) from later stages of spermatogenesis (Table 2).

PGK-2 represents about 0.5% of new protein synthesis in mature testes and cannot be detected in immature testes. The rate of synthesis detected after *in vitro* translation of RNA purified from mature testes was 2 orders of magnitude lower than that found *in vivo* but was at least 6-fold greater than the rate found after *in vitro* translation of RNA purified from immature testes (Table 3). Immunoprecipitation of *in vitro* translation products labeled with [^{35}S]methionine by a specific antisera to PGK-2 resulted in a single band of radioactive protein with the expected molecular weight of 47,000 (Fig. 3). Its synthesis was stimulated by RNA prepared from late spermatids (less effectively if the RNA was further purified by a second

Table 3. Incorporation of [^{35}S]methionine into PGK-1 and PGK-2 during *in vitro* translation of guanidine thiocyanate-purified RNA (n = 2)

Age of mice providing RNA	% of total acid-precipitable counts above background	
	PGK-1	PGK-2
17–19 days	0.00027	0.00084
> 10 weeks	0.00036	0.00488

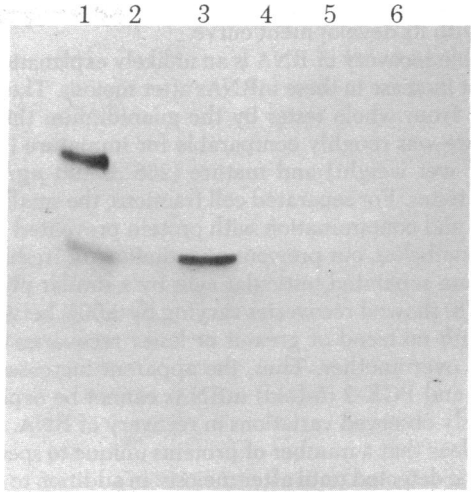

FIG. 3. Autoradiography, after NaDodSO$_4$ gel electrophoresis, of marker proteins and immunoprecipitates of *in vitro* translated, [^{35}S]methionine-labeled proteins by antisera to PGK-2. (Lane 1) [^{14}C]Methylated marker proteins: ovalbumin (lower) and bovine serum albumin (upper). (Lanes 2–6) Immunoprecipitates: lane 2, RNA prepared from late spermatids and precipitated twice with ethanol; lane 3, RNA prepared from late spermatids; lane 4, RNA prepared from early spermatids; lane 5, RNA prepared from spermatocytes; lane 6, β-globin mRNA.

precipitation with ethanol) but not by RNA prepared from early spermatids or spermatocytes (Fig. 3). As in the case of PLH, the detection of mRNA encoding for PGK-2 in cells from later stages of spermatogenesis was increased despite a lower total stimulation (on the basis of original number of cells) by RNA from the late stages compared to premeiotic stages.

DISCUSSION

Because PLH is unique in size and basicity, comigration of incorporated amino acids with the purified spermatozoal protein seems sufficient to identify newly synthesized protein. There are other basic proteins present in sperm in smaller amounts, but the amino acid analyses that we performed strongly suggest that the peak with an R_F of about 0.7 on these gels is PLH. The $\approx$3-fold increase of an mRNA in postmeiotic cells is of great interest regardless of the identity of the protein. Although the peaks of PLH found are small, they cannot be due to binding of isotope to proteins because a large excess of PLH and protamine was added to all samples (which included negative controls of β-globin mRNA and no RNA) prior to purification and electrophoresis.

The cell separations prepared by elutriation are not completely pure (18). Thus, the *in vitro* protein synthesis stimulated by early spermatid RNA, which might be PLH, could be entirely due to contamination of this fraction with late spermatids. Contamination of the spermatocyte fraction with postmeiotic cells is small; we could not detect any RNA in spermatocytes that could be translated *in vitro* to yield PLH, even when 25% of the amount present in late spermatids should be detectable. The microscopic appearance of the fractions also suggested that the spermatocytes were a highly uniform population, but we know that binucleate spermatids contaminate this fraction.

The potential problem of impure cell fractions is also not of consequence for the detection of mRNA for PGK-2; the only separated cell fraction from which RNA stimulated PGK-2 synthesis was the late spermatid fraction. Although the data on enzyme activity suggest that PGK-2 first appears in early spermatids (refs. 13 and 14 and unpublished data), those data also suggest that the concentration of PGK-2 increases in later

stages. Thus, the time of detection of the mRNA for PGK-2 agrees with its development curve.

Variable recovery of RNA is an unlikely explanation for the apparent increase in these mRNAs after meiosis. The recovery of RNA from whole testes by the guanidinium thiocyanate procedure was roughly comparable for immature (460 ± 15 μg/g of wet weight) and mature (268 ± 190 μg/g of wet weight) testes. For separated cell fractions, the small amounts of RNA and contamination with protein prevented quantitation. Nonetheless, our previous quantitations of freshly labeled RNA from separated testicular cells by a similar phenol procedure (8) showed recoveries varying by ±50% between fractions, with no trend of greater or lesser recoveries from one fraction over another. Thus, the apparent increases in PLH (3-fold) and PGK-2 (6-fold) mRNAs cannot be explained by previously observed variations in recovery of RNA.

It is clear that a number of proteins unique to spermatozoa cannot be detected until after meiosis: in addition to PLH and PGK-2, the sperm-specific hyaluronidase (30) and certain sperm autoantigens (31) do not appear until after meiosis. We are not aware of previous work that demonstrates increases in mRNA for such proteins after meiosis. It seems likely that these increases in mRNA correspond to the time of transcription of the PLH and PGK-2 structural gene(s). The methods used in preparing the RNA should have disrupted protein–RNA complexes such as informosomes, hypothesized to store mRNA for later use (32). We have also attempted to "activate" mRNA by denaturation with methyl mercury hydroxide before *in vitro* translation (33), but we find only inhibition of total incorporation (unpublished results). Although the synthesis of the message for protamine (in salmonoid fish) commences before meiosis (34), this is not in conflict with our results because cold-blooded vertebrates frequently use stored mRNA whereas mammals do not.

We thank Ken Harper for technical assistance, Miriam Meisler and John Valentine for useful discussions, and Rena Jones for excellent secretarial assistance. This work was supported by Research Grant HD 11738 and Genetics Training Grant GM 7544 from the National Institutes of Health.

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