Analysis of male sterile mutations in the mouse using haploid stage expressed cDNA probes

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ABSTRACT
A differential hybridization screening procedure has identified cDNAs which correspond to RNAs which are expressed in mouse testis and at lower levels in liver and spleen. The sensitivity of this procedure is such that approximately 0.5% of 1.4 x 10^4 cDNA clones are revealed as "testis specific". We have focused on ten cDNA clones which have been used to identify RNAs expressed in the haploid phase of spermatogenesis. Using Northern blots to analyse RNA isolated from the testes of mutant mice (Tfm/Y and Sxr/+ ) blocked at specific stages in spermatogenesis or RNA from sexually immature mice, 8 clones have been identified which correspond to RNAs expressed uniquely or at much higher levels in meiotic or post meiotic cells.

INTRODUCTION
It has been known for over a hundred years that the production of mature spermatozoa from diploid cells in the seminiferous tubule occurs via a number of histologically well characterised cellular intermediates (1). In the mouse this process takes about 35 days (2,3) and involves major biochemical and morphological alterations (4). Although evidence is available for alterations in the mRNA populations of the cell types involved in spermatogenesis (5,6,7) there is little data regarding changes in expression of specific genes (8). Studies on gene expression during spermatogenesis are of interest because they permit a molecular analysis of cellular differentiation and because particular problems in the regulation of gene expression are encountered. For example, during meiosis the primary spermatocytes are genetically diploid but connected to other cells by intercellular bridges thereby rendering them functionally polyploid (9), and at later stages the condensation of the chromatin presumably renders spermatids functionally enucleate even though they are undergoing a complex process of morphogenesis (10).

Of further interest are the implications of transcription of the haploid genome during the post-meiotic stages of spermatogenesis. If gene
expression were absent after meiosis, this would maintain functional equivalence between gametes. Yet occurrences of meiotic drive, the production of unequal numbers of gametes by heterozygotes (11), in Drosophila are best explained by gene action in haploid cells.

Two observations suggest that it is likely that haploid gene expression takes place in the mouse. The first observation is the identification of cytoplasmic connecting bridges between maturing spermatids (9,12) which could permit the sharing of most post-meiotic gene products and therefore could act as a mechanism for ensuring equality of gametes. The second observation is that some t-haplotypes are able to circumvent this process since they show abnormal transmission. This fact seems most satisfactorily explained by postulating that post-meiotic transcription occurs (13,12,14) resulting in components being unequally shared and thus the production of mature spermatocytes with different fertilizing capacities.

That post-meiotic gene expression does occur is supported by the following experimental evidence. Fujimoto and Erickson (15) using a translational assay, compared the meiotic and post-meiotic mRNA populations and their results strongly suggested that certain mRNAs are transcribed post-meiotically. Gold et al. (6) studying the testis specific isozyme of phosphoglycerate kinase (PGK-2) estimated a 5-fold increase in the mRNA for this protein in post-meiotic cells as compared to secondary spermatocytes. Most recently Kleene et al. (8) have used cDNA clones made from testis poly (A)+ RNA to detect transcripts present only in post-meiotic cells. The use of cDNA probes provides more reliable evidence as regards the presence or absence of mRNA in differing cell types and in addition is a much more sensitive approach than in vitro translation. In this paper we describe the isolation of cDNA clones from mouse testis poly (A)+ RNA, the selection of testis specific clones from the library and the initial stages of clone characterisation. We have made use of mice which have blocks in spermatogenesis at specific stages in combination with pre-pubertal mice to identify cDNA clones corresponding to mRNAs only transcribed in the later stages of spermatogenesis.

MATERIALS AND METHODS

Animals

All mice (CBA/Cbi, CBA/H-T6T6 and BALB/c) were routinely maintained in the animal house (Institute of Cancer Research) except for the mutant strains which were bred by Dr. M.F. Lyon and Dr. B. Cattanach (Harwell).
Preparation of RNA

RNA was prepared using the guanidine thiocyanate method (21). Poly (A)$^+$ RNA was selected on oligo dT cellulose (22). Total RNA was fractionated on 5-20% sucrose gradients in 10 mM NaOAc pH 5.2, 1 mM EDTA and 0.3% sarkosyl at 25,000 rpm for 17 h at 12°C in a Beckman SW28 rotor.

