The murine seminiferous epithelial cycle is pre-figured in the Sertoli cells of the embryonic testis

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SUMMARY

The seminiferous epithelial cycle and spermatogenic wave are conserved features of vertebrate spermatogenic organisation that reflect the need for the rigorous maintenance of sperm production. Although the cycle and the wave of the adult seminiferous epithelium have been well characterised, particularly in rodent species, their developmental origins are unknown. We show that the Sertoli cells of the pre-pubertal mouse, including those of the germ cell-deficient XXSxr mutant, exhibit coordinated, cyclical patterns of gene expression, presaging the situation in the adult testis, where Sertoli cell function is coupled to the spermatogenic cycle. In the case of the galectin 1 gene (Lgals1), localised differential expression in the Sertoli cells can be traced back to neonatal and embryonic stages, making this the earliest known molecular marker of functional heterogeneity in mammalian testis cords. In addition, the timing of germ cell apoptosis in normal pre-pubertal testes is linked to the temporal cycle of the Sertoli cells. These data show that the cycle and wave of the murine seminiferous epithelium originate at a much earlier stage in development than was previously known, and that their maintenance in the early postnatal cords depends exclusively on the somatic cell lineages.

Key words: Apoptosis, Cyclical gene expression, Galectin, Seminiferous epithelial cycle, Sertoli, Spermatogenic wave, Testis cords, Mouse

INTRODUCTION

The seminiferous epithelium of the mammalian testis is a complex and highly dynamic tissue in which germ cells proliferate, undergo meiosis and differentiate into spermatozoa. Male reproductive success ultimately depends on the ability of this tissue to produce prodigious numbers of sperm at a consistent rate, and these functional imperatives are reflected in its structure. The organisation of the seminiferous epithelium into long fine tubules serves to maximise its area and overall capacity for sperm production. In addition, the elaborate cellular structure of the epithelium, and the precise orchestration of spermatogenesis within it, ensure the optimal use of the available space and resources, and provide a timetable for the regular release of sperm.

In the mature seminiferous tubule, developing germ cells gradually traverse the epithelium from the basement membrane to the apical surface and are then released into the tubule lumen as spermatozoa. As each batch of germ cells progresses through this lengthy process, further rounds of spermatogenesis are initiated in its wake. As a result, the adult seminiferous epithelium always contains several layers of germ cells, at different stages of development. It has been known for over a century that the germ cells in a particular patch of epithelium at a given moment do not comprise a random assortment of spermatogenic stages, but one of a limited number of combinations. This is because the various generations, or layers, of germ cells all develop in a coordinated manner, in a repeating programme known as the seminiferous epithelial cycle. This has been studied most extensively in rodents, particularly the rat, in which fourteen distinct germ cell associations can be recognised cytologically. These are designated stages I-XIV of the seminiferous epithelial cycle (Leblond and Clermont, 1952). Twelve such stages (I-XII) have been described in the mouse (Oakberg, 1956a).

A second tier of organisation serves to maintain consistent sperm output, by coordinating the progress of the cycle in different regions of the tubule. Owing to the total duration of spermatogenesis, and the length of each epithelial cycle (35 and 8.5 days, respectively, in the mouse) (Oakberg, 1956b), synchronous or random phasing of the cycle throughout the tubule would result in infrequent or irregular production of sperm. This is prevented by the staggered timing of the cycle in adjacent regions of the epithelium, such that the temporal sequence of stages (I, II, III, etc.) is recapitulated in their
physical layout within the tubule. This dynamic spatial pattern, known as the spermatogenic wave (Perey et al., 1961), ensures that fully differentiated sperm are always available for release from a specific proportion of the epithelial surface.

The spermatogenic wave is readily apparent in the testes of rodents like the rat and mouse, in which successive stages of the epithelial cycle are arranged as a linear series of cylindrical segments along the seminiferous tubule. In other species, the stages are organised in more complex configurations. Nevertheless, the existence of the cycle and the wave has been demonstrated in many vertebrate species, from birds to humans (Kerr, 1995; Sharpe, 1994). Precise spatiotemporal patterning of the seminiferous epithelium is therefore a key conserved element of spermatogenic regulation in vertebrates, and plays an important role in male fertility.

While the physical layout of the mature seminiferous epithelium has been ingeniously exploited to investigate many aspects of spermatogenesis and its regulation (Kerr, 1995; Schlatt et al., 1997; Sharpe, 1994), relatively little is known about when or how this tissue acquires its pattern during development. This issue has been particularly intractable because of the reliance on germ cell morphology as the main criterion for distinguishing between different stages of the epithelial cycle. Standard staging methods depend on indicators such as differentiation and condensation of spermatid nuclei, and can therefore only be applied to post-pubertal stages of testis development. As these landmark cell types are not present before puberty, the spatial organisation of cellular function in early testis tubules, or testis cords, has rarely been addressed (Clément and Perey, 1957; Huckins and Clermont, 1968; Sung et al., 1986). We highlight the use of an alternative indicator of epithelial cycling that helps to overcome this problem.

Throughout spermatogenesis, the germ cells are entirely dependent on a population of highly specialised somatic cells known as Sertoli cells. In the adult tubule these large post-mitotic epithelial cells span the basal-apical axis of the seminiferous epithelium, providing a physical scaffold for the dividing and differentiating germ cells. They also generate a specialised microenvironment for each phase of spermatogenesis, which they sustain by the synthesis and delivery of appropriate metabolites (Byers et al., 1993). Moreover, the Sertoli cells are uniquely placed to play a major role in spermatogenic regulation. They are active participants in the seminiferous epithelial cycle, undergoing cyclical changes in gene expression, biochemical activity and morphology as the germ cells develop around them. They are the primary targets of the main hormonal regulators of spermatogenesis, follicle stimulating hormone (FSH) and testosterone, and remain intimately associated with each other, and with the developing germ cells, through a dynamic repertoire of specialised contacts and junctions (Enders, 1993; Jégou, 1993). As mediators of both endocrine and paracrine mechanisms of spermatogenic regulation in the adult testis, the Sertoli cells clearly play a central role in the maintenance of the epithelial cycle. They are also likely to be involved in effecting long range coordination of spermatogenesis through regulation of the spermatogenic wave.

