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Stage-specific gene expression during rat spermatogenesis: application of the mRNA differential display method

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ABSTRACT **Spermatogenesis is a complex differentiation process which requires the coordinate synthesis of diverse stage-specific proteins. In attempting a large-scale identification and characterization of those proteins, we have made use of the recently described mRNA differential display method (Liang and Pardee, *Science 257:* 967-971, 1992). This method is based on the reverse transcription of mRNAs obtained from two different cell populations (pachytene spermatocytes and spermatids in the present study) followed by a PCR reaction and comparison of the individual cDNA populations in a polyacrylamide gel system. Up to the present we have been able to identify 268 cDNA bands. Most of them (77%) are common to both cell stages. From the differentially expressed bands (23%) an ample majority was spermatid-specific (74%). According to our present results we conclude that the mRNA differential display is a promising approach for investigations on stage-specific gene expression during a differentiation process like spermatogenesis.**

KEY WORDS: *spermatogenesis, meiosis, spermiogenesis, gene expression, mRNA differential display*

Introduction

Spermatogenesis is a terminal differentiation process that results in the mature sperm. This process can be described as the coordinate execution of at least two individual programs of gene expression. One of these programs provides the primary spermatocyte with the meiosis-specific apparatus needed for chromosome pairing, molecular recombination and chromosome segregation. The second program provides the spermatid with the proteins necessary for the profound nuclear and cytoplasmic changes that take place during spermiogenesis. However, distinction between these programs is made difficult by the fact that they temporarily overlap. In rodents, for example, transcription of some spermiogenesis-relevant genes starts already during meiotic stages, while others are transcribed only post-meiotically by the round spermatid before transcription ceases in the elongating spermatid. In other species like *Drosophila*, post-meiotic transcription seems not to occur at all; transcription of genes coding for proteins needed during spermiogenesis is restricted to the spermatocyte stage. The complexity and execution of these gene expression programs explain the long duration of both meiotic prophase (as compared to the mitotic one) and spermiogenesis (for reviews see Hackstein, 1987; Willison and Ashworth, 1987; Erickson, 1990; Hecht, 1993; Stern, 1993).

Differential gene expression has been successfully investigated in a variety of model systems by using subtractive hybridization techniques (for an overview see Myers, 1993). More recently, however, an alternative strategy was described, called the mRNA differential display (Liang and Pardee, 1992; Liang *et al.*, 1993). The method is based on the reverse transcription of the mRNA of two different cell populations using oligo-dT primers anchored to the poly A^+ tail, and followed by a polymerase chain reaction (PCR) in the presence of a decamer arbitrary in sequence as a second primer. The resulting cDNA subpopulations are then visualized and compared using a polyacrylamide gel system (Liang and Pardee, 1992; Liang *et al.*, 1993). According to these authors the mRNA differential display method reveals several advantages in comparison to subtractive hybridization techniques. It is quicker, easier to reproduce and requires a lesser amount of starting RNA. Furthermore, it allows the simultaneous analysis of both groups of differentially expressed genes. The mRNA differential display method proved useful when applied to relatively simple models, i.e. the comparison of a transformed cell line vs. the corresponding non-transformed one (Liang and Pardee, 1992). Therefore, we were interested in testing its applicability to more complex situations, as in the case of a cell differentiation process like spermatogenesis. A prerequisite for the analysis of differential gene expression during spermatogenesis is the availability of methods that allow rapid separation of germ cell populations in sufficient amount

Abbreviations used in this paper: PCR, polymerase chain reaction.

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and purity. Among these methods centrifugal elutriation is the best suited (Meistrich, 1977). Here we report on our data, obtained by comparing mRNA populations isolated from meiotic and post-meiotic cells of the rat.

Results and Discussion

In an attempt to define different patterns of gene expression along spermatogenesis, we have applied the mRNA differential display method to RNA populations obtained from highly purified pachytene spermatocytes and round spermatids of the rat. Up to the present we have assayed eight different primer combinations, which, according to Liang *et al.* (1993), would cover about 10% of the poly A$^+$ RNAs expressed in a defined cell type. When the PCR products of these eight primer combinations were displayed on polyacrylamide gels we could clearly identify a total of 268 cDNA bands which appeared in duplicated experiments. The vast majority of them (77%) appeared to be common to spermatocytes and spermatids, while the remaining 62 bands (23%) were differentially expressed. Interestingly, from the differentially expressed bands 46 (74%) were specific for spermatids and 16 (26%) were found only in spermatocytes (Fig. 1 and Table 1). None of the 268 bands appeared in the negative controls without reverse transcriptase, which means that they corresponded to expressed RNAs and not to contaminant DNA.

