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Haploid Accumulation and Translational Control of Phosphoglycerate Kinase-2 Messenger RNA during Mouse Spermatogenesis

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The intracellular location of the mRNA for the testis-specific isozyme of phosphoglycerate kinase-2 (PGK-2) has been determined for two spermatogenic cell types. The mRNA activity for PGK-2 from the polysomal and nonpolysomal fractions of pachytene primary spermatocytes or round spermatids has been assayed by cell-free translation with the polypeptide products monitored by immunoprecipitation, followed by one-dimensional or two-dimensional electrophoresis and fluorography. The results reveal that the majority of PGK-2 mRNA activity of round spermatids was present in the polysomal fraction while the relatively less abundant PGK-2 mRNA of pachytene primary spermatocytes was present in the nonpolysomal fraction. No PGK-2 mRNA activity was observed in the cytoplasmic RNA from primitive type A spermatogonia or prepubertal Sertoli cells. These data indicate that mature PGK-2 mRNA first appears in the cytoplasm of spermatogenic cells during the prophase of meiosis and increases in amount after meiosis. Although mature PGK-2 mRNA is present in meiotic cells it is not actively translated until after meiosis has been completed. Thus, mRNA accumulation and translational mechanisms are involved in the control of phosphoglycerate kinase-2 synthesis during spermatogenesis.

INTRODUCTION

Phosphoglycerate kinase-2 (PGK-2, EC 2.7.2.3; ATP: 3-phospho-D-glycerate 1-phosphotransferase) is a testis-specific isozyme whose activity first appears during mouse development, 22 to 30 days after birth (VandeBerg, *et al.*, 1976, 1981; Kramer, 1981; Kramer and Erickson, 1981). Recent evidence has indicated that the levels of mRNA for PGK-2 in the mouse testis are low in pachytene primary spermatocytes and then increase dramatically following meiosis (Erickson *et al.*, 1980). Although these observations are consistent with the phenomenon of "haploid gene expression" during mammalian spermatogenesis (for review see Erickson *et al.*, 1981), good evidence also exists for the synthesis during meiotic prophase of long-lived RNA (Kierszenbaum and Tres, 1974; Söderstrom, 1976; Grootegoed *et al.*, 1977; Geremia *et al.*, 1977). In a lower vertebrate, Dixon and his colleagues have demonstrated that the testicular messenger RNA from protamine is synthesized in meiotic prophase, is stored in the cytoplasm and is not translated until considerably later during spermatogenesis (for review see Iatrou and Dixon, 1978). There is, however, little evidence for storage of mRNA in mammals. Nonetheless, recent studies of messenger RNA present in the polysomal and nonpolysomal fractions from mouse testis cytoplasm indicate that more than 50% of the total or the poly $(A)^+$ mRNA is present in the nonpolysomal fraction suggesting that cytoplasmic storage of mRNA may also occur during mammalian spermatogenesis (Gold and Hecht, 1981; Gold *et al.*, 1983; Stern *et al.*, 1983b).

In this report we have assayed PGK-2 messenger RNA activity in polysomal and nonpolysomal fractions from highly enriched populations of two testicular cell types, pachytene primary spermatocytes, and round spermatids. The amounts of PGK-2 mRNA activity in the total cytoplasmic RNA from pachytene primary spermatocytes, round spermatids, primitive type A spermatogonia, and prepuberal Sertoli cells have also been measured. The results indicate that mRNA for PGK-2 first appears during meiosis where it is found in a nonpolysomal fraction; an increased amount and polysomal mRNA for PGK-2 are found in postmeiotic cells.

MATERIALS AND METHODS

Mice. Random-bred, CD-1 mice (segregating for PGK-2A and PGK-2B) were used. In some cases, irradiation was used to enrich for spermatids (Grippo *et al.*, 1978).