In this report we show that, in addition to the vital role of Sertoli cells in the adult testis, cells of this lineage are involved in the establishment of temporal cycling and spatial pattern in the testis cords at an early stage of testis development. We describe the testicular expression of the murine gene encoding galectin 1, a 14 kDa β-galactoside-specific carbohydrate-binding protein. The galectin family currently includes 10 mammalian members (Cooper and Barondes, 1999), which are expressed in many different embryonic and adult tissues, where they may be found either in the intracellular or extracellular compartments, or both (Harrison and Wilson, 1992; Hughes, 1999). These proteins have been implicated in many different biological processes, including cell recognition (Puche et al., 1996), cell adhesion (Cooper et al., 1991; Hadari et al., 2000; Kuwabara and Liu, 1996), organisation of extracellular matrix (Hikita et al., 2000) and programmed cell death (Akahani et al., 1997; Colnot et al., 2001; Perillo et al., 1995).

We show that galectin 1 exhibits stage-specific regulation in the adult seminiferous epithelium, and localised differential expression in the Sertoli cells of the pre-pubertal and embryonic mouse testis. By correlating the galectin 1 expression pattern with that of other Sertoli cell genes, and with the distribution of apoptotic germ cells, we also show that the epithelial cycle and spermatogenic wave are prefigured in the pre-pubertal testis. In addition, we present evidence that the spatially coordinated, cyclical gene expression profile of the Sertoli cells can be maintained in the absence of germ cells.

MATERIALS AND METHODS

Northern blots
Total RNA was prepared by guanidinium extraction (Sambrook et al., 1989) from postnatal day (P) 7, 14, 28 and 56 XY testes, P56 XY epididymides and P56 XX♂♂ testes. All mice were from the same MFI YRIII genetic background. Northern blots were prepared using 10 μg of each RNA sample (Sambrook et al., 1989). To improve resolution of RNA species in the 0.5-1.0 kb range, the samples were electrophoresed through a discontinuous, or ‘two-step’, Mops/formaldehyde agarose gel, prepared as follows. A 200 ml mixture of 25% Mops/formaldehyde was prepared and 25% agarose was added at 0.8%. After heating the mixture to 60°C, 200 ml of 0.8% agarose was added and it was allowed to cool to room temperature before it entered the main body of the 2.0% agarose gel, was found to improve the resolution of smaller molecules. RNA was transferred to nylon membrane and probed with a digoxigenin labelled antisense cRNA, containing nucleotides 20-387 of galectin 1 (Poirier et al., 1992), using conditions recommended by the supplier of the labelling kit (Roche).

Staging of seminiferous tubule sections
Stages of the seminiferous epithelial cycle in sections of adult testis were determined by light microscopy with reference to the criteria outlined by previous authors (Leblond and Clermont, 1952; Parvinen, 1982; Russell et al., 1990). In some cases it was not possible to stain for the usual cytological markers, owing to prior processing of the tissue for in situ hybridisation and immunohistochemistry. For example, acrosome morphology and residual bodies could not be visualised in sections stained with Toluidine Blue after in situ hybridisation. In these cases, staging was based on other diagnostic criteria as follows: presence of meiotic figures (stage XII), absence of large pachytene nuclei (stage I), depth and bundling of elongating spermatids (stages I to VIII), absence of elongated spermatids (stage IX) and absence of round spermatids (stage XI).
In situ hybridisation

Tissues were from CBA/Ca, 129 or MFI YRIII strains of mice. Testes from MFI YRIII littermates, identified as XX5xr0 or XY by weight, were kindly provided by Maxine Sutcliffe and Paul Burgoyne (MRC, London). Tissues were processed for in situ hybridisation with 35S-labelled cRNA probes as previously described (Wilkinson and Green, 1990). A 1:1 dilution of HifoR K5 emulsion in 2% glycerol was used for autoradiography. Sections were counterstained with Toluidine Blue. The galectin 1 probe contained nucleotides 20-387 of the cDNA sequence (Poirier et al., 1992). Probes for Ahn (Münsterberg and Lovell-Badge, 1991), Cstl (Maguire et al., 1993), Rbp1 (Döll et al., 1990), Sgp2 (Collard and Griswold, 1987) and Wt1 (Armstrong et al., 1993) were kindly given by Andrea Munsterberg, Philippa Saunders, Pierre Chambron, Michael Collard and Jane Armstrong, respectively.

Immunohistochemistry

Adult mouse testes perfused with Bouin's fixative (Sigma) were kindly provided by Florence Bernex (École Nationale Vétérinaire d'Alfort, France). Other samples were fixed by immersion either in Bouin's fixative (Sigma) or paraformaldehyde (as for in situ hybridisation, above). All tissues were embedded in paraffin wax and sectioned at 6 μm. Sheep antiserum raised against purified mouse galectin 1 (Harrison and Wilson, 1992) was a generous gift from F. Lynne Harrison. Antibodies were purified from the crude serum by caprylic acid precipitation of non-IgG serum proteins, followed by ammonium sulphate precipitation and dialysis (Harlow and Lane, 1988). Immunoreactivity was detected by standard histochemical methods after incubation with alkaline phosphatase conjugated donkey anti-sheep secondary antibodies (Sigma). In addition to the initial characterisation of the antiserum (Harrison and Wilson, 1992), its specificity for galectin 1 under the conditions used here was confirmed by the following criteria (data not shown): (1) on mouse embryo sections the immunohistochemical staining pattern recapitulated the pattern of galectin 1 mRNA expression previously described (Poirier et al., 1992); (2) cross-reactivity with galectin 3 was excluded by incubating alternate serial sections from E10.5 embryos with antibodies against galectin 1 or galectin 3. Specific staining of the notochord was obtained as expected with anti-galectin 3 (Fowlis et al., 1995), but none was detected with anti-galectin 1; (3) the staining pattern obtained with the sheep anti-galectin 1 on testis sections was identical to that obtained with a rabbit anti-galectin 1 antiserum described previously (Colnot et al., 1998); and (4) no staining was obtained on sections of testis from galectin 1 homozygous null mice.

