SPERMATOGENESIS IN THE RAT:ISOLATION OF POST-MEIOTIC GERM CELLS AND CHARACTERISATION OF STAGE-SPECIFIC GENE EXPRESSION

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ABSTRACT

Spermatogenesis is largely dependent on stage-specific gene expression that takes place within the developing germ cell, and requires the close association between each spermatogenic cell stage and the supporting Sertoli cell. The aim of my thesis was to isolate purified populations of the post-meiotic germ cell (round spermatids) from the rat, and to characterise genes expressed at this cell stage which might encode proteins targeted to the acrosome. A novel panning technique was devised which allowed the isolation of 95% pure round spermatids from adult rat testis, exceeding purities obtained by sedimentation through a BSA gradient. Subsequently, two experimental strategies were devised which included the use of antiserum raised against mammalian acrosomal membranes to 1) immunologically isolate specific polysomal mRNA, or 2) to screen a human testis cDNA expression library. Although limitations were encountered using the polysome approach, a 2 kb cDNA clone (352) was isolated following library screening, which appeared to derive from a highly conserved, novel gene. Low levels of 352 mRNA expression were evident in a range of human tissues, but were clearly up-regulated in the testis. Before 15 days of age, 352 mRNA was not apparently expressed in the rat testis. Between 15 and 22 days of age, expression was at a very low level, coinciding with the initial accumulation of pachytene spermatocytes within the seminiferous epithelium. However, in males of 27 days or older, 352 mRNA was significantly elevated. This expression was concomitant with the appearance and subsequent differentiation of stage 1-5 spermatids. Surprisingly, Northern blot analysis indicated that round spermatids were not the cell type responsible for the elevated levels of expression of 352 mRNA in 27 day old rats. It was hypothesized that this up-regulation might be the result of predominant expression in Sertoli cells, such that, the appearance of round spermatids could induce Sertoli cells to synthesize 352 mRNA, and subsequently transfer the protein product to round spermatids where it may be targeted to the acrosome.

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Figure 1. Typical germ cell differentiation during mammalian spermatogenesis (from Bellve *et al.*, 1977)

INTRODUCTION

Spermatogenesis is a remarkable developmental achievement which, not surprisingly, has attracted much interest from researchers over the years. A multitude of complex cellular and molecular events converts an undifferentiated diploid stem cell (spermatogonium) into a highly specialized haploid spermatozoon (Leblond and Clermont, 1952; Alberts *et al.*, 1989; Sharpe, 1994). Within the seminiferous epithelium of the mammalian testis, germ cells at various stages of development divide, die, migrate, adhere to and interact with adjacent cells. For example, Sertoli cell-germ cell interactions involve signals produced from cell surface molecules of adjacent cells, or diffusible molecules such as growth factors and hormones which may bind to specific receptors on their target cells to bring about a cellular and molecular response.

A central question in biology is "How are specialized cells from less specialized ancestors generated and maintained?". In other words, the process of differentiation. Every cell, with the exception of B-lymphocytes, has the same genetic complement and so theoretically has the potential to make every gene-encoded protein. However, only specific sets of genes are switched on or off resulting in a fully differentiated cell with specialized functions. Male germ cell differentiation has been well characterised at a structural and ultrastructural level in the mouse and rat (Leblond and Clermont, 1952; Bellve, 1979; Hecht, 1986), see figure 1. Apart from being an important model for the study of mammalian cell differentiation, spermatogenesis provides a number of unique features:

1) Initiation and regulation of meiosis is restricted to germ cell differentiation. This allows the investigation of both diploid and haploid gene expression (Willison and Ashworth, 1987).

2) Intercellular communication via cytoplasmic bridges occurs between specific spermatogenic stages; germ cells therefore form a syncitium. The passage of various molecules such as proteins and messenger RNAs between the cells has been demonstrated with the use of transgenic animals (Braun *et al.*, 1989), see figure 2.

