Post-meiotic transcription in mouse testes detected with spermatid cDNA clones

Hirokazu FUJIMOTO*, Robert P. ERICKSON**, Marie QUINTO,
and Michael P. ROSENBERG

Department of Human Genetics, Box 015
University of Michigan School of Medicine,
1137 E. Catherine Street, Ann Arbor,
Michigan, U.S.A. 48109-0010

cDNA clones to poly(A)+ mRNA from spermatids have been obtained to study gene transcription in post-meiotic germ cells. Four cDNA clones detect mRNAs that increase in abundance in post-meiotic germ cells. One clone, pPM459, was shown to correspond to an mRNA that is transcribed after meiosis. Pulse-labelling experiments demonstrate transcription of the message in spermatids. These data constitute further evidence for post-meiotic gene transcription in spermatids.

Non-Mendelian genetic transmission ratios, which occur in a variety of orgamisms, are most easily explained by post-meiotic gene transcription. In plants, where one male pronucleus is activated during pollen formation, the occurrence of distorted transmission ratios is common, e.g., the *sugary* locus in maize (1). Other well-studied examples of non-Mendelian transmission ratios include *Segregation Distorter* in *Drosophila* (2) and *t*-alleles in mice (3,4). However, the syncytial nature of spermatogenesis has made it extremely difficult to prove post-meiotic gene transcription as a possible mechanism for non-Mendelian transmission. In recent years, the work of a number of investigators has provided evidence for mRNA synthesis after meiosis (5-11), but definitive evidence for post-meiotic transcription has awaited the availability of appropriate cDNA clones (12-14). We report here the isolation of four cDNA clones encoding spermatid poly(A)+ mRNAs which increase in abundance after meiosis. Transcriptional studies further demonstrate that one cloned cDNA encodes a gene transcribed in post-meiotic germ cells.

Current address: Mitsubishi-Kasei Institute of Life Sciences,
11 Minamiooya, Machida-shi, Tokyo 194, Japan.
**To whom reprint requests should be sent.*

Materials and Methods

Cell fractionation

Elutriation (centrifugation with a special cell-separation rotor) was performed on testicular germ cells from either adult testes or testes from 17-19-day-old CD-1 random-bred mice, which do not yet have post-meiotic cells, as previously described (7). Spermatids from irradiated (300 rads followed by two 100-rad treatments at 6-day intervals and killed 24 h following the third irradiation) CD-1 testes (7) were more than 90% pure.

Cloning and screening spermatid mRNA

mRNA was isolated from spermatids purified from irradiated mice by the Proteinase K-phenol method. Poly(A)+ RNA was purified on an oligo(dT)-cellulose column (7), from which single- and double-stranded cDNAs were synthesized (15) using avian myleblastosis virus reverse transcriptase (courtesy of J.W. Beard) and *E. coli* DNA polymerase I (Bethesda Research Laboratories). The double-stranded cDNA was then digested with S1 nuclease (Sigma), tailed with terminal transferase (P-L Biochemicals) in cobalt-cacodylate buffer and then purified on a Sephadex G-100 column (Pharmacia). dCMP-tailed double-stranded cDNA was then annealed to dG-tailed pBR322 (New England Nuclear). Clone pPM459 was prepared by annealing homopolymeric (dA)-tailed mRNA-cDNA hybrid molecules (16,17) to homopolymeric (dT)-tailed *Pst*-1 cleaved pBR322. All recombinant DNA molecules were transformed into the *E. coli* DH-1 strain. Transformants were grown in the presence of tetracycline (20 µg/ml) and ampicillin sensitivity was detected by the rapid iodometric identification assay method (18). For colony hybridization, bacterial colonies were placed on nitrocellulose filters in duplicate and grown on tetracycline (20 µg/ml) agar plates, and then amplified with chloramphenicol (170 µg/ml) (19). The clones were lysed in situ (20) and hybridized with 2×10^6 c.p.m. of ^{32}-labeled cDNA synthesized from poly(A)+ RNA.

Cytosolic dot blots

Cytosolic dot blots were prepared according to the method of White and Bancroft (21).

Transcriptional studies

Spermatids prepared by the elutriation (centrifugation) method were labellled in Dulbecco's minimal essential Eagle's medium, supplemented with 5% fetal calf serum, for 3 h at 32°C with the isotopes specified in the Table legends. They were then washed 3x and lysed by addition of an equal volume of lysis buffer (4.8% SDS/0.2-M NaCl/15-mM EDTA/50-mM Tris, pH 7.5/200 µg/ml heparin sulfate). The RNA was extracted by the proteinase K-phenol/chloroform method. DNA was removed by an additional DNase I (6330 DPFF from Millipore) treatment using vanadyl ribonucleoside complex (BRL) as an RNase inhibitor (22). Isolated RNA was hybridized (34) to Genescreen II® (New England Nuclear) filters in 50% formamide,

2 x SSC, 0.1% SDS, 10-mM triethanolamine, pH 7.0 for 24 h at 37°C. Following the incubation, filters were washed in 2x SSC and treated with pancreatic ribonuclease I (Sigma), further washed in 2x SSC and counted.