TUNEL analysis

P7 CBA/Ca testes were fixed, embedded and sectioned as for in situ hybridisation. Terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end labelling (TUNEL) assays (Gavrieli et al., 1992) were performed on four complete sections, taken from two separate regions of two different testes, using the Apoptag kit and protocol (Intergen). The sections were counterstained with Methyl Green (Merck). The adjacent pair of serial sections from each region were processed for galectin 1 in situ hybridisation and galectin 1 immunohistochemistry.

Assessment of galectin 1 mRNA and protein levels in testis cord sections

After in situ hybridisation and 4 days of autoradiographic exposure, the relative grain density over clear areas of Sertoli cell cytoplasm in the centre of the cords was assessed by visual inspection using standard brightfield microscopy. The signal over each individual cord was categorised, relative to the other cords in the same section, as either high, medium or low (see Fig. 6A). Similarly, sections treated by galectin 1 immunohistochemistry were assessed for the relative intensity of alkaline phosphatase staining. The score categories were recorded on digital images of the entire sections, to enable comparison of the TUNEL, in situ hybridisation and immunohistochemistry data for individual segments of cord. Images were superimposed and the data collated using Adobe Photoshop software.

To verify the validity and reliability of the visual scoring method, greyscale digital images covering one entire testis section after in situ hybridisation and one after immunostaining were analysed using the public domain NIH Image programme (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Mean density measurements were taken over the central area of the individual testis cord segments, and the values were then grouped into the three categories (high/medium/low) to which the segments had been independently assigned by visual inspection (Table 1). This analysis showed that under the conditions used, there was no significant overlap between the range of density measurements in the three score categories, either for mRNA or protein. This confirmed that the visual assessment method used to categorise relative signal intensities in individual cord segments was valid and sufficiently reproducible for the purposes of the present study.

Table 1. Quantitation of high, medium and low categories of galectin 1 mRNA and protein (see Materials and Methods)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Category</th>
<th>Mean density</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ hybridisation</td>
<td>High</td>
<td>158.67</td>
<td>20.98</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>88.11</td>
<td>15.90</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>45.72</td>
<td>10.04</td>
<td>100</td>
</tr>
<tr>
<td>Immunostaining</td>
<td>High</td>
<td>199.91</td>
<td>10.58</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>161.62</td>
<td>14.00</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>124.92</td>
<td>13.36</td>
<td>30</td>
</tr>
</tbody>
</table>

For the purposes of the present study.

Statistical analysis of in situ hybridisation, immunohistochemistry and TUNEL data

To test the statistical significance of the frequencies of TUNEL-positive cord segments in the high, medium and low score categories for galectin 1 mRNA and protein, the observed frequencies (percentage of the total number (n=129) of TUNEL-positive segments that fell within each score category) were compared with the frequencies that would be expected on a random basis, according to the number of segments in each category (see Table 3). χ2 values were calculated for each mRNA and protein score category, using the formula χ2 = (observed percentage–expected percentage)/expected percentage. The values were then added together to give an overall measure of the disagreement between the sets of observed and expected frequencies. To assess whether this value (2χ2) was large enough to indicate a significant deviation of the observed from the expected frequencies, the significance probability (P) was obtained from tabulated values of a χ2 distribution with two degrees of freedom.

RESULTS

Galectin 1 transcripts in the postnatal mouse testis

Following our earlier observation of galectin 1 mRNA in the postnatal mouse testis (Poirier et al., 1992), we investigated the timecourse of expression in this tissue from postnatal day 7 (P7) to adulthood (P56) by northern blot analysis. Preparations of total RNA from P7, P14, P28 and P56 testes (Fig. 1, lanes 1-4) all contained galectin 1 transcripts that migrated as a broad band of approximately 0.6 kb, as found in adult epididymis (Fig. 1, lane 6) and other somatic tissues (Poirier et al., 1992). Resolution of the transcripts in this size range was optimised by electrophoresing the samples through a two-step agarose gel (see Materials and Methods), which separated the broad 0.6 kb band into a doublet or triplet of major species as shown in Fig. 1. In several experiments on different RNA preparations (not
shown), the 0.6 kb species were always more abundant in samples from P7 testes than older stages. This reflects the fact that the majority of galectin 1 transcripts in the testis are expressed in Sertoli cells (see later experiments). In the mouse, these cells cease dividing during the second postnatal week, in contrast to the germ cell population, which expands rapidly between birth and adulthood (Vergouwen et al., 1991). Consequently, the Sertoli cells make up a much greater proportion of testicular tissue in prepubertal stages than in the adult, and their transcripts are accordingly more highly represented in prepubertal whole testis RNA.

In addition to the 0.6 kb species of galectin 1 message, a discrete band of slower migrating transcripts was found in RNA from P28 and P56 testes (lanes 3 and 4 in Fig. 1). As these larger transcripts were not found in somatic tissues or less mature testes, we reasoned that they may correlate with the more advanced germ cell stages present in the testis at P28 and P56. This was supported by the absence of a corresponding band in RNA from the testes of P56 XX

*Sxra mice (Fig. 1, lane 5), which do not contain germ cells (Burgoyne, 1989; Cattanach et al., 1971). The larger sized transcripts therefore appear to be a novel testis-specific, age-dependent size variant of galectin 1 message. The time when this variant first appears, between 2 and 4 weeks after birth, corresponds to the appearance of the first pachytene spermatocytes and round spermatids in the seminiferous tubules (Vergouwen et al., 1993). The variant transcripts may therefore be synthesised specifically in germ cells that have reached or passed a particular stage of spermatogenesis. Alternatively, they may be produced by other testicular cells, such as the Sertoli cells, when in the presence of particular germ cell stages.