3) Gross morphological changes occur during the process of spermiogenesis, the post-meiotic shaping of the round spermatid into the distinctive spermatozoon. This involves: species-specific shaping of the sperm nucleus and condensing of the nuclear chromatin in the formation of the acrosome; the loss of cytoplasm (figure 3), and the formation of the flagellum.
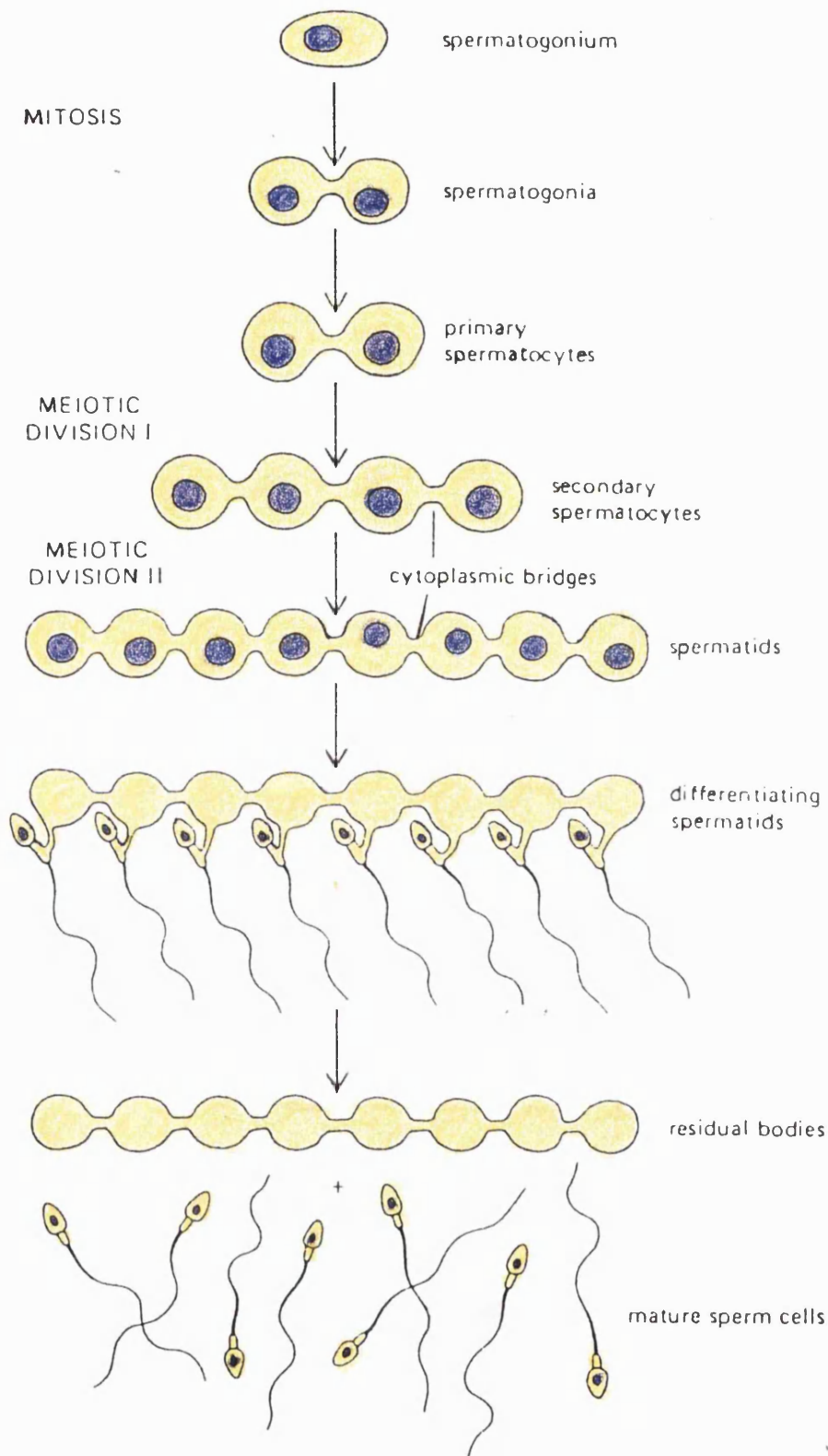
Figure 2. Syncitial organization of spermatogenic cells (from Alberts *et al.*, 1989).

4) The presence of a blood-testis barrier. This is formed as a result of tight junctions between adjacent Sertoli cells (the somatic elements of the seminiferous epithelium), and was first demonstrated in the rat by Dym and Fawcett (1970). Such junctions form a barrier that is impermeable to certain macromolecules, thus creating two distinct compartments within the seminiferous epithelium: the basal and the adluminal compartments, each having a characteristic cellular, molecular and ionic composition (figure 4).

5) Spermatogenic cells remain in close association with Sertoli cells and are extremely dependent on these somatic cells for their survival and development through cell-cell interactions (de Kretser and Kerr, 1988; Jegou, 1991; Jegou *et al.*, 1992; Sharpe, 1994). A diagrammatic representation of a cross-section through a seminiferous tubule is illustrated in figure 4.

Each spermatogenic cycle within the seminiferous epithelium of the rat consists of 14 stages of germ cell development (Leblond and Clermont, 1952). Over a given period of time, there is a particular set of germ cell associations each at different stages of development. This feature of mammalian spermatogenesis is referred to as the spermatogenic wave (figure 5), and is described in more detail on page 20. It has been established for some time that the structure and function of Sertoli cells changes according to the stage of the spermatogenic cycle and therefore, the germ cell complement with which they are associated (Sharpe 1993; Parvinen, 1993). Moreover, these functional changes, particularly secretory function, are in accordance with the requirements of each germ cell stage and are thought to be the result of the direct influence of germ cells on Sertoli cells. Evidence of functional interactions between the Sertoli and germ cells derives from *in vitro* and *in vivo* studies and is discussed on pages 40 and 32, respectively.

For a more complete understanding of mammalian spermatogenesis, it is necessary to describe the organization of the spermatogenic cycle within the seminiferous epithelium.