Preparation and initial screening of testis cDNA library

Poly (A)$^+$ RNA extracted from CBA testes was used for cDNA synthesis. cDNA was prepared using the double linker method (23). The double stranded cDNA was inserted into Hindlll EcoRI double digested pUC9 (24) which had been agarose gel purified. Recombinants were transformed into JM83 using the procedure of Hanahan (25) and selected on ampicillin plates (40 μg/ml) containing X-gal (60 μg/ml). All clones were colony purified before being used as probes. Starting with 20 μg of testis poly (A)$^+$ RNA we obtained $1.4 \times 10^6$ clones. To identify those clones which contained inserts corresponding to mRNAs only expressed in testis we transferred $2 \times 10^4$ amp colonies to Whatman 541 paper (26). The transferred colonies were then amplified on chloramphenicol plates. The filters were probed with randomly primed cDNA made against an 18s sucrose gradient fraction of testis poly (A)$^+$ RNA. After autoradiography the filters were washed in 70% formamide (75°C, 2 hours) to remove the testis cDNA probe reprobed with an equivalent 18s fraction of liver poly (A)$^+$ randomly primed cDNA probe and autoradiographed. By comparison of the autoradiographs we selected 600 clones which hybridized strongly to the testis probe but only weakly or not at all to the liver probe (fig. 1).

Preparation of highly radiolabelled cDNA probes.

One to three micrograms of sucrose gradient fractionated poly (A)$^+$ RNA was reverse transcribed using random DNA primers in the presence of 0.5-1.0 mCi of $^{32}$P-dCTP in a reaction volume of 125 microlitres. The reaction mix also contained 125 μg each of dGTP, dATP and dTTP, 750 μg calf thymus primer (see below), 200 units of AMV reverse transcriptase (Beard) and 40 units RNAsin in 60 mM Tris HCl pH 8.1, 2.5 mM DTT, 6 mM DTT, 6 mM MgCl$_2$, 50 mM KCl. The reaction was carried out at 37°C for 1 hr and stopped by addition of NaOH to 0.3M. This reaction was incubated overnight at room temperature and then applied to a G-50 Sephadex column equilibrated with 50 mM Tris pH 7.6, 10 mM EDTA, 100 mM NaCl. The excluded peak contained in the region of 10-30% of the total input counts. This was used as probe without further treatment. The calf thymus primer was made by dissolving 1 gram of DNA in 20 mM Tris HCl pH 7.4, 10 mM MgCl$_2$ at a concentration of
LIVER TESTIS

Fig. 1 Hybridisation of 32p labelled randomly primed reverse transcribed 18S poly (A)\(^+\) RNA to cDNA clones. DNA from bacterial colonies containing recombinant cDNA plasmids was immobilised on Whatman 541 paper, as described in Materials and Methods, and hybridised to a 32P labelled probe made by reverse transcription of 18s poly (A)\(^+\) RNA isolated from mouse testis. After autoradiography the filters were washed to remove the testis probe and reprobed with a probe made from liver 18s poly (A)\(^+\) RNA. Small arrows (→) on both filters indicate colonies containing cDNA clones corresponding to mRNAs expressed in both liver and testis. Large arrows (••) on the right hand filter indicate cDNA clones corresponding to mRNAs expressed more highly in testis than in liver.

30 mg/ml\(^-1\). 2 mg of DNAse was added and incubated for 30 min at 37°C. SDS was then added to 1% and pronase to 1 mg/ml\(^-1\) and the mixture was further incubated at 37°C for 45 min. After two phenol/chloroform extractions the DNA was denatured at 100°C for 10 min and brought to 100 mM NaCl before being applied to a 20 ml DEAE column equilibrated in 5 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl. The column was washed extensively in equilibrated buffer before the bound primer was eluted in 5 mM Tris pH 7.4, 1 mM EDTA, 300 mM NaCl. The yield was approximately 15% and the primer was concentrated by ethanol precipitation and resuspended in 5 mM Tris pH 7.4, 1 mM EDTA at 50 mg/ml\(^-1\).

**Northern Blotting and Southern Blotting**

Analysis of RNA on Northern blots was carried out as described by Thomas
and analysis of DNA on Southern filters was as described by Southern (27). Nick translated probes were prepared as described by Rigby et al. (28).