Testis-specific mRNA size variants have been documented for many other genes, and can result from differential
Cyclical function of early Sertoli cells

splicing, the use of alternative promoters or polyadenylation sites, or altered length of the poly(A) tail. (Eddy and O’Brien, 1998; Wolgemuth and Watrin, 1991). Preliminary RNase H digestion experiments suggest that the increased size of the testis-specific galectin 1 transcripts is due to additional polyadenylation (data not shown). Hyperadenylation is a relatively common modification of testicular mRNAs, and is particularly associated with the delayed translation of messages that are transcribed in pachytene spermatocytes and round spermatids (Kleene, 1996).

Localisation of galectin 1 mRNA and protein in the adult mouse testis

The distribution of galectin 1 transcripts in the testis was investigated by in situ hybridisation of paraffin wax embedded sections with a 35S-labelled cRNA probe. In the fully mature testis (8 weeks post partum), low levels of galectin 1 mRNA were detected in the tunica albuginea and the walls of some blood vessels (not shown), and stronger signal was localised over the interstitium (Fig. 2B,D). Expression was also observed in the seminiferous epithelium, where the level of mRNA was high in some individual tubule cross sections and undetectable in others (Fig. 2A-D). In positive tubule sections the entire epithelium appeared to be labelled, although the most intense signal was localised in the basal region, specifically over the Sertoli cells (Fig. 2B,D,F). The more diffuse labelling over the adluminal part of the epithelium suggested that galectin 1 mRNA may also be expressed in germ cells, as already indicated by our northern data (see Fig. 1). Although we were not able to assign this signal specifically to germ cells, rather than the apical processes of Sertoli cells, it is likely that galectin 1 mRNA is expressed in both cell types, as the broad distribution of signal over the epithelium was distinctly different from that of Sertoli cell-specific markers such as Ctsl (Fig. 2E) and Sgp2 (not shown), in which a much greater proportion of the signal was concentrated around the periphery of the tubules. Alternatively, the distinctive hybridisation patterns of Ctsl and Sgp2, versus that of galectin 1, could represent differential localisation of their respective transcripts within the Sertoli cell cytoplasm.

Histological examination of the germ cell populations in tubule cross sections (see Materials and Methods) revealed a consistent correlation between stages in the seminiferous epithelial cycle and the level of galectin 1 mRNA expressed. The overall strength of the galectin 1 hybridisation signal was maximal at stages X-XII of the cycle, weaker at stages I-VI, very low or undetectable at stages VII-VIII and stronger again at stage IX (Fig. 2C,D). This suggests a single pulse of galectin 1 transcription during each epithelial cycle, initiated between stages VIII and IX. Fig. 2F shows a patch of epithelium that is identifiable as stage VIII by the closely packed spermatozoa lined up at the luminal surface. In contrast to most stage VIII tubules, which were negative for galectin 1 mRNA (see Fig. 2C,D), transcripts were clearly detectable in the Sertoli cells in this particular cross section. The adjacent epithelium in the same tubule contained no
spermatozoa (see asterisk in Fig. 2A), indicating that they had been released into the lumen. The presence of galectin 1 mRNA in this group of Sertoli cells suggests that the onset of galectin 1 transcription is marked by a rapid accumulation of transcripts, and occurs at a very precise point in the epithelial cycle, coinciding with spermiation.

Galectin 1 protein was detected by immunohistochemistry in the tunica albuginea (not shown), some interstitial cells and blood vessel walls (Fig. 3A,B), resembling the distribution of the mRNA. However, in the seminiferous tubules galectin 1 protein was found at all stages of the epithelial cycle (Fig. 3C-F). The strongest staining was associated with the nuclei and cytoplasm of the Sertoli cells. In addition, many of the germ cells, including spermatogonia, spermatocytes and elongating spermatids, were surrounded by weak staining. Although this was not unequivocally localised to the cytoplasm of the germ cells rather than the Sertoli cells, it was noteworthy that this staining was excluded from groups of round spermatids (Fig. 3F).

Expression of galectin 1 during testis development

The distribution of galectin 1 transcripts in the mouse testis at P14 and P28 (not shown) was very similar to the adult pattern, including localised differential expression in the developing tubules. Although these pubertal testes did not contain the full complement of spermatogenic intermediates, it was possible to distinguish groups of germ cells at different developmental stages by the size and Toluidine Blue staining intensity of their nuclei. The in situ hybridisation signal intensity over individual tubule sections revealed a consistent correlation between the level of galectin 1 mRNA and the spermatogenic stages present within the local germ cell population, as in the adult testis.

Fig. 4A,B shows sections of P7 testis probed for galectin 1 mRNA, and stained for galectin 1 protein respectively. Both
mRNA and protein were detected in the peritubular cells, a subset of blood vessels, some interstitial cells and the tunica (Fig. 4A,B and data not shown). At this pre-pubertal stage, the tubules have not yet formed a true lumen and are referred to as testis cords. The centre of the cords is filled with the cytoplasm of the Sertoli cells. The Sertoli cell nuclei and most of the germ cells are situated basally, at the periphery of the cords. No galectin 1 mRNA was detected in the germ cells, using probes labelled with either $^{35}$S (Fig. 4A) or $^3$H (not shown). Galectin 1 mRNA and protein were preferentially localised in the centre of the cords, with marked variability in the signal intensity for both mRNA and protein in different cord cross sections (Fig. 4A,B). By contrast, no significant local variation was detected in the expression level of galectin 7, a marker of immature Sertoli cells (Timmons et al., 1999), or of Wt1 (Wilms’ tumour) mRNA (Fig. 4C,D). Wt1 is expressed in developing and mature Sertoli cells, with no apparent stage-relatedness (Pelletier et al., 1991).