In mammals, spermatogenesis begins with the reactivation at puberty, of interphase germ cells of the immature testis, to enter a repetitive cycle of mitosis. Henceforth they are known as stem cells or A_0 spermatogonia. The stem cells proliferate slowly in the basal compartment of the tubule, serving as a reservoir of stem cells from which, at intervals, spermatogonia with a distinct morphology appear: A_1 spermatogonia. Each A_1 spermatogonium undergoes a limited number of mitotic divisions depending on the species (eg. in the rat there are 6 divisions resulting in a maximum clone size of 64 cells). The morphology of the daughter cells
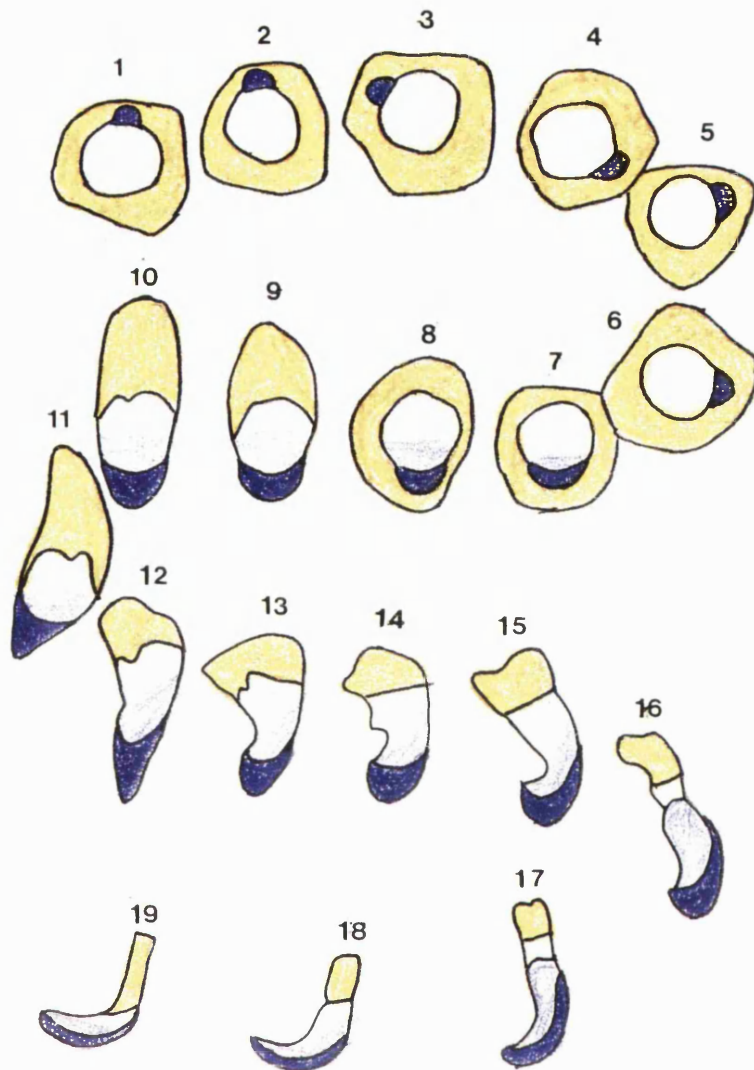
Figure 3. Spermiogenesis in the hamster (from Leblond and Clermont, 1952).

1-4   Golgi phase

5-8   Cap phase

9-15   Acrosome phase

16-19   Maturation phase
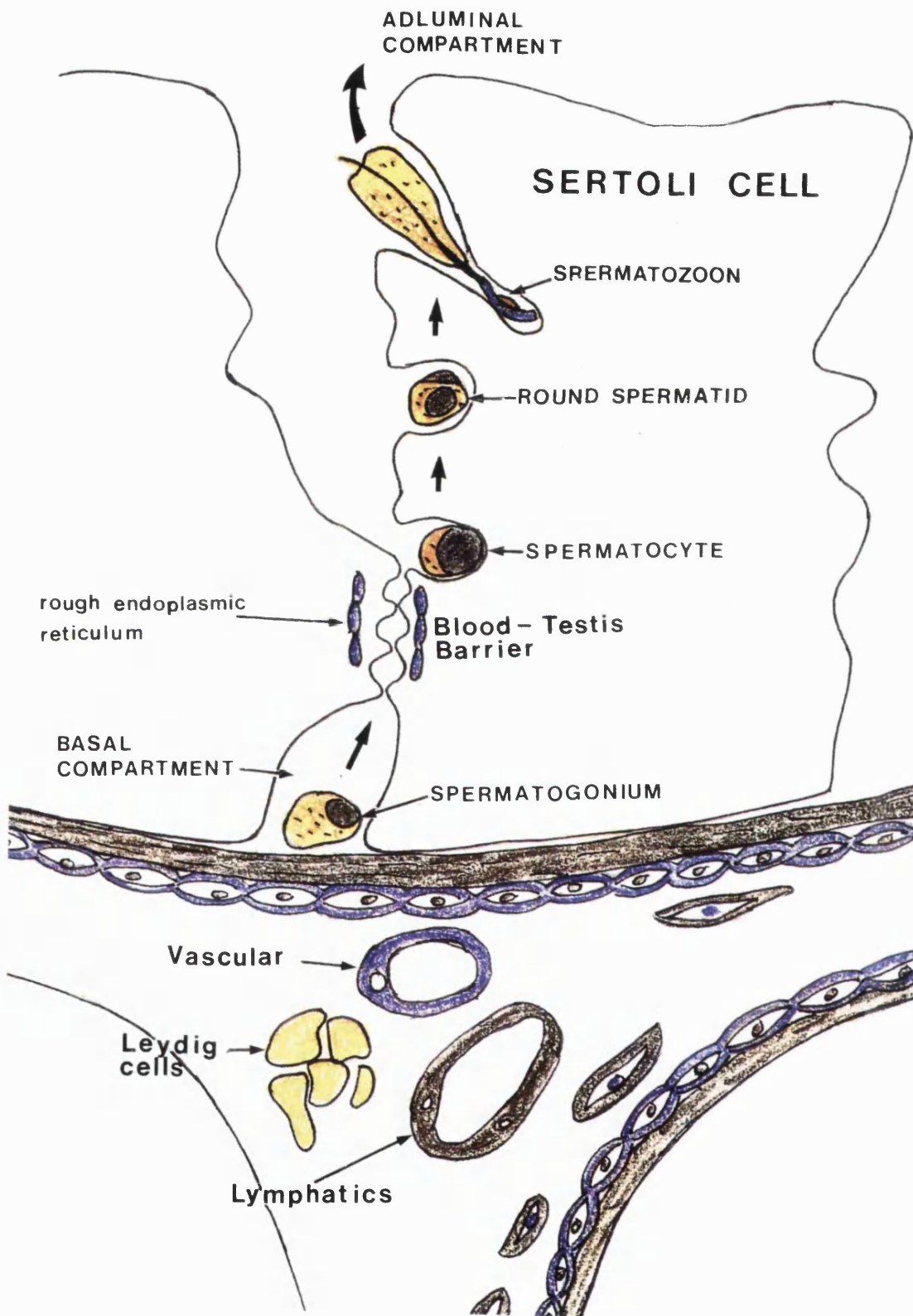
Figure 4. Cross-section through seminiferous epithelium (from Johnson and Everett, 1988).