RESULTS

Screening for testis specific cDNAs

In the Materials and Methods section we describe the initial screen of the testis cDNA library which identified 600 cDNAs corresponding to mRNAs expressed at higher levels in testis than liver. As a further selection these 600 cDNA clones were re-screened with a random primed cDNA probe of the 18S fraction of spleen poly (A)$^+$ RNA. The pattern of hybridization was compared to an identical series of filters made by replica plating and probing with the testis 18S poly (A)$^+$ RNA randomly primed cDNA probe. After controlling for variations in the amount of plasmid DNA present in each colony (by re-probing each set of filters with nick translated pUC9 probe) 48 colonies out of 192 were selected which corresponded to RNAs more abundant in the testis and absent or present in low amounts in spleen. Of these 48 colonies, 10 were selected, colony purified and used in subsequent experiments. We carried out Northern blotting experiments in order to determine the size of RNA corresponding to each of the 10 clones (Table 1). Total RNA was prepared from testis and liver and equal amounts of the two RNAs were loaded onto denaturing gels and blotted (16). RNAs were detected by hybridizing nitrocellulose strips to nick translated probes. The results are shown in Table 1 and figure 2). Of the 10 clones, 6 (clones 9,14,33,20,A10.2 and C9.4) hybridized to RNAs present in testis but not detectably to liver RNA. Of the remaining 4, 3 (clones Cl, 6 and B1.4) hybridized to both but much less strongly to liver, and 1 (clone 2) hybridized with equal intensity to both. All the RNAs detected on the Northern had a size of between 15s and 20s, as expected since an 18s fraction of poly (A)$^+$ RNA was used for the colony screening, and all except clone 6 hybridized to one band. Clone 6, when used as a probe, hybridized to 2 bands, an upper one at about 23s of similar intensity in liver and testis, and a lower one at 18s which was much stronger in testis. Clone C6.4 which appears in figure 2, was originally selected as testis positive liver negative, but failed to hybridize detectably to any RNAs on Northern blots and was not further investigated. We performed densitometry tracings on autoradiographs (differing exposures) of Fig. 2. With two clones, 6 and B1.4, there is a detectable signal in liver and testis RNA. Hybridization
Table 1 Characteristics of the 10 clones studied. The first column identifies the clone. The columns headed 'Liver RNA', 'Testis RNA', '2 week RNA', '3 week RNA', 'Tfm RNA' and 'Sxr RNA' show the results of using the individual clones in nick translated form as probes on Northern blots: a '+' indicates hybridisation of the probe to an RNA species on the Northern filter, a '-' means no band was detectable and a 'weak +' indicates hybridisation was only barely detectable. For clone 6 two entries are given in each of the 6 columns because the probe hybridised to two RNAs. The size of the RNA to which each clone hybridised was determined by comparing the position of the band on the Northern with the position of ribosomal RNA markers and is approximate. The insert size was determined by digesting each of the clones with EcoRI and HindIII followed by electrophoresis of the DNA on a 1.2% agarose gel alongside molecular weight standards.

<table>
<thead>
<tr>
<th>LIVER RNA</th>
<th>TESTIS RNA</th>
<th>Tfm RNA</th>
<th>Sxr RNA</th>
<th>SIZE OF RNA</th>
<th>CLONE INSERT SIZE (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>weak+;+</td>
<td>+;+</td>
<td>+;+</td>
<td>+;+</td>
<td>+;+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
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<td>14</td>
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<td>-</td>
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<tr>
<td>33</td>
<td>-</td>
<td>+</td>
<td>very weak+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A10.2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B1.4 weak+</td>
<td>+</td>
<td>weak+</td>
<td>+</td>
<td>weak+</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>weak+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>C9.4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

With clone B1.4 is 22.4 times greater to testis RNA than to liver RNA. In the case of hybridization with clone 6, the lower molecular weight transcript is 8.6 times more abundant in testis RNA than in liver RNA and the higher molecular weight transcript is 0.83 times as abundant. In summary, it seems that the screening procedure has differentiated between RNAs which show approximately ten-fold and greater differences in expression between testis and liver.