To find out when differential galectin 1 expression in the cords could first be detected during testis development, in situ hybridisation was carried out on testis sections from early postnatal and embryonic stages. Multiple domains of differential expression within the cords were found at all postnatal stages including the day of birth (P0, Fig. 4E,F). The earliest stage at which galectin 1 mRNA was localised in discrete regions of the cords was approximately 1 day before birth on embryonic day (E) 18 (Fig. 4G,H). At earlier stages, for example E16 (Fig. 4I,J), localised expression in the cords could not be detected, although at this stage the cords were obscured by the relatively strong signal over the interstitial cells. These results show that the cyclical pattern of galectin 1 expression in Sertoli cells first arises during embryonic development, before there are any overt signs of developmental heterogeneity within the germ cell population. To test whether this distinctive pattern of gene expression in the Sertoli cells is dependent on the presence of germ cells, we performed in situ hybridisation on sections of P8 XXSxr<sup>a</sup> testes, in which the germ cells are severely depleted or absent (Cattanach et al., 1971). The distribution of galectin 1 transcripts in the XXSxr<sup>a</sup> testis cords (Fig. 4K) was indistinguishable from that in wild-type cords (Fig. 4A), suggesting that viable germ cells are not required for the maintenance of this expression pattern. Fig. 4M,N illustrates a further detail of the galectin 1 expression pattern that was particularly clear in XXSxr<sup>a</sup> cords, owing to the absence of germ cells. Longitudinal sections of cord occasionally revealed sharp demarcations between regions of weak and strong galectin 1 mRNA expression.

Regional variation in the level of specific mRNAs in pre-pubertal Sertoli cells has previously been reported for other genes, including anti-Müllerian hormone (Amh) (Münsterberg and Lovell-Badge, 1991). We also found this to be the case in XXSxr<sup>a</sup> testes, as shown in Fig. 4L. In addition, we found that the relative intensity of the Amh signal in individual testis cord cross sections was inversely related to the level of galectin 1 mRNA. This was observed consistently in wild-type testes (not shown) and in XXSxr<sup>a</sup> testes, as shown in Fig. 4K,L, which
are consecutive serial sections of P8 XXY Sxra testis probed for galectin 1 and Amh respectively.

**Patterns of gene expression in pre-pubertal Sertoli cells prefigure the adult seminiferous epithelial cycle**

To determine whether the localised expression of galectin 1 in developing testis cords was associated with its cycle-related expression in adult Sertoli cells, we compared the distribution of galectin 1 mRNA in P7-P8 testis cords with that of four other Sertoli cell marker genes. Consecutive serial sections of wild-type and XXY Sxra testes were probed by in situ hybridisation for expression of either galectin 1, or one of the marker genes, to directly compare the relative levels of these mRNAs in the same testis cord regions. The results of this analysis were the same in wild-type and XXY Sxra testes, and the latter are illustrated in Fig. 5.

In the adult testis, the genes encoding cathepsin-L (or CtsL) and sulphated glycoprotein 2 (Sgp2) have a similar expression profile, in which their mRNA levels in the Sertoli cells peak at stages VI-VII and VII-VIII, respectively, of the rat seminiferous epithelial cycle (Erickson-Lawrence et al., 1991; Morales et al., 1987). As shown in Fig. 5A,B, these genes also exhibited regional variation in mRNA expression within the cords of the P8 XXY Sxra mouse testis. Moreover, the patterns of relative signal intensity for both of the probes were superimposable, indicating that the mRNA expression of these genes is regulated in parallel in the pre-pubertal testis cords, as it is in adult tubules.

Fig. 5C,F shows adjacent sections probed for galectin 1 and cellular retinol binding protein (Rbp1). In contrast to CtsL and Sgp2, these genes show maximal mRNA expression in adult Sertoli cells at stages X-XII and X-XIII of the mouse and rat seminiferous cycles, respectively (Fig. 2) (Rajan et al., 1990). Fig. 5C,D,F show that, like the previous pair of markers, galectin 1 and Rbp1 are expressed in a coordinated fashion in the pre-pubertal testis cords, as they are in adult tubules.

Comparison of all four probes shows that the expression patterns of CtsL and Sgp2 are complementary to those of galectin 1 and Rbp1, reinforcing the correlation with the adult testis, in which these two pairs of genes are expressed in alternate phases of the seminiferous epithelial cycle. The coordinated, cyclical gene expression profile exhibited by adult Sertoli cells during the seminiferous epithelial cycle therefore appears to be established before the onset of spermatogenesis, and can be maintained in the absence of germ cells.

This analysis also included a probe for Amh, which is not expressed in the adult testis and does not therefore show classical stage-related regulation during spermatogenesis. As shown in Fig. 5E, the pattern of Amh expression in P8 testis cords was directly comparable with that of CtsL and Sgp2, and complementary to galectin 1 and Rbp1 (also see Fig. 4K,L). Amh expression is therefore coordinated with the cyclical programme of the immature Sertoli cells, even though it is rapidly downregulated after birth (Münsterberg and Lovell-Badge, 1991).

**The timing of pre-pubertal germ cell apoptosis is linked to the Sertoli cell gene expression cycle**

The in situ hybridisation studies described above demonstrated the cyclical regulation and wave-like organisation of gene expression in the Sertoli cells of the pre-pubertal testis cords. This prompted us to look for associated patterns of functional organisation within the germ cell population. In rat testis cords, type A and type B spermatogonia are found in discrete groups by the second postnatal week, suggesting rudimentary spatial organisation of germ cell activity by this stage (Clermont and Perey, 1957). However, even though the equivalent spermatogonial subtypes were expected to be present in P7 mouse testes (Bellvé et al., 1977), the staining methods needed to distinguish these cells could not be used on tissue...
that was processed for in situ hybridisation. As no molecular markers were available for specific stages in pre-pubertal germ cell development, we sought an alternative indicator of regional differences within the germ cell population, and chose to investigate the distribution of apoptotic germ cells in the testis cords.