Cell separation: General. Cells were separated by (1) sedimentation at unit gravity using a modification of

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the method of Romrell *et al.* (1976) or (2) by elutriation; both methods were sometimes followed by density gradient centrifugation in Percoll (Meistrich *et al.*, 1981; Stern *et al.*, 1983a). Briefly, CD-1 male mice, after irradiation or unirradiated, were sacrificed by cervical dislocation, the testes were dissected, and the tunica albuginea was removed with forceps. The seminiferous tubules were then placed in bicarbonate buffered RPMI Medium 1640 (Gibco, Grand Island, N. Y.) and incubated in the presence of 1 mg/ml of collagenase (Sigma, St. Louis, Mo.) with agitation from bubbling 5% CO_2 95% air at 34°C for 15 min. The seminiferous tubules were then washed and resuspended in RPMI 1640 containing 10 μg/ml of DNase I (Sigma, St. Louis, Mo.) and 250 μg/ml of trypsin (Sigma). After reincubation at 34°C for 10 min, followed by dispersal with a Pasteur pipet, the cells were filtered through 80-μm Nitex nylon mesh (Tetco, Inc., Elmsford, N. Y.) and centrifuged at 1500*g* for 10 min at 4°C. The cells were then resuspended in RPMI 1640 and filtered through 80-μm nylon mesh one additional time. After counting an aliquot, between 5 and 10 $\times 10^8$ cells were loaded into a Staput chamber (Johns Scientific, Toronto, Ontario, Canada) and a 2–4% bovine serum albumin w/v (BSA, Fraction V, Sigma) gradient in RPMI 1640 (total volume = 1200 ml) was formed beneath the loaded cells. After approximately 2.5 hr, the gradient was fractionated into 11-ml fractions and the cells, which distribute according to size, were examined by phase-contrast microscopy. The entire period of cell loading, gradient formation, and fractionation took approximately 4 hr. Cells were identified according to the criteria discussed by Romrell *et al.* (1976) and pachytene primary spermatocytes and round spermatids retained. These combined populations were centrifuged at 1500*g* for 10 min in preparation for cell fractionation or further purification. At this stage of purification, populations of cells 85–90% pure were routinely obtained.

Cell separation: Spermatogonia and Sertoli cells. To obtain spermatogonia and Sertoli cells, 6-day-old CD-1 male mice (Charles River Breeding Labs, Wilmington, Mass.) were sacrificed by exposure to ether and their testes were dissected as described above. The cell separation was as described above except that the RPMI 1640 media contained 250 μg/liter of Gentamicin (Schering, Kenilworth, N. J.) to prevent microbial contamination. Cells were identified according to the morphological criteria discussed in Bellvé *et al.* (1977).

Cell separation: Further purification. Following Staput separation, cells were separated by density differences in Percoll (Pharmacia, Uppsala, Sweden) with a modification of the procedure developed by Meistrich *et al.* (1981). Populations of pachytene primary spermatocytes or round spermatids which had been separated at 1*g* in BSA gradients (as above) were resuspended in 0.5 ml of 20% Percoll v/v. Pachytene primary spermatocytes and round spermatids in 0.5 ml were layered onto preformed 10-ml Percoll gradients generated in thick-walled Oak Ridge polycarbonate tubes by centrifugation for 15 min at 23,000 rpm in a Type 65 rotor (Spinco Div. of Beckman Instruments, Inc., Palo Alto, Calif.) These gradients were recentrifuged at 10,000 rpm for 10 min at 4°C, with the centrifuge brake off. After centrifugation, fractions were collected from the bottom of the gradient and cells were identified by phase-contrast microscopy. Purities of greater than 90% pachytene primary spermatocytes or round spermatids were routinely obtained.

To obtain 98% pure populations of spermatocytes, cell suspensions were first separated by centrifugal elutriation and further separated by equilibrium density centrifugation on Percoll gradients. Cell suspensions from mature testes were suspended in 0.5% BSA, 0.002% DNase I (Sigma DN-25), 5 m*M* naphthol disulfonic acid (Eastman-Kodak, Rochester, N. Y.), Dulbecco's phosphate-buffered saline (PBS), and loaded into an elutriator rotor (Beckman). Elutriation was performed as reported by Grabske *et al.* (1975). Percoll density gradients were performed by the method of Meistrich *et al.* (1981). Briefly, 25–37% gradients were constructed in 25 ml in 36-ml tubes. Enriched cells were suspended in 5% Percoll/PBS containing DNase I (100 μg/ml). The gradients were placed in an SS-90 vertical superspeed rotor (Dupont, Newton, Conn.) and spun for 10 min at 11,000*g*. Highly enriched pachytene primary spermatocyte fractions were withdrawn, washed with PBS, and frozen in liquid nitrogen.