produced at each mitotic division differs slightly from the parent cell, and are subclassified as type A_{1-4} for the first 3 divisions, and type intermediate after the fourth division. The mitotic proliferation of spermatogonia ends with the formation of type B spermatogonia, cells which are now committed to a spermatogenic pathway. The committed cells mitotically divide to become resting primary spermatocytes, the meiotic cells. The onset of puberty sees the surge of testosterone from the Leydig cells, and follicle stimulating hormone (FSH) from the pituitary, which enables resting primary spermatocytes to enter the first meiotic division to form secondary spermatocytes. These cells then undergo the second meiotic division to form haploid round (or early) spermatids. The process of spermiogenesis occurs as outlined previously, to form mature spermatozoa (figure 3). The marked morphological and biochemical changes that accompany spermiogenesis include the formation of the acrosome from a Golgi apparatus vesicle situated at the anterior end of the spermatid nucleus; the flagellum derived from a centriole; and nuclear compaction resulting from a high degree of chromatin condensation. The major implication of nuclear condensation is that transcription of genes ceases in the rat in step 8 spermatids. As a result, many unusually long-lived mRNAs are made during early spermiogenesis or even earlier at mid-pachytene when RNA synthesis is maximal (Hecht, 1990). Before the mature spermatozoa are released from the Sertoli cell into the seminiferous tubule (spermiation), the majority of cytoplasm, now termed the residual body, is phagocytosed by the Sertoli cell and pulled down through the epithelium to the base of the Sertoli cell where it is degraded lysosomally.

Spermatogonia reside in the basal compartment of the seminiferous tubule in association with the Sertoli cell. As primary spermatocytes enter meiosis, they move away from the basement membrane and pass through the tight junctions formed between adjacent Sertoli cells that establish the blood-testis barrier. Therefore, meiosis and subsequent differentiation takes place in the adluminal compartment, exposed to different environmental factors compared to pre-meiotic germ cells. In the rat, the spermatogenic cycle takes 48 days: 12 days for stem cells to complete mitosis and become primary spermatocytes, 24 days for the completion of meiosis and the formation of round spermatids, and a further 12 days for the process of spermiogenesis and the formation of spermatozoa. Degeneration of germ cells during mitotic and meiotic phases is a natural process that occurs during sperm development. For example, cell loss in spermatogonial stages probably exceeds 75% in the rat, while degeneration of meiotic germ cells results in 4-6% less spermatids formed. For unknown reasons, such

Figure 5. The fourteen types of cellular association observed at cross-section in the rat seminiferous epithelium (from Perey *et al.*, 1961).