Twenty-eight cDNA bands of the display, that were clearly stage-specific, were selected for reamplification. As shown by agarose gel electrophoresis (Fig. 2A), almost every band could be reamplified by the procedure. Remarkably, reamplified cDNA bands were in the same size range as the original bands of the display. In a subsequent phase, Northern-blot assays were performed in order to verify the stage-specificity of the amplified

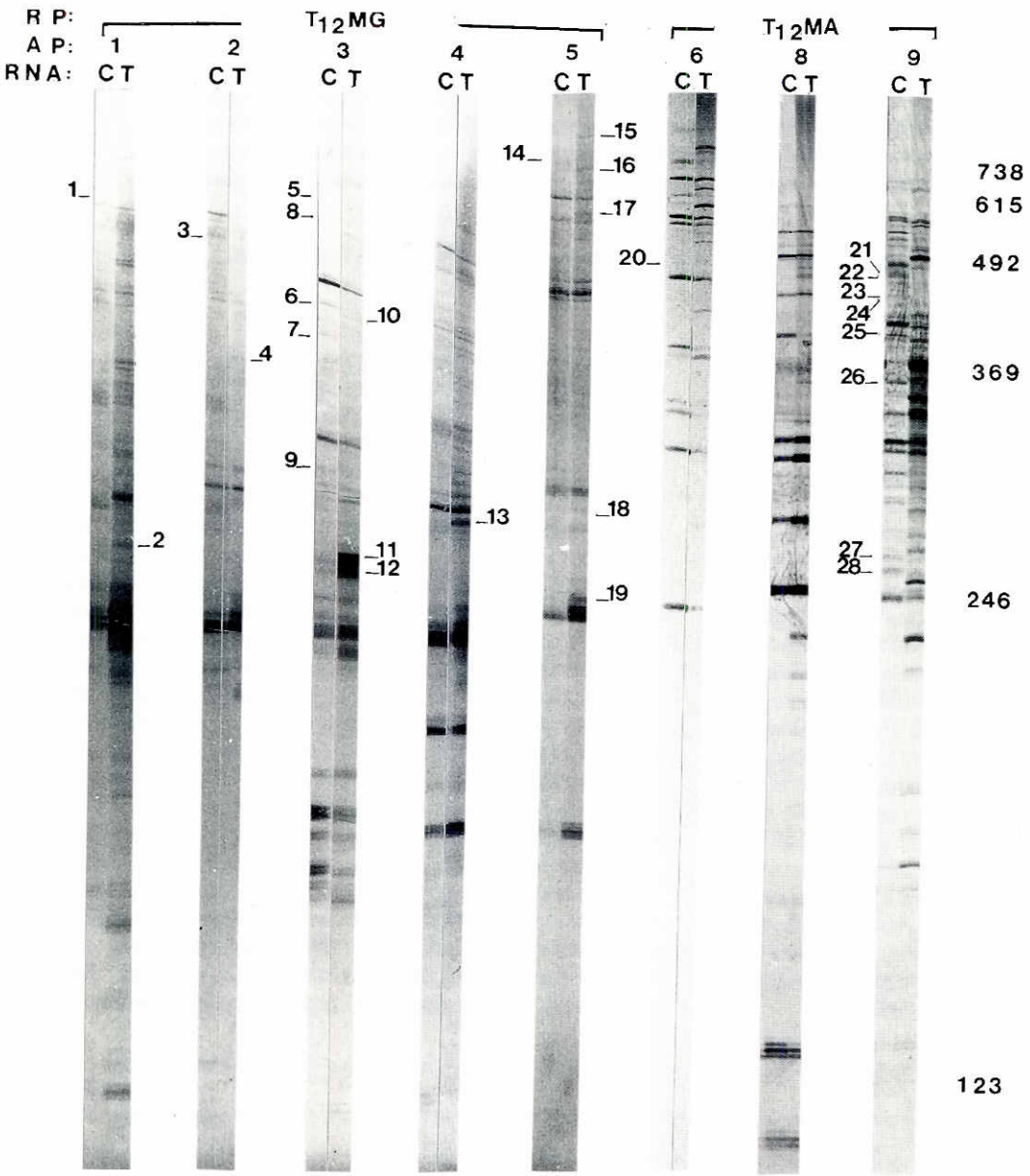


Fig. 1. Differential display of mRNAs from spermatocytes and spermatids using eight combinations of primer sets made of T12MG and T12MA and a group of eight arbitrary decamers. *C: spermatocytes; T: spermatids; RP: right primers (i.e., primers for the poly A$^+$ tail); AP: arbitrary primers. cDNA bands selected for reamplification are numbered from 1 to 28. A DNA marker (123 bp ladder) was used as molecular weight standard (BRL).*