Results

Screening for clones containing abundant messages with increased signals in spermatid (post-meiotic) cells

E. coli DH-1 cells were transformed with either double-stranded cDNA (clones 1-99) or mixed cDNA-mRNA hybrids (clone 459) cloned into the *Pst* 1 site of pBR322 by dG-dC tailing respectively. This yielded 100 colonies with cloned inserts. These clones were initially screened in a colony hybridization assay to detect the clones representing mRNA synthesized preferentially in spermatids, thus, presumably transcribed post-meiotically. Duplicate nitrocellulose filters were prepared with the 100 recombinant clones and a pBR322 clone, and hybridized with ^{32}P-labeled cDNA to poly(A)$^{+}$ mRNA prepared from either spermatids or immature (18-19-day) testes (Fig. 1). Since immature testes have spermatogonia and spermatocytes, but no spermatids, they serve as a source of RNA synthesized in pre-meiotic cells. As shown in Fig. 1, four clones (69, 72, 94 [unnumbered in the Fig.], 459) yielded strong signals with the cDNA probe from spermatids, and weak or non-detectable signals with the cDNA probe from
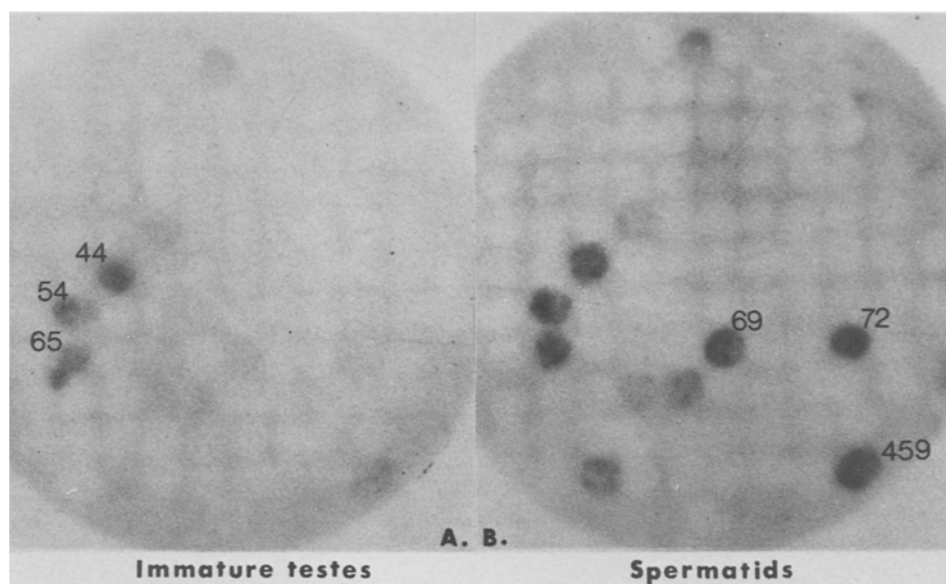

Fig. 1. Screening by filter colony hybridization for cDNA clones encoding spermatid poly(A)$^{+}$ mRNA. **(A)** Filter hybridized with ^{32}P-cDNA from immature (18-19-day-old) testicular poly(A)$^{+}$ RNA; and **(B)**, filter hybridized with ^{32}P-cDNA from poly(A)$^{+}$ RNA purified from spermatids isolated from irradiated mice.
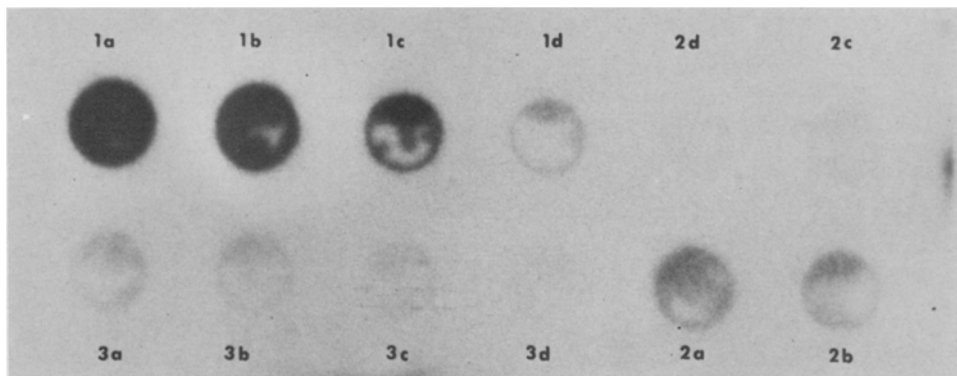
Fig. 2. Cytosolic dot hybridizations of testes from
mice of different ages hybridized with pPM459.
Samples corresponding to **(a)** 286, **(b)** 143, **(c)**
28.6, and **(d)** 5.7 µg of wet wt. of tissues were
applied. **1a–d,** day 21; **2a–d,** day 14; **3a–d,** day 7.