Ribonucleoprotein fractionation. Cells, purified by sedimentation at unit gravity and density gradient centrifugation in Percoll, without being frozen, were fractionated into polysomal and nonpolysomal fractions as previously described (Gold and Hecht, 1981). Nonpolysomal and polysomal fractions obtained from sucrose gradients were precipitated by the addition of sodium acetate to a final concentration of 2% and 2 1/2 vol of absolute ethanol and stored overnight at −20°C.

Extraction of RNA. Phenol extraction of RNA was performed as previously described (Gold and Hecht, 1981) except that it was found necessary to add 20 μg of carrier *Escherichia coli* tRNA to the postmitochondrial supernatants of primitive type A spermatogonia and Sertoli cells in order to improve the yield of poly(A)$^+$ and translatable RNA. In some cases total cytoplasmic poly(A)$^+$ RNA was prepared using proteinase K. Frozen, separated cells were suspended in 0.15 *M* NaCl and lysed by the addition of an equal volume of lysis buffer (25 m*M* Tris-HCl, pH 7.5) with 1.5 mg/ml heparin (Sigma). The postmitochondrial supernatant was in-

cubated at 37°C for 2 hr after the addition of 0.1 vol of 10% SDS, 50 m*M* EDTA, and 100 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, Ind.). The digested sample was extracted with phenol-chloroform and the aqueous phase was precipitated with ethanol containing 0.2 *M* sodium acetate overnight at −18°C. RNA was pelleted and washed with 3 *M* sodium acetate (pH 6.0), 5 m*M* EDTA to remove DNA, small RNA, and heparin. RNA was reprecipitated with ethanol and finally dissolved in application buffer for oligo(dT)-column chromatography (0.5 *M* NaCl, 10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 0.1% SDS). To isolate poly$(A)^+$ RNA, the RNA fraction had one cycle of oligo(dT)-cellulose (T-3, Collaborative Research, Waltham, Mass.) column chromatography after heating at 65°C for 3 min. The poly$(A)^+$ fraction was eluted with low salt buffer (10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 0.1% SDS) and precipitated with ethanol as previously described (Fujimoto and Erickson, 1982).

Cell-free translation and product analysis. Cell-free translations using reticulocyte lysate or wheat germ lysate and measurements of poly$(A)^+$ RNA were carried out as previously described (Erickson *et al.*, 1980; Gold and Hecht, 1981).

In order to assay PGK-2 mRNA activity, aliquots of reticulocyte or wheat germ translation mixtures of equal volume were combined with 1 or 2 μl of antiserum against PGK-2 (Erickson *et al.*, 1979) and precipitated as previously described (Erickson *et al.*, 1980). Washed precipitates were analyzed by SDS–urea polyacrylamide gel electrophoresis or two-dimensional polyacrylamide gel electrophoresis.

One-dimensional electrophoresis was performed on 13% polyacrylamide slab gels (0.8 mm thick) containing 0.1% SDS and 5.5 *M* urea, with a 5.5% polyacrylamide stacking gel containing 2.7 *M* urea (Storti and Rich, 1976). Equal volumes of labeled *in vitro* reaction mixtures were applied to the gel after adjusting to 50 m*M* Tris–HCl, pH 6.8, 2% SDS, 35 m*M* 2-mercaptoethanol, and 10% glycerol. Electrophoresis was carried out at 200 V for 24 hr. The gels were stained with Coomassie brilliant blue R (Sigma), destained, and dried onto 2MM Whatman paper *in vacuo.* Two-dimensional gel electrophoresis was carried out as reported by O'Farrell (1975). The isoelectric focusing in the first dimension formed a pH gradient from 4.3 to 6.8, and a 10% acrylamide–SDS slab gel was used for the second dimension. After electrophoresis, two-dimensional gels were prepared for fluorography and fluorographs of both kinds of gels were obtained by exposure to Kodak AR film (Eastman-Kodak) as previously described (Gold and Hecht, 1981). Quantitation of relative abundance of PGK-2 mRNA was performed by cutting out the autoradiogram-identified PGK-2 spots and control spots from the dried two-dimensional gels, rehydrating them in 100 μl water, digesting them overnight in 1.0 ml NCS (Amersham, Arlington Hts., Ill.) at 46°C, adding scintillation fluid and counting for 50 min.