A, type A spermatogonia; In, intermediate type spermatogonia; B, type B spermatogonia; R, resting primary spermatocyte; L, leptotene primary spermatocytes; Z, zygotene primary spermatocytes; P, pachytene spermatocytes; Di, diakinesis of primary spermatocytes; II, secondary spermatocytes; 1-19, steps of spermiogenesis. Subscript m indicates the occurrence of mitosis.
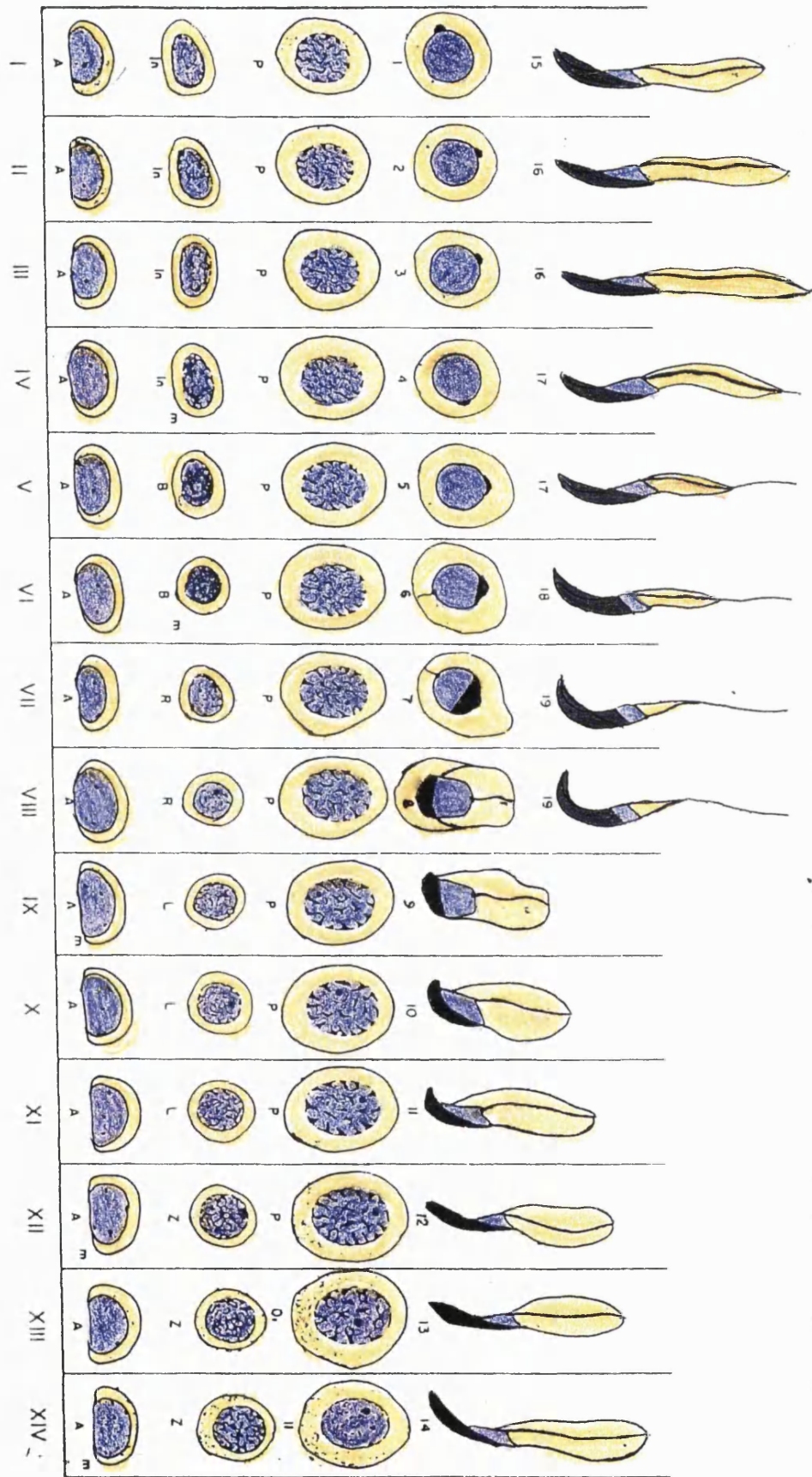
degeneration is particularly prevalent in man, such that only approximately 60% of spermatids are formed from spermatocytes (Roosen-Runge, 1973). Although it is not known what causes or controls degeneration of meiotic spermatocytes, it may represent some sort of selection process which prevents aberrations from progressing further through spermatogenesis.

Each spermatogenic cycle within the seminiferous epithelium of the rat consists of 14 stages of germ cell development, and within each stage there is a particular set of germ cell associations each at different stages of development. This particular feature of spermatogenesis is referred to as the spermatogenic wave and has been studied in beautiful detail by Perey et al. (1961). In rodents, cross sections of testis taken at intervals along a seminiferous tubule, contain different sets of cell associations which characterise the various stages of the cycle (figure 5). It seems as though certain segments of the tubule were activated first at puberty, and then a hypothetical 'activator message' spread slowly along the tubule in both directions, progressively initiating mitosis, and thus, the first cycle of clonal growth of spermatogonia. It has been established for some time that the depletion of vitamin A in adult rats leads to failure of spermatogenesis, and the disappearance of all germ cell types apart from A_0 spermatogonia, A_1 spermatogonia and a few preleptotene spermatocytes (reviewed in Sharpe, 1994). However, when these animals are supplemented with retinol, the remaining A_1 spermatogonia divide synchronously and spermatogenesis is reinitiated in all of the seminiferous tubules at more or less the same time. Therefore, only 3 or 4 stages of the seminiferous cycle exist rather than the normal 14.

The following section presents a review of developmental research that exists in the field of mammalian sperm development

REVIEW OF EXPERIMENTAL APPROACHES FOR INVESTIGATING SPERMATOGENESIS

The differentiative events of spermatogenesis have been investigated using various techniques outlined below. Each experimental design is set up to investigate a particular area of developmental research within the mammalian testis, and advantages as well as disadvantages are mentioned.

1) DIFFERENTIAL GENE EXPRESSION

There are 14 germ cell stages that have been characterized during the development of sperm, from the spermatogonial stem cell to the mature spermatozoa. These various cells differ in size and morphology, as the result of differential gene expression, most likely in response to extracellular signals. To understand the molecular processes that occur during spermatogenesis, these differentially expressed genes must be identified, as well as the proteins they encode. Obviously there will be genes expressed that are common to all cell stages, including the house-keeping genes necessary for the survival of any cell, for example, esssential enzymes or structural proteins, such as actin and tubulin. However, genes that are specific to just one cell stage, and/or expressed at different levels also exist (Willison and Ashworth, 1987).

Gene expression during spermatogenesis can be divided into two categories for simplification: i) genes expressed predominantly (that is, exclusively or almost exclusively) during spermatogenesis, and ii) switching of isotypes or isozymes.