Germ cells are eliminated by apoptosis during embryonic and postnatal development, and in the mature testis (Billig et al., 1995; Wang et al., 1998). This process is controlled by various factors at different times, and may serve numerous purposes; for example, in tubulogenesis, regulation of the germ cell to Sertoli cell ratio and the elimination of defective meiotic products (Coucouvanis and Martin, 1995; Kierszenbaum, 2001; Print and Loveland, 2000; Rodriguez et al., 1997). It has also been suggested that germ cell degeneration may play a part in, or be an indicator of, the establishment of the spermatogenic wave, based on the well spaced, focal distribution of surviving cells following the extensive death of gonocytes in developing rat testis cords (Huckins and Clermont, 1968).

To determine whether germ cell apoptosis occurred randomly, or in a pattern that might be related to the gene expression cycle of the Sertoli cells, we labelled apoptotic cells in sections of P7 CBA testes by TUNEL, and probed the adjacent serial sections by in situ hybridisation for galectin 1 mRNA. Discrete clusters of TUNEL-positive germ cells were distributed at intervals throughout the testis cords, as illustrated in Fig. 6B. Preliminary experiments showed that most of the TUNEL-positive germ cells occurred in segments of testis cord in which the galectin 1 in situ hybridisation signal was very weak. However, it was also apparent that weakly expressing cord segments were relatively common in the testis sections. Therefore, to establish whether there was a significant correlation, we performed TUNEL on individual sections from two separate regions of two different testes, and treated the adjacent serial sections by galectin 1 in situ hybridisation and immunostaining (Fig. 6A-C).

All cross sections of testis cord in each of the testis sections were examined by light microscopy for the presence of TUNEL-positive germ cells, the intensity of in situ hybridisation signal, or immunostaining. The level of galectin 1 mRNA or protein in each individual section of cord was classified as either high, medium or low, relative to the other cords in the same testis section (see Materials and Methods). The scores were recorded on digital images of the respective sections, which were then superimposed so that the three parameters could be collated for each individual segment of testis cord. Any cord segments that were not present and intact on all three serial sections were excluded from the analysis. The four triplets of sections that were analysed contained a total of 803 intact testis cord segments. The collated TUNEL, mRNA and protein expression data are shown in Table 2.

Table 2. Segments of P7 mouse testis cords scored for TUNEL-positive germ cells, galectin 1 mRNA and protein

<table>
<thead>
<tr>
<th>Protein</th>
<th>RNA</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>30</td>
<td>197</td>
<td>130</td>
<td>253</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>93</td>
<td>81</td>
<td>49</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>17</td>
<td>145</td>
<td>223</td>
<td>342</td>
<td></td>
</tr>
</tbody>
</table>

803 segments of testis cord were scored for relative levels of galectin 1 mRNA and protein, and the presence or absence of TUNEL-positive (apoptotic) germ cells. Figures in bold show the number of cord segments in each of the nine mRNA/protein expression classes. The total number of segments in each mRNA or protein category is shown in plain type. Figures in brackets indicate the number of segments that contained TUNEL-positive germ cells.

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Clusters of apoptotic germ cells were found in 129 of the 803 testis cord segments analysed. To determine whether they were distributed randomly, or otherwise, with respect to galectin 1 expression, we compared the number of testis cord segments that were TUNEL positive in each of the score categories for galectin 1 mRNA (high, medium and low), with the numbers that would be expected in a random distribution. If apoptotic clusters were scattered randomly through the cords, the number falling within each mRNA score category would be expected to be proportional to the size of that category. Thus, for example, since 54% (435 out of 803) of cord segments were in the low mRNA category (see Table 2), this category would be expected to include 54% of the total number of TUNEL-positive segments.

Table 3A shows the number of TUNEL-positive segments that were observed in each mRNA category, expressed as a percentage of the total (n=129), versus the percentage expected in a random distribution, based on the size of the category (also see Table 2). A higher proportion of the TUNEL-positive segments than expected (67% versus 54%) were found in the low mRNA category, with correspondingly lower numbers in the medium and high categories. The divergence from the expected (random) distribution was shown to be statistically significant by χ² analysis (Σχ²=8.78; P=0.01-0.02). This demonstrates that the spatial distribution of TUNEL-positive germ cells in the testis cords was non-random, and was skewed in favour of regions with low galectin 1 mRNA expression. It also suggests that the timing of germ cell apoptosis is linked to the temporal cycle of the neighbouring Sertoli cells.

To investigate this further, we examined the immunohistochemical staining data from the same set of cord segments. This aspect of the analysis revealed an important feature of galectin 1 expression in the P7 testis. Many of the segments of testis cord that contained high levels of galectin 1 mRNA stained relatively weakly with galectin 1 antibody, and vice versa (Fig. 6A-C; also see Fig. 4A,B). This indicates a time lag between transcription and translation of galectin 1 message in the P7 Sertoli cells.

The spatial (and temporal) disparity between galectin 1 mRNA and protein expression in the P7 Sertoli cells, and the ability to distinguish major quantitative differences in their levels in situ, enabled us to examine the dynamics of the galectin 1 expression cycle in more detail. Collation of the mRNA and protein scores of individual cord segments resulted in nine different combined-score classes (Table 2, Table 4), each representing a particular galectin 1 expression state, and corresponding to one or more specific periods in the temporal
Table 3. Correlation of the incidence of germ cell apoptosis with expression of galectin 1 mRNA and protein

<table>
<thead>
<tr>
<th></th>
<th>RNA</th>
<th>Expected (%)</th>
<th>χ²=(O-E)^2/E</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>8.53</td>
<td>18.06</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>24.03</td>
<td>27.77</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>67.44</td>
<td>54.17</td>
<td>3.25</td>
<td></td>
</tr>
</tbody>
</table>

The observed number of TUNEL-positive testis cord segments in each score category for galectin 1 mRNA (A) or protein (B) is given as a percentage of the total number of TUNEL-positive segments (n=129). The figures in the expected column give the size of each score category as a percentage of the total sample (n=803). These values correspond to the percentage of the total TUNEL-positive segments that should occur in each category if apoptotic germ cells were spread randomly throughout the cords. The χ² values indicate the extent of deviation of observed (O) from expected (E) values. The significance probability (P) values show that the sums of the χ² values exceed the upper 1% and 2% points of the χ² distribution with 2 degrees of freedom in A and B, respectively.