RESULTS

Comparisons of PGK-2 mRNA activity are based on fluorographs of gels in which PGK-2 has been identified by immunological methods. Adding increasing amounts of exogenous RNA resulted in a linearly increasing stimulation of the cell-free systems over a broad range followed by decreased incorporation. At the level of input RNA which annealed to 400 cpm of $[^3H]$poly(U) all the incorporations were in the linear part of the incorporation curve and this input of RNA is used throughout the following studies.

In assays of mRNA from the total cytoplasm of whole testis, the majority (82% by densitometric comparison) of the mRNA activity for PGK-2 is found in the polysomal fraction while a smaller amount is found in the nonpolysomal fraction (Fig. 1). There were no indications of a cross-reaction of any radiolabeled polypeptides in our cell-free translation mixtures with normal rabbit serum. However, there may be some proteolytic breakdown of assayed PGK-2 subsequent to the immunoprecipitation which results in a low-molecular-weight smearing in the gel. When the total cytoplasmic RNA from pachytene primary spermatocytes (lane 1) and from round spermatids (lane 2) was assayed for activity, round spermatids contained several times more activity than was found in pachytene primary spermatocytes (Fig. 2).

When the mRNA activity for PGK-2 was assayed in polysomal and nonpolysomal fractions from pachytene primary spermatocytes and round spermatids, all of the PGK-2 mRNA in the fractionated pachytene primary spermatocytes was found in the nonpolysomal fraction (lane 4) while in round spermatids more than half of the PGK-2 mRNA was present on polysomes (lane 5) (Fig. 2). Although no activity was detected in the polysomal fraction derived from pachytene primary spermatocytes (lane 3), we were able to detect a substantial amount of activity (about one-third of the amount present in total round spermatid cytoplasm as estimated by densitometry of the fluorograms) in the nonpolysomal fraction of round spermatids (lane 6).

To establish whether the absence of PGK-2 mRNA in the polysomal fraction of pachytene primary spermatocytes was physiologically significant or a technical artifact, the relative levels of mRNA per cell and cellular compartment were considered. Compared to round spermatids, pachytene primary spermatocytes contain approximately four times more total cytoplasmic RNA

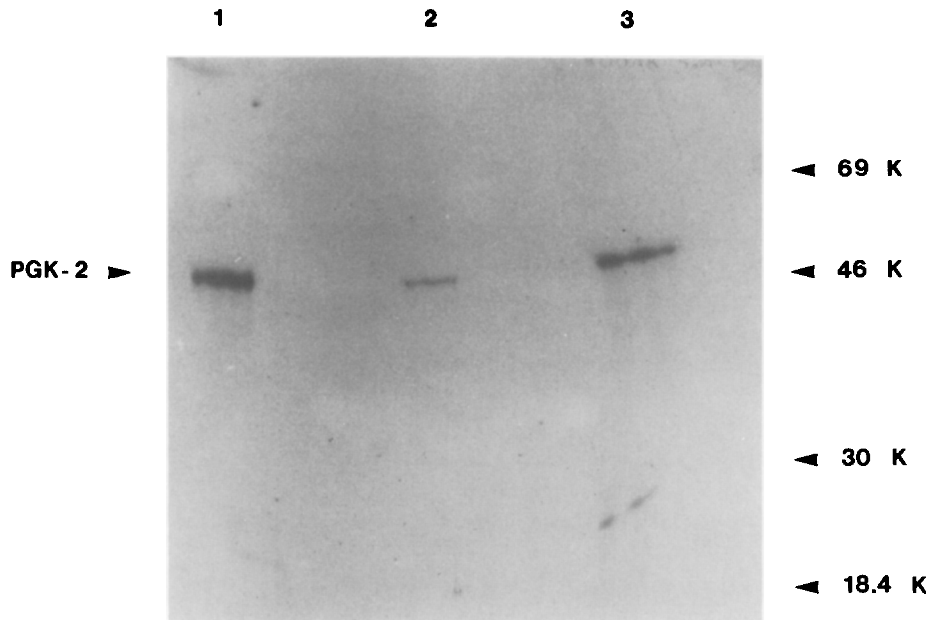

FIG. 1. Fluorograph of an SDS-urea polyacrylamide gel containing immunoprecipitates of [^{35}S]methionine-labeled polypeptides from a rabbit reticulocyte cell-free translation. Lane 1: products from mouse testis total cytoplasmic RNA. Lane 2: products from nonpolysomal mouse testis RNA. Lane 3: products from polysomal mouse testis RNA. Normal rabbit serum controls have been run to the right of each of the corresponding numbered lanes. Parallel experiments carried out using the cell-free wheat germ *in vitro* translation system gave similar results.