immature testes. Three colonies (44, 54, 65) had a very strong signal
with the spermatid probe; however, they were also found to have a
much stronger signal than the other four when hybridized with the
immature testicular cDNA probe. Because pPM459 (PM, post-meiotic)
had the largest insert (630   20), and because it gave clearer
indications of being expressed post-meiotically in Northern blots, this
clone was characterized in greater detail.

Evidence for transcription of pPM459 in post-meiotic cells

To demonstrate that messenger RNA corresponding to pPM459 is
more abundant in post-meiotic germ cells, cytosolic dot hybridizations
were performed using testes from mice of different developmental
ages. The use of testes from immature mice to generate a develop-
mental curve is based on the appearance of specific cell types during
the onset of spermatogenesis in the testicular tubules. Thus, differ-
ential positive signals with pPM459 correlated with the appearance of
specific cell types during spermatogenesis, and meiosis, in a develop-
mental time course (Fig. 2). No signal, above that obtained with
pBR322 alone (data not shown), was detected in cytosolic dots of
day-7 neonatal testes. The testes at this stage have all the somatic
elements, i.e., Leydig and Sertoli cells, as well as vascular and
connective tissues present in adult gonads, thus demonstrating that
pPM459 was not significantly expressed in these somatic cells.
Spermatocytes first appear on day 13 in the testes. pPM459
hybridized to cytosolic dot blots from day-14 testes yielded a positive
signal with 286 and 143 µg of wet wt. testicular tissue. Finally, a
strongly positive signal with pPM459 was detected in day-21 testes in
as little as 5.7 µg of wet wt. tissue. Early spermatids have made
their appearance in the testes at about 20 days. Thus, we found
approx. a 15-fold increase in the amount of pPM459 mRNA sequences
when spermatids first appear in testes from 21-day-old mice compared
to those from 14-day-old mice.

Transcriptional assay for post-meiotic transcription of clone pPM459

Our data thus far have shown that pPM459-mRNA increases in abundance in spermatids. In order to determine if this tissue-specific regulation occurs at the transcriptional level, elutriator-purified spermatids were incubated in media containing [5',6'-^{3}H]uridine for 3 h and then total RNA was isolated. Spermatid fractions that were at least 90% pure were used in these studies, with the major contaminant having been cytoplasmic fragments from late spermatids. We were able to obtain 190 µg of RNA with a specific activity of 3.08 x 10^3 c.p.m./µg (Table 1). Although our level of incorporation was low, the amount of RNA we obtained was proportional to that obtained in previous isolations for an equivalent number of cells. The ^{3}H-labeled RNA was then hybridized to filters containing 2.5 µg of either *Eco*-RI-digested pPM459 or f7 plasmid DNA. f7 plasmid DNA (courtesy of C. Jahn) contains the cDNA of the β-globin major gene cloned into pBR322, and served as a negative control for subtraction of background. We were able to clearly detect a hybridized message to the filter containing pPM459 plasmid DNA when corrected against background, i.e., hybridization to f7 filters.

Because total isolated spermatid RNA was used for hybridization, the values we obtained indicate that pPM459 mRNA only represents 246 parts per million, and therefore is of relatively low abundance (20% of newly synthesized spermatid RNA contains poly(A) (5)). However, this was probably an underestimate of the amount actually present in spermatids.

We have also performed transcriptional studies using RNA labellled with adenine and uridine. Although cytoplasmic adenylation of pre-existing mRNA is described in fertilized eggs (23-25), mammalian somatic-cell mRNA adenylation is predominantly nuclear (26). There is little evidence of nuclear storage of transcripts and mammalian testicular nuclei turn over mRNA as rapidly as, if not more rapidly than, somatic nuclei (27). Thus, the double-labelled RNA most likely reflects newly synthesized and polyadenylated RNA. Using the double label, although the specific activity of RNA was lower, we found a

Table 1.   Hybridization of [^{3}H]uridine-labeled
spermatid RNA to pPM459

1.1 x 10^8 spermatids were incubated in 500 µCi of [5',6'-^{3}H]uridine (42 Ci/mmol) in 10 ml of Dulbecco's minimal essential medium with 5% heat-inactivated fetal calf serum for 3 h at 32°C. The isolated RNA had a specific activity of 3.08 x 10^3 c.p.m./µg; 23.75 µg of RNA (73.25 x 10^3 c.p.m.) was hybridized to filters containing 2.5 µg of *Eco*-RI-digested pPM459 and f7 (mouse major β-globin probe in pBR322, negative control) plasmid DNAs.