Before going on to discuss the progress made so far, it is worth mentioning that, until recently, post-meiotic gene expression was thought not to occur in animals (Erickson, 1990). This arose due to evolutionary arguments that genetic selection should only be zygotic; gene expression in gametes could result in phenotypic differences affecting function and could potentially be subject to selection. At the same time that the first evidence for post-meiotic gene expression in plants was being obtained, over half a century ago, an opposite conclusion was being obtained in animal gametes by Muller and Settles (reference within Erickson, 1990). However, evidence that genes were being expressed post-meiotically, in spermatids, was provided by Erickson *et al.* (1980). As spermatids exist in a syncitium, connected to each

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other by large intercellular bridges, gene products can be shared making each spermatid phenotypically similar. This has now been shown in heterozygous transgenic mice (Braun *et al.*, 1989). At present, numerous haploid-expressed genes have been identified, and the list is still growing.

i) *Genes expressed predominantly during spermatogenesis*

A number of germ-cell specific genes have been identified that are thought to be important during spermatogenesis, although the actual role of many of the corresponding proteins is not clear. During spermiogenesis, the nucleoproteins undergo several dramatic changes as the spermatids differentiate to produce mature sperm. With nuclear elongation and condensation, the histones are replaced by spermatid-specific basic transition proteins, TP1 and TP2 (Grimes, 1986). The displacement of histones (in particular H4) from the chromatin of rat spermatids was found to be associated with the presence of highly acetylated forms of the protein during steps 11 and 12 of sperm development (Meistrich *et al.*, 1992). The testis-specific histone H1t gene contains a unique DNA element within the proximal promoter which has been shown to bind specifically to nuclear proteins from pachytene spermatocytes and early spermatids (Wolfe and Grimes, 1993). Therefore, this provides evidence of the onset of histone H1t transcription in pachytene spermatocytes with the appearance of the testis-specific DNA binding proteins. Another testis-specific histone gene TH2B of the rat is expressed during meiosis. Hypomethylation of the promoter region of the gene was present in male germ cells but not somatic cells, and was subsequently shown to play an important role in germ cell-specific transcription allowing the binding of transcription factors to their recognition sequences (Choi and Chae, 1991). Despite the presence of the hypomethylated TH2B gene in pre-meiotic cells, the mRNA levels are very low in these cells and rises sharply in pachytene spermatocytes. The low levels of expression appear to be the result of transcriptional repression of the gene by a pre-meiotic cell-specific protein, which binds to a site between the TATA element and the transcription initiation site of the gene (Lim and Chae, 1992).

The transition proteins are removed later from the condensing chromatin and replaced by protamines, the prodominant class of nuclear proteins in the mature spermatozoa which performs the very specialized function of DNA packaging in the sperm head (Hecht, 1986). The mouse protamine 1 gene, MP1, is translationally regulated during spermatogenesis. The

gene is initially transcribed in round spermatids where it is stored as an inactice ribonucleoprotein particle for up to a week before it is translated (Braun, 1990). From transgenic experiments carried out in male mice, it was proposed that sequence-specific RNA-binding proteins interact with the 3'-untranslated region and thereby mediate temporal translational control (Braun, 1990). In addition, transcriptional regulation of the gene was restricted to round spermatids (Zambrowicz and Palmiter, 1994). It was established that a testis-specific protein that appears after day 12 of post-natal development in male mice, interacts with a sequence within the promoter region essential for the initiation of transcription.

The proto-oncogenes *int*-1 and c-*mos* are both expressed in the germ cells as well as during embryogenesis and oncogenesis, respectively (Shackleford and Varmus, 1987; Van der Hoorn *et al.*, 1991; Goldman *et al.*, 1987). Given that oncogenes can modify the growth characteristics of cells, it is reasonable to assume that their progenitors, the proto-oncogenes, play a role in growth and possibly development. A large body of evidence supports this general hypothesis: some proto-oncogenes encode growth factors or their receptors and are implicated in cell cycle control, prevention or promotion of differentiation, and are expressed in a temporally regulated or spatially restricted manner during development (Varmus, 1987). It is therefore not surprising to find expression of these genes during spermatogenesis. *Int*-1 is expressed post-meiotically during spermatid differentiation, and also in the central nervous system of the mouse embryo. This protein may be secreted, and if so, may serve as a ligand for one or more receptors on cells in the vicinity. The c-*mos* gene is specifically transcribed in the testis and ovary of adult mice. A testis-specific 1.7 kb transcript is abundantly expressed in early spermatids (Goldman *et al.*, 1987). In rat testis, three c-*mos* transcripts of 5, 3.6 and 1.7 kb are detectable by Northern blot analysis before and after meiosis, peaking in early spermatids. However, western immuno-blot analysis reveals a 43 kD c-*mos* protein in total testis and pachytene spermatocytes, but not in post-meiotic cells, suggesting that c-*mos* is under translational as well as transcriptional regulation (Van der Hoorn *et al.*, 1991; Wolgemuth *et al.*, 1992). The function of c-*mos* in murine oocytes has been investigated by injection of c-*mos* antisense oligonucleotides (O'Keefe *et al.*, 1989). These oocytes failed to arrest at metaphase II, and often cleaved into two cells. These results suggest that c-*mos* is needed for oocyte maturation and important in subsequent stages of oocytes differentiation. More recently, Sheets *et al* (1995) demonstrated that cytoplasmic poly (A+) addition of c-