and twofold more cytoplasmic poly(A) per cell (Kleene *et al.*, 1983). In both pachytene primary spermatocytes and round spermatids, approximately two-thirds of the total polyadenylated RNA is found in the nonpolysomal cellular compartment (Gold *et al.*, 1983). Thus, although the nonpolysomal fraction of pachytene primary spermatocytes contains substantially *more total* polyadenylated RNA, no mRNA for PGK-2 was detected. Analysis of the total population of polypeptides encoded by the polysomal and nonpolysomal mRNA fractions from pachytene primary spermatocytes and round spermatids revealed that over 200 polypeptides ranging in molecular weight from 11,000 to over 100,000 daltons were synthesized in a cell-free system (Gold *et al.*, 1983). These results demonstrated that the absence of PGK-2 mRNA in the nonpolysomal fraction of pachytene primary spermatocytes cannot be ascribed to the inability of this fraction to synthesize polypeptides.

No detectable level of PGK-2 was synthesized *in vitro* by total cytoplasmic RNA preparations from primitive type A spermatogonia or from Sertoli cells isolated from 6-day-old mice. The Staput, Percoll prepared spermatocytes were about 90% pure but the major contaminants were multinucleate spermatids which have high concentrations of PGK-2 mRNA. Since we had not previously been able to detect PGK-2 mRNA in pachytene primary spermatocytes (Erickson *et al.*, 1980), we used the elutriator, Percoll density gradient method to obtain 98% pure pachytene primary spermatocytes from which poly(A)$^+$ RNA was prepared. This preparation, enriched for mRNA, was translated with the rabbit reticulocyte system. When the immunoprecipitates were analyzed by two-dimensional gel electrophoresis, the two spots corresponding to PGK-2A and PGK-2B were detected (Fig. 3). Estimation of the relative abundance of PGK-2 mRNA by elution and counting of the spots (after subtraction of counts from gel blanks) indicated about a sixfold increase from primary spermatocytes (46 ppm) to spermatids (267 ppm).

DISCUSSION

In these studies we have used antibody directed against PGK-2 to detect the amount of PGK-2 messenger RNA present in several testicular cell types. Our assumption is that the amount of PGK-2 present in cell-free translations of equal volume and equal RNA input (based on input RNA which annealed to 400 cpm of [^{3}H]poly(U)) truly reflects the relative abundance of PGK-2 mRNA in the cell.

Several investigators have reported that phosphoglycerate kinase-2 is predominantly synthesized during spermiogenesis (VandeBerg *et al.*, 1976, 1981; Kramer, 1981; Kramer and Erickson, 1981). Previous studies on the time of appearance of PGK-2 mRNA readily detected PGK-2 mRNA in spermatids while none was de-

