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FUNCTIONAL ASSAYS FOR mRNA DETECT MANY NEW MESSAGES AFTER MALE MEIOSIS IN MICE

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We have investigated the changes occurring in the pattern of translatable mRNA species in germ cells during spermatogenesis of mice. Highly homogeneous cell populations of spermatocytes or spermatids were purified. Poly(A)$^+$ mRNA was isolated from each cell population by oligo(dT)-cellulose chromatography. A comparison of meiotic and post-meiotic mRNAs was made by two-dimensional gel analyses of their *in vitro* synthesized translation products. Among spots identified in the fluorograms of two-dimensional gels, a number of qualitatively new proteins appeared after meiosis. The results suggest that some of poly(A)$^+$ mRNAs are transcribed post-meiotically in haploid germ cells.

It is an unsolved problem whether genetic transcription occurs after meiosis during mammalian spermatogenesis (1,2). Sperm differentiation could be explained by the synthesis and accumulation of stable mRNA molecules in diploid spermatocytes and their subsequent translation during spermiogenesis (3). However, the transmission ratio distortion of *t*-alleles in the mouse seems to be preferentially interpreted by post-meiotic gene function in haploid cells (4-6). Recent biochemical studies reveal the presence of newly synthesized RNA containing poly(A) sequences in purified post-meiotic cells (7,8). Moreover, it has been shown, using two-dimensional gel electrophoresis, that there are quantitative and qualitative differences in the pattern of protein synthesis during the diploid and the haploid phases of spermatogenesis and that a number of proteins are detectable only in post-meiotic cells (9,10). In order to examine if these new proteins are synthesized on newly transcribed mRNAs, we have isolated poly(A)$^+$ RNA from highly homogeneous cell populations of meiotic and post-meiotic testicular germ cells, and characterized them using an *in vitro* translation system followed by two-

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dimensional gel analyses. The results demonstrate specific changes in the populations of mRNAs detectable during spermatogenesis and that there are a number of mRNA species only detected after meiosis.

EXPERIMENTAL PROCEDURES

Mice. Random bred CD-1 mature males between 9 and 15 weeks of age and immature males between 17 and 19 days of age were used.

Irradiation. In order to deplete the testes of spermatogonia and meiotic cells, mature males were irradiated using a ^{60}Co γ-source with 300, 100, 100 rads at intervals of 6 days (7). They were killed 24 hr after the third irradiation to prepare spermatid rich fractions.

Preparation of cell suspensions. The irradiated or non-irradiated testes were decapsulated and seminiferous tubules free of the interstitial tissue were obtained by 0.1% collagenase (Gibco) treatment in phosphate-buffered saline containing 0.1% glucose (PBS-G) (34°C, 20 min). Following incubation in PBS-G containing 0.1% trypsin (Gibco, 2X crystallized) and 0.002% DNAse I (Sigma, DN-25) (31°C, 20 min), newborn calf serum (Gibco) was added to a concentration of 8%. The cell suspension was gently pipetted and filtered through glass wool and a nylon screen mesh. The cells were centrifuged (500 *g*, 10 min) and washed with PBS-G containing 0.02% soybean trypsin inhibitor (Worthington) and 0.001% DNAse I.

Centrifugal elutriation and Percoll density gradients. In order to fractionate testicular germ cells, cell suspensions from mature testes were resuspended in PBS containing 0.5% Bovine Serum Albumin (Sigma, Fraction V), 0.002% DNAse I and 5 mM naphthol disulfonic acid (Eastman Kodak), and loaded into an elutriator rotor (Beckman). Elutriation was performed as reported (11). The enriched pachytene spermatocytes and enriched late spermatids were separated. The pachytene spermatocyte fraction was also separated from immature testes by this procedure. To obtain highly purified populations of pachytene spermatocytes from mature testes, fractions enriched by centrifugal elutriation were further separated by equilibrium density centrifugation on Percoll (Pharmacia) gradients (25-37%) as described previously (12).