mos mRNA in *Xenopus*, controlled by specific sequences in the 3' untranslated region (which include the highly conserved sequence AAUAAA), stimulates translation of c-*mos* mRNA. This polyadenylation step is essential for the meiotic maturation of *Xenopus* oocytes, and is prevented by selective amputation of polyadenylation signals from c-*mos* mRNA. Cytoplasmic polyadenylation has also been shown to be required for c-*mos* mRNA translation and meiotic maturation in mice (Gabaner *et al.*, 1994). Unfortunately, functional studies of c-*mos* during spermatogenesis are lacking. Given that high levels of c-*mos* expression in pachytene spermatocytes, it is likely that this serine-threonine kinase plays a role in male germ cell meiosis comparable to that in oocytes (Wolgemuth *et al.*, 1992).

A novel mouse gene, meg 1, has been isolated and shown to be expressed exclusively in adult testis (Don and Wolgemuth, 1992). The pattern of expression in the testis was revealed by *in situ* hybridization analysis, as well as Northern blot analysis using RNA from purified populations of spermatogenic cells. The 0.75 kb transcript encodes a 10.8 kD protein, and was found to be most abundant in pachytene spermatocytes suggesting a role for meg 1 during germ cell differentiation, possibly during meiotic prophase.

The p53 suppressor gene was found to be expressed predominantly in the testis of adult mice (Almon *et al.*, 1993). More specifically, RNA transcripts were confined to primary spermatocytes suggesting that p53 may play a role in the meiotic process of spermatogenesis.

Another member of the mammalian endoprotease family, designated PC4, was isolated from mouse testis and found to be expressed only in the testis of 20 day old animals concomitant with the appearance of spermatids in the seminiferous epithelium (Nakayama *et al.*, 1992). Subsequently, PC4 RNA transcripts were detected predominantly in round spermatids as established from Northern blot analysis. Thus, PC4 represents an endoprotease involved in the processing of precursor proteins at a specific stage of testicular germ cell development.

A gene encoding a new zinc finger protein, Zfp-37, was isolated from an adult mouse testis cDNA library, and was found to be specifically expressed at high levels in the adult testis following Northern blot analysis (Burke and Wolgemuth, 1992). Zfp-37 RNA transcripts were also expressed at low levels during embryogenesis and in the placenta. The major testicular transcripts were 2.3 and 2.6 kb, with a 4 kb transcript present at lower levels. Northern blot and *in situ* hybridization analysis showed most abundant expression in post-meiotic germ cells indicating that this putative zinc finger protein might play a role in regulating spermiogenesis.

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Similarly, another gene encoding a protein with 14 copies of the zinc finger motif, Zfp-29, was isolated and shown to be expressed only in the testis of adult mice and during embryogenesis (Denny and Ashworth, 1991). Zfp-29 RNA transcripts were present at highest levels in round spermatids suggesting that the encoded protein may regulate particular genes expressed in post-meiotic germ cells.

The Rex-1 gene also encodes a zinc finger protein which is expressed in preimplantation embryos, trophoblast, and in adult testis where *in situ* hybridization and Northern blot analysis indicated that Rex-1 transcripts are restricted to spermatocytes (Rogers *et al.*, 1991).

The gene encoding for a POU domain protein (Tst-1) was identified in the rat testis, which was also found to be expressed in embryonic rat brain (He *et al.*,1989). The Tst-1 gene shows 94% homology to the POU domain. The POU domain in mammals contains similar sequence to the homeodomain of drosophila regulatory gene products, such as transcription factors. Whether Tst-1 has a regulatory role in spermatogenesis has yet to be established. Another POU domain factor referred to as sperm 1 (Sprm-1) has been identified by Anderson *et al.* (1993). This DNA-binding protein is expressed during a 36 to 48 hour period immediately preceding meiosis I in male germ cells, and was shown to preferentially bind to a specific variant of the classic octamer DNA-response element. These results suggest that Sprm-1 may regulate gene expression during meiosis.

Multiple transcripts of the mouse homeobox-containing gene Hox-1.4 are present at high abundance in the adult testis (Wolgemuth *et al.*, 1987). Expression of the gene occurs in male germ cells during the meiotic and post-meiotic phases, from pachytene spermatocytes to elongated spermatids, and is also present in the central nervous system during embryogenesis. More recently, *cis*-acting control elements necessary for the expression of Hox-1.4 gene were defined by Behringer *et al.* (1993), which is discussed in section 5 (i) on page 34. *Int*-1 exhibits the most striking parallel with Hox-1.4 in it's pattern of expression. A new rat gene, RT7, isolated using differential cDNA cloning procedures, is specifically expressed during spermatogenesis at very high levels in early spermatids (Van der Hoorn *et al.*, 1990). The gene encodes a putative 90 amino acid protein, whose N-terminus is predicted to fold as an amphipathic alpha helix with features resembling the leucine zipper structure found in a family of transcription factors. Additionally, the promoter sequence indicates that it contains a putative testis-specific regulatory sequence also found in protamine P1 and P2 promoters (Van der Hoorn *et al.*, 1990). Furthermore, the RT7 promoter is activated by cAMP-

responsive element modulator (CREM). The CREM gene encodes a family of transcriptional regulators (activators and repressors) that bind to promoter sequences activated by increased intracellular cAMP levels. During development of male germ cells, there is a switch in the transcripts generated by CREM as a result of alternative splicing and alternative translational initiation, such that, expression of the CREM tau activator protein is restricted to post-meiotic germ cells (Delmas *et al.*, 1993).