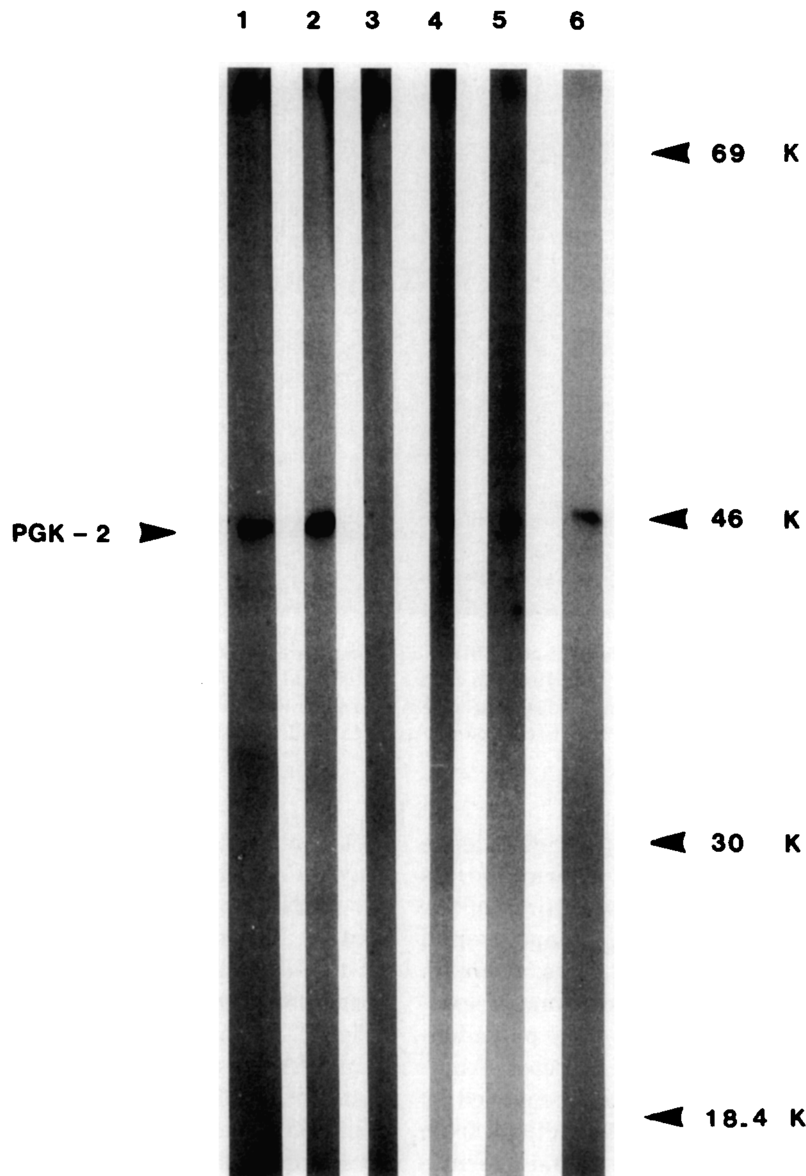

FIG. 2. Fluorograph of an SDS-urea polyacrylamide gel containing immunoprecipitates of [^{35}S]methionine-labeled polypeptides from a rabbit reticulocyte cell-free translation. Lane 1: products of pachytene primary spermatocyte total cytoplasmic RNA. Lane 2: products of round spermatid total cytoplasmic RNA. Lane 3: products of pachytene primary spermatocyte polysomal RNA. Lane 4: products of pachytene primary spermatocyte nonpolysomal RNA. Lane 5: products of round spermatid polysomal RNA. Lane 6: products of round spermatid nonpolysomal RNA. Quantitative comparisons cannot be made directly between PGK-2 mRNA from total cytoplasm and from fractionated cytoplasm because they are prepared from different batches of cells.

tected in spermatocytes (Erickson *et al.*, 1980). Careful quantitation of the amount of PGK-2 mRNA present at various stages of spermatogenesis led to the conclusion that the amount of PGK-2 mRNA increased at least sixfold from meiotic to postmeiotic stages (Erickson *et al.*, 1980). The current work differs from the previous work in that more highly purified RNA, derived either by sucrose gradient fractionation or by separation of poly(A)$^+$ RNA, was used for *in vitro* translation. Using these methods, we find a meiotic appearance of PGK-2 mRNA in a nonpolysomal compartment. This PGK-2 mRNA was detected in the poly(A)$^+$ fraction prepared from highly purified pachytene primary spermatocytes. Nonetheless, postmeiotic transcription must also occur since we now estimate a fivefold relative increase in PGK-2 mRNA after meiosis (see below).

Evidence is beginning to accumulate that the synthesis of testicular proteins can be regulated at the level of selective translation or selective production of mature messenger RNA. In one of the best characterized

FIG. 3. Fluorograph of a two-dimensional gel containing immunoprecipitates of [^{35}S]methionine-labeled polypeptides from a rabbit reticulocyte lysate cell-free translation. Poly(A)$^+$ RNA was purified from 98% pure primary spermatocytes prepared by elutriation and Percoll density gradient centrifugation. This mRNA was translated *in vitro* and the products immunoprecipitated with antisera to PGK-2. The isoelectric focusing in the first dimension formed a pH gradient from 4.3 to 6.8, and a 10% acrylamide-SDS slab gel was used for the second dimension. The small arrows indicate PGK-2A and B; the large arrow indicates a spot endogenous to the reticulocyte lysate; i.e., one which is found when incubation is performed without added RNA.