Isolation of poly(A)$^+$ mRNA. RNA was prepared from each cell fraction by proteinase K (Boehringer-Mannheim) digestion and phenol-chloroform extraction, and poly(A)$^+$ RNA was isolated using oligo(dT) cellulose column chromatography as previously described (8). In the case of spermatocytes separated from immature testes, the post-mitochondrial supernatant digested with proteinase-K was directly applied for oligo(dT) cellulose column chromatography to enhance recovery from small numbers of cells.

In vitro translation and product analysis. Translation was performed with the RNA-dependent reticulocyte lysate prepared from phenylhydrazene-treated rabbits and digested with micrococcal nuclease (Boehringer-Mannheim) to destroy endogenous message (13,14). Poly(A)$^+$ RNA samples were dissolved in a small amount of water and added to the translation mixture at a sub-saturating level after heating at 65°C for 3 min. [^{35}S]-methionine (sp. act. 1060 - 1490 Ci/mM, Amersham) was used at concentrations of 1.5 - 2.0 μCi/μℓ of translation reaction. Incubation was for 60 min at 37°C and aliquots were processed in order to quantitate protein synthesis (15). Translation products were frozen at -70°C until used for two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to O'Farrell (16) with slight modifications. The isoelectrofocusing in the first dimension was carried out in 5% acrylamide gels containing 9 M urea, 2%

Nonidet P-40 and 6% ampholines (LKB; pH 5-7, pH 3.5 - 10; 4:1). For the second dimension, a 10% acrylamide-SDS slab gel was used as described by Laemmli (17). After electrophoresis, gels were prepared for fluorography (18, 19) and radioactive proteins were detected using Kodak XR-5 or XAR-5 film.

RESULTS AND DISCUSSION

Representative gels of translation products of meiotic and post-meiotic stage mRNA are compared in Fig. 1. Approximately 100 spots were easily detectable on each fluorograph, and their patterns were generally reproducible in independent experiments. Many qualitatively lesser spots were also found, but it was hard to characterize them. There are great differences in quantity and quality between both stages. The meiotic division stage pattern is characterized by the stage-specific proteins clustered around the basic side, while the post-meiotic stage-specific proteins are located at more acidic pIs. Especially, several post-meiotic proteins shown by arrows on the figure are strikingly conspicuous in their intensity and shape of their spots. Although several prominent spots are present at both stages, some of them also undergo a quantitative increase after meiosis, for example, PGK-2. Fig. 2 shows the schematic comparison between the meiotic and the post-meiotic stages. This is presented by means of a map which indicates the positional distribution of proteins which are qualitatively either common or specific. If only the most obvious changes are considered as being significant, 40 - 60% of message species were stage-specific.

One of the important points in the study concerns the purity of the cell populations used. The germ cells within the seminiferous epithelium occur in clusters of synchronously differentiating cells connected by intercellular cytoplasmic bridges (20). During cell separation, these intercellular bridges can "open up" to form multinucleate symplasts. Thus, one pachytene spermatocyte is equivalent in cell volume to a tetranucleated early spermatid symplast, which is a descendant of the pachytene spermatocytes. This causes a significant contamination in the pachytene spermatocyte fraction separated by velocity sedimentation, in which cell volume is a major factor responsible for separations. In this study, cell purity has been improved by the two-step