TABLE 1

COMMON AND DIFFERENTIALLY EXPRESSED cDNA BANDS OF SPERMATOCYTES AND SPERMATIDS

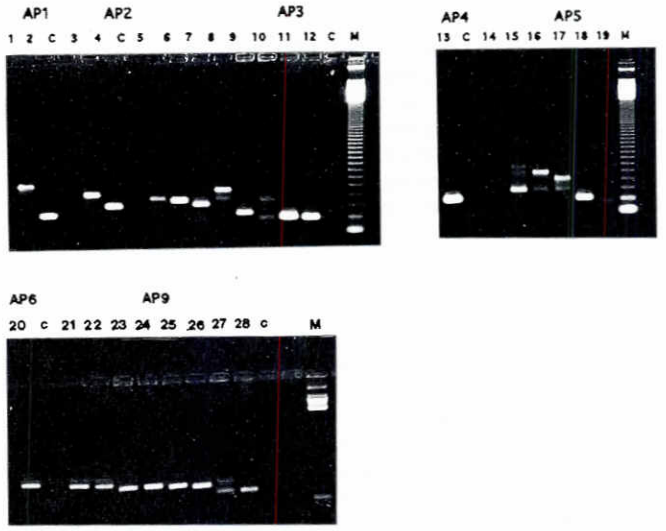
right primer	left primer	total number of bands*	differentially expressed bands	spermatocyte-specific bands	spermatid-specific bands
$T_{12}MG$	1	33	2	1	1
$T_{12}MG$	2	20	1	0	1
$T_{12}MG$	3	32	9	5	4
$T_{12}MG$	4	26	4	0	4
$T_{12}MG$	5	19	5	1	5
$T_{12}MA$	6	26	4	1	3
$T_{12}MA$	8	38	8	0	8
$T_{12}MA$	9	74	28	8	20
Totals		268	62	16	46
Percentage from total		100%	23%	6%	17%
Percentage from the differentially expressed bands				26%	74%

* Only bands clearly distinguishable from the background were considered

bands. Figure 2B shows three examples in which cDNA from spermatocyte-specific (filter a) and spermatid-specific bands (filter b; lanes 1 and 2) were used as a probe. In agreement with the display signals were restricted to the lanes respectively containing RNA isolated from pachytene spermatocytes or round spermatids.

Considered together, the results presented here allow some preliminary conclusions. Our results indicate that the mRNA differential display method is a promising strategy in studies aiming at a large-scale characterization of genes expressed in a complex differentiation process. Interestingly, the number of cDNA bands obtained in the display was within the range as described by Liang and Pardee (1992). The application of this method to spermatogenesis allowed the isolation of several cDNA bands corresponding to mRNAs present in round spermatids but absent form spermatocytes. Thus, these cDNAs in all probability correspond to genes transcribed by the round spermatid (for discussions on the sensitivity of the method see Liang and Pardee, 1992). Concerning the cDNA bands differentially expressed in spermatocytes, more information is needed in order to definitively establish whether the corresponding genes are in fact transcribed by the spermatocyte, or earlier during spermatogenesis. The isolation of a high percentage of spermatid-specific bands as compared to spermatocyte-specific ones is not surprising, and agrees with previous large-scale attempts to isolate spermatogenic specific cDNAs using the differential screening of testis libraries as a strategy (e.g. Thomas *et al.*, 1989; Höög, 1991). Finally, our results lend further support to the notion that active transcription takes place during post-meiotic stages of mammalian spermatogenesis (for reviews see Erickson, 1990; Hecht, 1993). In experiments currently in progress, selected differentially expressed cDNA bands are being sequenced and used as probes in order to obtain full-length clones.

Experimental approaches like the mRNA differential display appear to be well suited for studies aiming at a large-scale identification and characterization of stage-specific spermatogenic proteins. This does not seem to be the case for other strategies, such as those based on the comparison of the two-dimensional protein spot patterns obtained from two different cell populations. In fact, in previous attempts we had prepared protein fractions from highly purified pachytene spermatocytes and round spermatids, and compared them after separation in a two-dimensional gel system. Despite the fact that clear differences in protein composition were observed between these two spermatogenic stages, the outcome of these experiments was frustrating. For most of the interesting protein spots of the two-dimensional gels it was not feasible to obtain enough material for protein microsequencing or production of polyclonal antibodies. An important limiting factor here is the amount of spermatocytes