Table 4. Modulation of germ cell apoptosis during the galectin 1 expression cycle

<table>
<thead>
<tr>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
</tr>
<tr>
<td>High</td>
<td>16.67</td>
</tr>
<tr>
<td>Medium</td>
<td>21.51</td>
</tr>
<tr>
<td>Low</td>
<td>24.62</td>
</tr>
</tbody>
</table>

The figures shown are the percentages of segments in each class that contained TUNEL-positive germ cells. Values are derived from the data in Table 2.

cycle of the Sertoli cells. In addition, the number of cord segments in each class gave an indication of the overall proportion of time that was spent in that expression state. Thus, a more detailed picture of the galectin 1 expression profile could be obtained from the degree of overlap between galectin 1 mRNA and protein in the same segments. For example, galectin 1 mRNA was detected in 46% of all segments, indicating that transcripts were expressed during approximately 46% of the temporal cycle. However, the level of protein was low during part of this time (12% of the cycle), even though protein expression was medium or high for 75% of the cycle as a whole. One straightforward interpretation of these data, assuming that the sensitivities of our detection methods for RNA and protein were broadly equivalent, would be that the lag time between the onset of galectin 1 transcription and translation in P7 Sertoli cells approximates 12% of the temporal cycle, and that galectin 1 protein persists for 40% of the cycle after the degradation of the mRNA.

The combined-score classes also revealed additional detail about the timing of germ cell apoptosis. Table 4 gives the percentage of segments in each class that were TUNEL-positive. Analysis of the TUNEL data in terms of mRNA expression alone showed that the overall frequency of apoptosis was higher during the 54% of the cycle when mRNA was not expressed (Table 3A). However, when the protein scores were also taken into account, it was evident that the highest frequency of apoptosis occurred in the 16% of the cycle when low mRNA expression coincided with high levels of protein.

DISCUSSION

In the adult testis spermatogenesis is controlled by a complex interplay of endocrine and paracrine signals acting on and within the seminiferous epithelium (Jégou and Pineau, 1995; Schlatt et al., 1997). The elaborate regulatory system within this tissue is reflected in its structural complexity, and in the coordinated progress of the entire cellular community through regular cyclical changes, in a pattern that is echoed across species. One of the least understood aspects of testis biology is how the structural and regulatory complexity of the seminiferous epithelium is generated during development. In this study, we have shown that the framework for the spatiotemporal organisation of spermatogenesis is laid down early in development, when only a few immature cell types are present. Among these, cells of the Sertoli cell lineage play a key role in the establishment of physical and functional pattern in the developing seminiferous epithelium.

Galectin 1 is a marker of cyclical function in adult Sertoli cells

Galectin 1 is widely expressed in embryonic and adult tissues, but its specific functions in vivo remain obscure. Targeted mutation of the galectin 1 gene has produced homozygous null animals that survive and reproduce normally in the laboratory environment (Poirier and Robertson, 1993). However, these mutant animals may benefit from compensatory effects of other members of the galectin family. In addition, in the case of the olfactory system, careful analysis has revealed a subtle effect of the mutation on the distribution of a specific population of cells (Puche et al., 1996). This suggests that the detection of phenotypic effects in other organ systems may depend on the design of specific assays and the availability of cell-specific markers appropriate for each tissue. So far, simple histological analysis has not revealed any abnormalities in the testes of galectin 1 mutant mice (P. M. T. and F. P. unpublished), even though the gene is expressed in a complex pattern in this tissue, indicating regulation at the levels of transcription, transcript processing and translation.

Although there is no clear indication of a defined function for galectin 1 in the testis, its expression pattern has provided new insight into the cyclical function of mature and developing Sertoli cells. In adult Sertoli cells, the level of galectin 1 mRNA is regulated in concert with the spermatogenic cycle, following a biphasic pattern in which transcripts are most abundant at stages X-XII, and fall to undetectable levels at stages VII to VIII. The onset of galectin 1 transcription is marked by a rapid accumulation of transcripts between stages VIII and IX. This feature of the expression profile may also be apparent in the cords of the prepubertal testis, in which we have observed abrupt discontinuities in the level of galectin 1 mRNA.
Anti-galectin 1 immunohistochemistry shows that the protein is present throughout the epithelial cycle, although this does not preclude stage-specific functions for galectin 1, which may depend on features that have yet to be addressed, such as the extent of expression in germ cells, post-translational modification of the protein, and its subcellular localisation. The latter issue is likely to be particularly important, as galectin 1 and other members of this family are known to change their intra-extracellular distribution according to growth conditions and cellular differentiation state (Cooper and Barondes, 1990; Harrison and Wilson, 1992; Hikita et al., 2000). The function of galectin 1 in the adult seminiferous epithelium could thus depend on its cellular and subcellular distribution at different stages of the spermatogenic cycle.

Evidence for a pre-pubertal epithelial cycle

In situ hybridisation analysis revealed that galectin 1 mRNA is expressed in a striking spatial pattern in the Sertoli cell population throughout postnatal development. In view of the cyclical expression of galectin 1 in the mature seminiferous epithelium, the highly regulated transcript pattern in early testis cords suggests that Sertoli cell function is temporally and spatially organised, or patterned, well before the cells acquire their fully differentiated adult phenotype. As we were also able to detect local variation in the transcript levels of other genes in pre-pubertal cords, we tested whether these patterns of expression could be correlated with the cyclical gene expression profile of adult Sertoli cells. By detailed comparison of the relative mRNA levels for galectin 1, Rbp1, Ctsl and Sgp2, we showed that galectin 1 and Rbp1 are maximally co-expressed in the same spatial domains within the testis cords, while Ctsl and Sgp2 are expressed in a complementary pattern. The collective expression pattern of these four marker genes in pre-pubertal (P7) cords therefore presages their eventual profile in adult seminiferous tubules, demonstrating that the rudiments of the epithelial cycle are already established in the murine Sertoli cell population by the end of the first postnatal week. Furthermore, the cyclical mode of Sertoli cell function at this stage was reflected not only in the expression of genes that show stage-specific expression during spermatogenesis, but also in the expression of Amh, which is rapidly downregulated at puberty (Münsterberg and Lovell-Badge, 1991). It will be interesting to discover how many more genes exhibit cyclical modes of expression in these cells, and whether these will include, for example, genes that are involved in general cell metabolism.