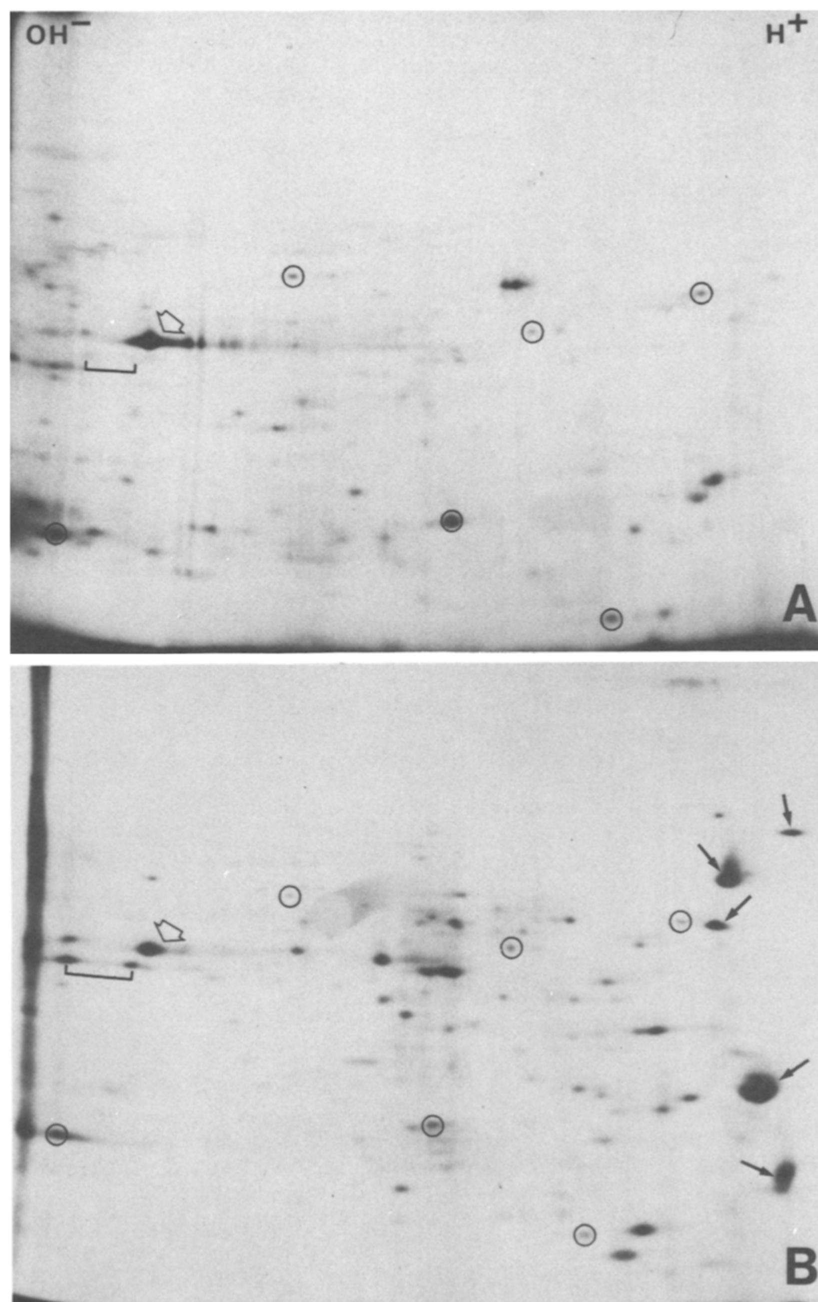

Figure 1. Fluorographs of two-dimensional gels of [^{35}S]-methionine-labelled proteins synthesized in a rabbit reticulocyte translation system stimulated by poly(A)$^{+}$ mRNA isolated from highly homogeneous cell populations of spermatocytes (A) and spermatids (B). Translation products from the two RNA populations are qualitatively very different. Small arrows indicate conspicuous spots in the spermatids. The spots of PGK-2A and B, which have been identified by immunoprecipitation with antisera to PGK-2 (25) are denoted by brackets. Circled spots were used for facilitating comparison of spots between both cell types with the reference constellation procedure. Open arrows show spots endogenous to the reticulocytes lysate.