Northern analysis of prepubertal mouse testis RNA

The clones were used as probes to screen RNA isolated from the testis of 2-week old and 3-week old mice. Histological analysis of the testis of
Fig. 2 Northern blot analysis of cDNA clones. RNA was isolated from liver and testis, electrophoresed on a 1.2% agarose gel and transferred to nitrocellulose paper, as described by Thomas (16). 'L' indicates a lane containing RNA from liver; 'T' indicates a lane containing RNA from testis. Nitrocellulose strips with one lane of liver RNA and one of testis RNA were hybridised to nick translated cDNA clones identified by the numbers at the bottom of the figure. The results using only 5 of the clones as probes are shown in this figure.

young mice shows that primary spermatocytes do not appear until about day 10, while the second meiotic division is not complete until about day 21 (4). By using RNA from these mice of different ages it should prove possible to select clones which correspond to RNAs produced during or just after the completion of the second reduction division. The results of this experiment are shown in Table 1 and figure 3. Six of the nick translated clones hybridized to RNA from the testis of 3-week old mice but not 2-week old mice and 5 of these (clones 9, 14, 20, A10.2 and C9.4) had also shown no hybridization to RNA from liver. Clones B1.4, 33, 6 and 2 hybridized to 3-week old and 2-week old testis RNA but both B1.4, 33 and 6 hybridized more
Fig. 3  Northern blot analysis of cDNA clones using RNA isolated from 2-week and 3-week old mice. Strips of nitrocellulose with one lane of 3-week RNA (3) and one lane of 2-week RNA (2) were hybridised to nick translated cDNA clones identified by the numbers at the bottom of the figure. Data from only 5 clones are given in this figure.

strongly to 3-week old testis RNA. Clone 6 again hybridized to 2 bands, the lower one less intensely in the 2-week old testis RNA.

Northern analysis of testis RNA from mutant mice

As an alternative way of obtaining information about the time during spermatogenesis when the clones were expressed, we isolated RNA from the testis of testicularly feminised (Tfm) mice. These mice, genetically Tfm/Y, appear outwardly to be female but have small testes and no male accessory glands (17). This has been shown to be due to the absence of an androgen binding receptor in the Tfm/Y mice (18). The testes contain Sertoli and Leydig cells, spermatogonia and spermatocytes, but spermatogenesis does not proceed past the first meiotic division. The results of the experiments using RNA from the testes of these mice are shown in Table 1 and figure 4. The results are consistent with the results from the prepubertal RNA experiment in the sense that those clones not expressed in
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Fig. 4  Northern blot analysis of cDNA clones using DNA isolated from normal testis (T) or the testis of testicularly feminised mice (Tfm). Strips of nitrocellulose with one lane of each RNA were hybridised to nick translated cDNA clones identified by the numbers at the bottom of the figure. The arrow indicates the position of a band in lane '3, T' not present in lane '3, Tfm', which is poorly visible in the photograph. No size markers are given because this is a composite photograph of filters made from different original gels.

2-week old mice were also not expressed in Tfm/Y.

As a further screen we analysed RNA isolated from sex-reversed mice. X/X, Sxr/+ mice have small testes, few, if any spermatogonia, and no germ cells past this stage. They are consequently sterile, like the Tfm/Y mice (19). The results of these experiments are shown in Table 1 and confirm our previous findings. Only clones 2 and 6 hybridized to the Sxr testis RNA, although clone 14 was not tested in this experiment.

Throughout these experiments clones 2 and 6 proved to be particularly useful as probes for internal standardisation since they presumably correspond to RNA expressed in non-germ cells of testis. For example, in figure 4 we attempted to load equal quantities of the normal and mutant testis RNAs. It appears that, using clone 2, slightly more RNA was in fact loaded onto the normal (T) track as opposed to the Tfm. We know that clone 2 corresponds to a mitochondrial RNA by hybridizing to a cloned mouse mito-
chondrial genome (20) (data not shown). Mitochondria are present in all cells and transcripts would be expected to be present at the same relative levels in the various testes which we have examined. In addition, on every occasion throughout the course of these experiments filters were washed to remove a probe and reprobed with clone 2 or 6 to ensure our results could not be caused by RNA degradation.

Southern analysis of male and female DNA with cDNAs

It would be of considerable interest to identify Y chromosome specific transcripts since they might be expected to play a role in sex determination. Therefore we hybridized the 10 clones to male (XY) and female (XX) mouse liver DNA. In no case did we detect restriction fragments which were present in male but not female DNA and dosage effects were not observed either. We have, however, observed polymorphisms when comparing DNAs of different strains of mice of the same sex (data not shown). This fact will facilitate genetic mapping of some of these clones.