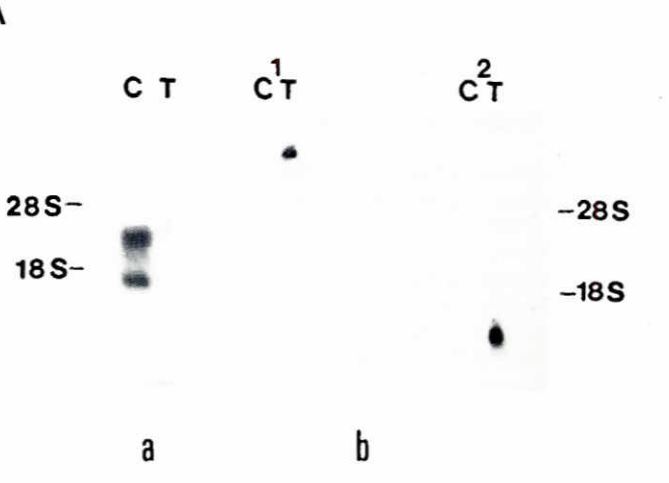


Fig. 2. Characterization of differentially expressed bands. (A) *The cDNA bands indicated in the former figure were reamplified and run on three 1.5% agarose gels. The numbers on top of each lane correspond to those of Figure 1. Lanes named C are the negative controls without cDNA for each primer set. The molecular weight standard used (M) was the same as in Figure 1.* **(B)** *Reamplified cDNA bands #25 (filter a), #2 (filter b; lane 1) and #18 (filter b; lane 2) were labeled with α-[^{32}P]dATP and used as probes in Northern blots. Each lane contains 30 μg of total RNA from spermatocytes (C) or spermatids (T).*

and spermatids that can be isolated from a rat (D. Treichel, M. Alsheimer and R. Benavente, unpublished observations).

Materials and Methods

Obtention of stage-enriched cell populations of rat testis

Wistar rats were obtained from Charles River Wiga (Sulzfeld, Germany). A cell suspension of testicular cells was prepared and submitted to centrifugal elutriation essentially as described (Meistrich, 1977; Meistrich *et al.*, 1981; Heyting and Dietrich, 1991; Smith and Benavente, 1992). Fractions with the highest concentration of pachytene spermatocytes and round spermatids were collected separately. After a centrifugation at 450g for 10 min the cell pellets were washed three times in sterile PBS.

RNA isolation and removal of DNA contamination

Isolation of total RNA was performed with the RNA isolation kit from Stratagene (Heidelberg, Germany). 50 µg of total RNA from pachytene spermatocytes and 50 µg from round spermatids were respectively treated with 10 units of RNase-free DNase I (GenHunter, Brookline, MA, USA) as previously described (Liang *et al.*, 1993), except that 20 units of RNase block I (Stratagene) were added to the incubation mix.

Differential display

The mRNA differential display was carried out in its essentials according to Liang *et al.* (1993). 0.2 µg of total RNA from pachytene spermatocytes and round spermatids were used for reverse transcription. The cycling parameters were as described, but a 2 min. initial denaturation cycle was included. The amplified cDNAs were displayed on a 6% DNA sequencing gel. All the reverse transcription and PCR reactions were performed in duplicate and the results were compared. In order to demonstrate that the display was independent from chromosomal DNA contamination RNAs were incubated in a reverse transcriptase reaction in the absence of reverse transcriptase followed by PCR reactions for each primer combination. AmpliTaq DNA polymerase was purchased from Perkin-Elmer (Applied Biosystems, Darmstadt, Germany). α-[^{35}S]dATP was obtained from Amersham (Braunschweig, Germany). Further reagents for the mRNA differential display including the oligo-dT primers and the arbitrary decamers 1 to 6, 8 and 9 (GenHunter's denomination) were obtained from GenHunter.

Recovery and reamplification of cDNA probes

The 6% sequencing gel with the displayed cDNA bands was blotted onto a piece of Whatman 3 mm paper, dried, and exposed for up to five days. cDNA bands that appeared to be specific to pachytene spermatocytes or spermatids in duplicate experiments were cut from the acrylamide gel and eluted as described (Liang *et al.*, 1993). Briefly, 4 µl of an eluted cDNA probe were reamplified in a 40 µl reaction volume using the same primer set and PCR conditions as in the differential display, except that the dNTP were at 20 µM and no radioactive isotopes were added. 30 µl of reamplified samples were run in a 1.5% agarose gel and stained with ethidium bromide. Control reamplification experiments without cDNA were performed for each primer combination.

Northern blot analysis

Reamplified cDNA probes were purified from the agarose gel by means of the Qiaex kit from Qiagen (Chatsworth, CA, USA) and labeled with α-[^{32}P]dATP (Amersham) using a Random-Prime DNA labeling kit from Gibco (Eggenstein, Germany). Labeling was performed essentially as instructed except that 1 µl of 10 µM corresponding T12MN primer was included in the reaction. Northern blot analysis was carried out according to Sambrook *et al.* (1989). Prehybridization and hybridization were done at 42°C in 50% formamide. Washes were carried out twice with 1x SSC containing 0.1% SDS at room temperature and 0.25x SSC containing 0.1% SDS at 55-60°C for 15-30 min. Filters were exposed overnight at -70°C with intensifying screen.

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