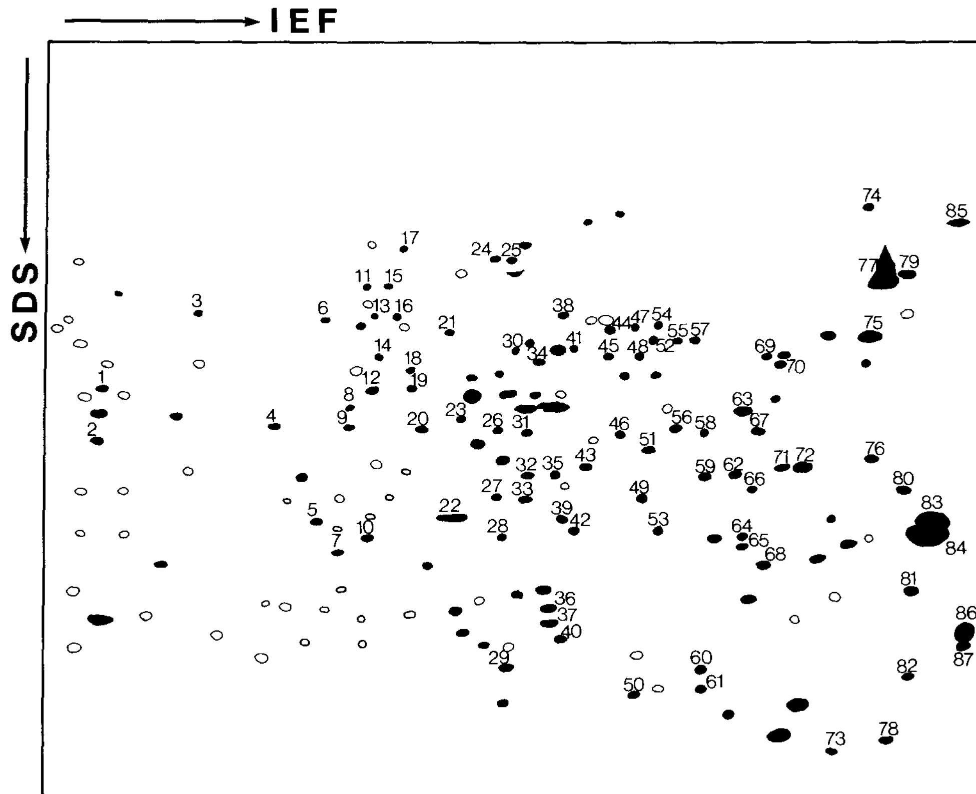

Figure 2. Schematic representation of protein patterns on two-dimensional gels in spermatocytes/spermatids comparison based on multiple gels of each cell type. Spermatid specific proteins: filled, numbered traces, spermatocyte specific proteins: unfilled, unnumbered traces; proteins common in both cell types: filled, unnumbered traces.

procedure. In a final fraction, 98% purity was achieved for the pachytene spermatocytes. Alternatively, the pure pachytene spermatocyte fraction was also obtained from immature testes, in which post-meiotic cells have not yet differentiated (21). The virtual similarity of the results from both separations confirms the stage specificity of the identified proteins. A minimum of 90% purity of the post-meiotic germ cells was found by phase contrast microscopy using the criteria described by Romrell *et al*. (22). As the predominant contaminants in these fractions were cytoplasmic fragments from spermatids, they would not contribute significantly to lowering the purity. Even though there is small contamination of meiotic cells in the post-meiotic cell fractions, intense spots for several proteins specifically found on the gels of the post-meiotic stage clearly indicate new appearance of translatable mRNAs after meiosis.

Detection of an mRNA species by the present approach depends on the sensitivity of the gel analysis. Some differences may be missed because certain proteins may not contain methionine residues and particular proteins, such as basic proteins like protamine-like histone, do not focus in the pH range used in this experiment. Although we have tried to compare *in vitro* products in the present study with proteins synthesized *in vivo* in early studies (9,10,23, 24), it was difficult to find exact correspondences except for PGK-2 which was identified by immunoprecipitation (25). This may be the result of differences in experimental techniques of cell separation and gel analysis and the lack of any post-translation modifications of synthesized proteins. The correspondence of PGK-2 spots between *in vivo* labelled and *in vitro* translated samples for both stages demonstrates that differences between the stages is unlikely to be caused by degradation of mRNA during sample preparation.

Our results strongly indicate that some of poly$(A)^+$ mRNAs may be transcribed post-meiotically in haploid germ cells. The possibility is not excluded, however, that some messages are present before meiosis at very low levels or in an untranslatable form. This should be clarified by the use of specific cDNA probes.

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