cases for a specific testicular protein, protamine in trout, the mRNA is known to be a meiotic transcription product which is preserved in the nonpolysomal fraction of trout testis cytoplasm until it is expressed during spermatogenesis (Iatrou and Dixon, 1978). In studies on another testis-specific isozyme, the C subunit of lactate dehydrogenase-X (LDH-X), Wieben (1981) concluded that functional LDH-X mRNA activity coincided with the appearance of LDH-X catalytic activity. This had earlier been demonstrated to occur at Day 14 after birth in the mouse (Goldberg and Hawtrey, 1967). Wieben's data (1981) revealed that elongated spermatids contained significantly less LDH-X mRNA than either pachytene primary spermatocytes or round spermatids, a pattern paralleling that of the rate of synthesis of LDH-X. In contrast, increasing levels of mRNA and protein synthesis are observed for PGK-2 as spermiogenesis proceeds (Erickson *et al.*, 1980).

Regulation of gene expression can be readily modulated by the distribution of mRNA between polysomal and nonpolysomal compartments. At least 50% of poly(A)$^+$ and total mRNA is found in the nonpolysomal fraction of cytoplasm from total testis, pachytene spermatocytes, round spermatids, or elongating spermatids, indicating that much of testicular mRNA at any one time is not actively involved in protein synthesis (Gold and Hecht, 1981; Gold *et al.*, 1983; Stern *et al.*, 1983b). Clearly this estimate varies markedly for individual proteins since more than 80% of the mRNA encoding PGK-2 from total testis is associated with the polysomes.

We have been able to detect PGK-2 messenger RNA activity in the nonpolysomal fraction from pachytene primary spermatocytes, and in both the polysomal and nonpolysomal fraction from round spermatids. At the level of detection possible in these studies we have not

observed any PGK-2 messenger RNA activity in the polysomal fraction of pachytene primary spermatocytes. Similarly, an electrophoretic technique sensitive enough to detect less than 1% of normal PGK-2 activity does not detect any PGK-2 activity in normal testes from 17- to 20-day-old mice, an age in which both spermatogonia and spermatocytes are present (Oakberg, 1955).

Though it is possible that the PGK-2 mRNA that we have detected in the total and nonpolysomal fraction of the cytoplasm from pachytene primary spermatocytes is due to a small number of contaminating cells such as binucleate round spermatids, we feel this is unlikely to account for our results because (1) the cell preparations have been purified using separation criteria of both sedimentation and density, (2) round or binucleate spermatids in the pachytene primary spermatocyte preparations would be expected to contribute PGK-2 mRNA primarily to the polysomal compartment, a fraction lacking any detectable activity; and (3) highly purified populations of pachytene primary spermatocytes obtained after elutriation and Percoll density gradient purification gave results in agreement with Staput purified cells.

Pachytene primary spermatocytes contain approximately twice as much cytoplasmic poly(A)$^+$ RNA per cell as do round spermatids (Kleene *et al.*, 1983) and *in vivo* incorporate about five times more radioactive amino acids into protein than round spermatids (Kramer and Erickson, 1982; Stern *et al.*, 1983a). To normalize this difference, we have titrated our cell-free translation mixtures with equal amounts of poly(A)$^+$ RNA from these two cell types. Since the amount of DNA has decreased fourfold, the amount of poly(A)$^+$ RNA relative to the amount of DNA in spermatids has doubled. Based on the data from counted PGK-2 spots, PGK-2 mRNA increased sixfold in absolute amount or threefold relative to the amount of poly(A)$^+$ RNA corrected to a constant amount of DNA. These results are consistent with the hypothesis that PGK-2 messenger RNA first appears in the cytoplasm during prophase of meiosis, that its synthesis continues, and that its appearance in the cytoplasm increases in a manner analogous to that of protamine mRNA. Further, we wish to suggest that the mRNA encoding PGK-2 may be stored in the form of an inactive mRNP in a mammalian system as has been demonstrated for the protamine messenger RNA in trout (Iatrou and Dixon, 1978). Definitive proof of the transcriptional and translational control for this gene product awaits a suitable DNA probe to measure PGK-2 mRNA.

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