Plasmid	C.p.m./filter*	C.p.m. - control	P.p.m. hybridized
f7	4.35 ± 1.50	–	–
pPM459	22.37 ± 0.33	18.02	246

*Mean ± standard error.

nearly 10-fold greater hybridization signal when pBR322 alone was used as the control, but a 5-fold greater hybridization signal when ^{32}P-labelled pBR322 was used as an internal control (Table 2) for the expected variability in amounts of DNA on filters.

Discussion

Autoradiographic studies of early mouse spermatids were originally interpreted as showing little RNA synthesis (28). Quantitative autoradiographic analyses showed, however, that the rate of RNA synthesis per cell decreased only four-fold during meiosis, i.e., the RNA-synthesis-to-DNA ratio was essentially unchanged across the meiotic divisions (11). Such studies depend upon the incorporation of exogenous [3H]uridine into RNA. If the intracellular uridine pool size was greatly decreased in post-meiotic cells, added [3H]uridine would be more quickly taken up into the pool. Consequently the label would be incorporated into RNA more rapidly, inflating the apparent rate of RNA synthesis. However, pool-size estimates indicate that the intracellular uridine pool is increasing, rather than decreasing, after meiosis (29), thus resulting in a tendency to underestimate total RNA synthesis. With this consideration in mind, and considering the fact that total RNA was hybridized to the filters, our estimates of the amount of pPM459 mRNA transcribed may be artificially low.

Table 2. Hybridization of [3H]uridine- and [3H]adenine-
labelled spermatid RNA to pPM459

3.7×10^8 spermatids were labelled with 800 μCi of [5',6'-^{3}H]uridine (42 Ci/mmol) and 800 μCi of [2,8-^{3}H]adenosine (31 Ci/mmol) in 12 ml of media. The resultant RNA had a specific activity of 1.42×10^3 c.p.m./μg.

	Plasmid	C.p.m./filter*	C.p.m. – background	% Hybridized
a) Without internal standard[†]	pBR322	2671 ± 86	–	–
	pPM459	3524 ± 213	853	0.284
	Plasmid	^{3}H – c.p.m.	% Hybridization corrected for pBR322**	% Hybridized
b) With internal standard[††]	pBR322	1149.5 ± 22.2	0.134	
	pPM459	312.6 ± 39.6	0.284	0.15

*Mean ± standard error.

$$**\quad \frac{[^3H]RNA\ hybridized}{[^3H]RNA\ input} \quad \times \quad \frac{[^{32}P]pBR322\ maximum\ bound}{[^{32}P]pBR322\ hybridized}$$

[†]212.5 μg of total RNA containing 300.8×10^3 c.p.m. was hybridized to 2.5 μg of plasmid DNA.
[††]80.2 μg of total RNA containing 113.5×10^3 c.p.m. and 0.25 ng of ^{32}P-pBR322 was hybridized to 2.5 μg of plasmid DNA.

The data we have obtained with cytosolic dot blots, colony hybridization, and a transcriptional assay are compatible with post-meiotic expression of a gene containing homology to pPM459. Interestingly, all four clones we have obtained are expressed to a minor extent in spermatocytes, but their corresponding message is more abundant in spermatids. Recently, Kleene et al. (12) have found that 17 cDNA clones from testicular poly(A)$^+$ RNA preferentially hybridized to spermatid RNA, while Dudley et al. (14) found that the messages detected by 6 out of 10 testes-specific cDNA clones studied showed increased abundance after meiosis. The message for a sperm-specific tubulin has also only been found in post-meiotic cells (13). However, mRNA for *Pgk*-2 first appears during meiosis, where it appears in a non-polysomal fraction, and increases in amount in round spermatids where it is found in the polysomal fraction (19). These workers have suggested that *Pgk*-2 mRNA may be stored in the form of an inactive mRNP, and that its accumulation in spermatids is consistent with post-meiotic gene expression. Enrichment of mRNA by the selective degradation of other mRNAs would not be an adequate explanation for both our results and the results obtained by others (12-14) since the amount of mRNA per amount of DNA decreases about 30% (30) in early spermatids. Also, our cloning screening methods would select for highly abundant spermatid mRNA. Therefore, our data and that of others (12-14) are more compatible with transcription after meiosis.

Acknowledgements

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