It has previously been suggested that heterogeneity in the appearance or distribution of germ cells within the testis cords, such as the physical separation of distinct spermatogonial stages in early postnatal stages (Clermont and Perey, 1957), and local clustering of germ cells that survive apoptosis in the P7 rat (Huckins and Clermont, 1968), may indicate regional organisation of early germ cell development. Early spatial patterning of the cords could also underlie asynchronous behaviour of gonocytes in the newborn testis, for example, in the timing of their exit from mitotic arrest (Sung et al., 1986), and attachment to the basement membrane (McGuinness and Orth, 1992). Clustering of germ cell activity is largely explained by the synchronous development of clonally related cells, which remain connected by intercellular cytoplasmic bridges, but in the absence of molecular markers, it has not been possible to show whether heterogeneity between these clusters is random or based on an underlying pattern. To investigate whether pre-pubertal germ cell activity could be correlated with cyclical gene expression in the Sertoli cells, we mapped the distribution of apoptotic germ cells with respect to local levels of galectin 1 mRNA and protein. This experiment demonstrates a significant correlation between the frequency of TUNEL-positive germ cell clusters and the level of galectin 1 in the surrounding Sertoli cells. The non-random spatial distribution of TUNEL-positive clusters shows that the timing of germ cell apoptosis is coupled to the cycle of gene expression that we have demonstrated in the Sertoli cell population at this stage of development. The murine epithelial cycle at 1 week of age therefore includes functional coordination of the Sertoli cell and germ cell lineages.

The epithelial cycle may be driven by post-transcriptional regulation

Our in situ studies have shown that the gene expression profile of Sertoli cells provides a convenient and biologically relevant readout of local, transient, functional states within the developing seminiferous epithelium. This offers the potential to build a detailed molecular profile of the Sertoli cell temporal cycle, and to address issues such as cellular differentiation, organisation and regulation within the epithelium. In the present case, we observed novel and unexpected features of galectin 1 expression that highlight the potential importance of post-transcriptional mechanisms in the regulation or modulation of cyclical cell function. In pre-pubertal Sertoli cells, galectin 1 message and protein both show marked cyclical regulation, but are out of step, presumably reflecting a temporal delay in the translation of each pulse of galectin 1 transcripts. Adult Sertoli cells also exhibit dramatic cyclical fluctuations in the level of galectin 1 message, but the protein is present throughout the epithelial cycle. Galectin 1 expression in Sertoli cells therefore appears to be strictly controlled at the post-transcriptional level, and differentially modulated during development. Furthermore, a strikingly similar mode of regulation in Sertoli cells has recently been reported for Rbp1 (Eskild et al., 2000).

The distinct profiles of galectin 1 mRNA and protein in pre-pubertal cords show that the temporal cycle of the Sertoli cells, even at this immature stage, is more complex than it appears from mRNA expression alone. As many other genes are probably regulated in similarly complex modes, it is possible to envisage a model in which the epithelial cycle develops, and is driven, by a combination of transcriptional and post-transcriptional regulation. In keeping with the observations of Linder et al. (Linder et al., 1991), the underlying biphasic rhythm of the cycle may be set by the synchronised transcription of two groups of genes triggered at different points in the cycle. Each pulse of transcription might then result in a cascade of combinatorial effects, owing to different gene-specific rates of transcript processing, translation, protein maturation and degradation. This type of scenario may account for the generation of multiple distinct stages in the adult epithelial cycle and the maintenance of cycle progression.

Are germ cells required to establish the epithelial cycle?

There is ample evidence that germ cells at various stages of...
differentiation participate in the regulation of spermatogenesis in the adult seminiferous epithelium (Jégou, 1993; Sharpe, 1994). However, germ cells are not required for morphogenesis of the embryonic testis cords (McLaren, 1981; Merchant, 1975), and their role, if any, in establishing the epithelial cycle, is not known. Localised expression of galectin 1 in murine testis cords suggests that functional patterning of the developing epithelium occurs at or before E18, and therefore appears to coincide with a period (from E16 to P0), in which the gonocytes are arrested in G1 of the cell cycle (Vergouwen et al., 1991). The quiescent state of the gonocytes at the time when regional differences are appearing in the Sertoli cells is that the germ cell lineage is unlikely to play an instructive role in the initial patterning process, but this issue requires further investigation.

We found the same coordinated, cyclical patterns of gene expression in P8 XXsXmSertoli cells as in the wild-type XY testis. As most of the germ cells in these mutant testes have been eliminated by the time of birth (Cattanach et al., 1971), this shows that the somatic epithelial cycle can be maintained in the absence of germ cells, at least for the first week of postnatal life. As some gonocytes may survive in the embryonic XXsXmSertoli testis until the time when the cords are first patterned, we cannot rule out the possibility that these cells could play a part in the initial patterning process. Nevertheless, our observations provide strong support for the alternative possibility that establishment of the epithelial cycle depends exclusively on the somatic cell lineages. The Sertoli cells undoubtedly play a major role in this process, but the underlying molecular mechanisms have yet to be uncovered. One intriguing possibility raised by the early regulated gene expression in these cells, is that the Sertoli cell lineage may have an intrinsic capacity for temporal cycling, thereby fulfilling one of the essential pre-requisites for the development of functional pattern in the seminiferous epithelium.

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