The hybridization signals on these Southern blots suggested that none of the 10 clones consists predominantly of sequences which are highly repeated in the mouse genome. This result was endorsed when a nick translated total mouse DNA probe was hybridized to the excised inserts of each of the 10 clones on a Southern blot. Only DNA corresponding to sequences repeated several hundred times in the genome would be expected to show detectable hybridization. Hybridization was only detected to clone 2, the mitochondrial clone.

DISCUSSION

In the work described in this paper we have had two major objectives. Firstly, we wanted to determine how effectively we could identify recombinant cDNA clones which corresponded to mRNAs expressed solely or predominantly in the testis. Secondly, we wished to select from this sub-set of the original library clones corresponding to mRNAs transcribed only in germ cells, and to determine at which stage during spermatogenesis they were expressed.

The testis cDNA library which we have constructed contained 1.4 x 10^6 clones and we found that approximately 0.5% of the cDNA clones showed at least an order of magnitude greater levels of expression in testis than in liver as measured by densitometry. This is of the same order as observed in differential cDNA screening experiments with pairs of normal and SV40 transformed murine fibroblasts (29,30). A similar experimental protocol to
the one described here was used by Barth et al. (31) to isolate cDNAs of developmentally regulated mRNAs in mouse liver and whilst a direct comparison of the data is not possible a similar order of frequency of specific clones was observed. In practice it is extremely difficult to obtain absolute values with respect to the screening sensitivity in these types of differential screening experiments, particularly since in our experiments we used partially purified RNA to make probes with. We rescreened 1.3 x 10⁴ clones from the pooled library (isolated from the primary transformants and not subsequently amplified) with the purified insert of two clones (6 and 33); 0.47% of the clones cross-hybridized with 6 and 0.09% with 33. This result illustrates the fact that we are failing to detect numerous clones which are more abundant in testis RNA than in liver RNA.

The data from the experiments involving the mouse mutants (sex reversed and testicularly feminised) and the sexually immature 2-week old mice suggest very strongly that apart from numbers 2 and 6, the clones we have isolated code for mRNAs expressed during or after meiosis. This may seem to imply that there is little or no expression of testis specific mRNAs in the cells involved in the pre-meiotic stages of spermatogenesis. However, it seems more likely that because the cDNA library was made from mature testis in which, according to Kleene et al. (8), up to 70% of the germ cell poly (A)⁺ RNA is derived from post-meiotic cells, our library is heavily weighted towards poly (A)⁺ RNAs which are accumulated during the later stages of spermatogenesis. As a precaution we have routinely overexposed our Northern blots to allow for any discrepancy in the amount of germ cell RNA, as opposed to total testis RNA, present in the different samples. This is necessary because a greater percentage of the RNA isolated from the testis of a mutant blocked in the early stages of spermatogenesis will be of non-germ cell origin when compared to RNA isolated from the testis of a wild type mouse.

All of the cDNA clones we have investigated so far code for RNAs larger than 15S (1600 nucleotides) and this reflects the use of a size fractionated poly (A)⁺ RNA probe in the cDNA cloning. We have identified a series of cDNA clones coding for germ cell specific sequences which are different from those previously reported by Kleene et al. (8). In their experiments a cDNA library was constructed from poly (A)⁺ RNA isolated from trypsin dissociated mouse seminiferous tubules and screened with cDNA probes made from poly (A)⁺ RNA isolated either from pachytene spermatocytes or round
spermatids. They identified 17 clones which coded for sequences enriched in the poly (A)+ RNA fraction of round spermatids as compared to pachytene spermatocytes and all of these clones hybridised to one of two or three RNA species of 900 nucleotides or less on Northern blots.

We intend to use the cDNA clones we have isolated to study the mechanisms by which genes are expressed during spermatogenesis, in particular in the haploid phase of the pathway. In addition, it is hoped to use this approach to study the role of cell surface antigens during germ cell morphogenesis by synthesising peptides corresponding to germ cell specific cDNA clones. Anti-sera raised against the synthetic peptides can then be used to screen isolated germ cells and thereby identify clones coding for cell surface polypeptides.

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REFERENCES