More recently, a cDNA (ODF27) encoding the major 27 kDa protein of rat sperm outer dense fibres was isolated following the screening of a testis λgt11 cDNA library with an anti-27 kDa ODF polyclonal antibody (Morales *et al.*, 1994). The cDNA recognized RNA transcripts of 1.2 and 1.5 kb in the rat testis first detected in round spermatids. *In situ* hybridization analysis revealed a peak in ODF27 RNA transcripts in steps 8-10 of spermiogenesis at the time transcription ceases, which remained at high levels from steps 11-15 and diminished in steps 16-18, at the time ODF protein synthesis and assembly are shown to be maximum. This provides another example of translational regulation in the testis. Furthermore, sequence analysis showed the ODF27 cDNA to be homologous to the rat gene RT7 outlined above.

The sperm-specific organelle, the acrosome, is a vesicle overlying the anterior aspect of the sperm head. It has been described as lysosomal-like because it contains many acid hydrolases (Alison and Hartree, 1970). However, it is an exocytotic vesicle that secretes it's contents upon stimulation by egg-derived factors during fertilization (Meizel, 1985). Acrosin is a serine proteinase located in the acrosome in a zymogen form, proacrosin. Studies have determined that proacrosin is first detectable by immunofluorescence in early spermatids of several species (Phi-van *et al.*, 1983; Florke-Gerloff *et al.*, 1984; Arboleda and Gerton, 1988; Kashiwabara *et al.*, 1990). However, it was later shown that proacrosin is immunodetected as early as primary spermatocytes in the human (Escalier *et al.*, 1991). In mammals, proacrosin is activated to the mature enzyme acrosin during the acrosome reaction. However, in humans it has been shown that acrosin activation follows it's surface exposure and precedes membrane fusion (the outer acrosomal membrane and the plasma membrane), that accompanies the acrosome reaction (Tesarik, 1990). This serine proteinase has long been thought to facilitate penetration, by hydrolysis, of spermatozoa through the zona pellucida. An additional function of proacrosin, in the recognition and binding of the spermatozoa to specific glycoproteins of the zona pellucida has been shown in the boar (Jones *et al.*, 1988).

As mentioned previously, acrosin is localized on both inner and outer acrosomal membranes in the mouse prior to the acrosome reaction, and is a potential egg-binding protein for ZP2, the secondary receptor present on the the zona pellucida, in order that sperm remain bound to the egg following the acrosome reaction (Wassarman, 1992).

Another acrosomal glycoprotein originally isolated from guinea pig testes is acrogranin. This cysteine-rich glycoprotein is synthesized during meiosis in pachytene spermatocytes and is found later in the developing acrosomes of spermatids and spermatozoa (Anakwe and Gerton, 1990). Immunoblotting experiments using several tissues isolated from the guinea pig suggested that acrogranin was a germ cell-specific glycoprotein. However, Northern blot analysis showed that acrogranin RNA transcripts were present in all guinea pig and mouse tissues examined (Baba et al., 1993). Furthermore, the nucleotide sequence revealed that guinea pig and mouse acrogranins were homologues of the precursor of the human and rat epithelin/granulin peptides, previously demonstrated to have growth-modulating properties. However, the role of this glycoprotein during spermatogenesis and/or fertilization has yet to be investigated.

Galactosyltransferase has been implicated as a sperm receptor in the mouse (Shaper, 1990). It is one member of a functional family of intracellular, membrane-bound enzymes that participate in the biosynthesis of carbohydrate moieties of glycoproteins or glycolipids. The 4.1 kb mRNA transcript present in spermatogonia is replaced by 2.9 kb and 3.1 kb transcripts in spermatids. Blocking the enzyme with antibodies, which has been localized on the murine sperm surface overlying the acrosome, has prevented sperm-egg binding *in vitro*. This has led to the hypothesis that the catalytic site functions in the binding of sperm to the appropriate carbohydrate substrates on the zona pellucida.

A 95 kD antigen present on the plasma membrane overlying the acrosomes of rodent and human spermatozoa, has been implicated as the primary receptor for the zona pellucida (Moore et al., 1987; Leyton and Saling, 1989).

ii) *Switching of isotypes or isozymes*

The second category of genes expressed during spermatogenesis includes those encoding proteins for which there is switching of isotypes or isozymes. PGK-2 encodes the testis-specific isozyme of phosphoglycerate kinase, which converts glucose (or fructose) to pyruvate. It is an autosomal gene expressed in all mammals. In the mouse, PGK-2 mRNA is

expressed at low levels during meiosis, but then becomes abundant in spermatids. However, translation occurs in the late spermatid. Gebara and McCarrey (1992) have investigated differences in protein-DNA interactions associated with the promoter of the PGK-2 gene, using nuclear protein extracts from adult testis cells versus somatic cells and pre-pubertal testis cells that do not express the gene. Results indicated that tissue-specific transcription of PGK-2 gene is associated with changes in protein-DNA interactions occurring within a 40 bp enhancer region. PGK-1, an X-linked gene, is expressed in all somatic cells and in pre-meiotic germ cells. It was suggested that the cessation of X-linked PGK-1 is associated with X-inactivation (Gold et al., 1983; Goto et al., 1990). In vitro experiments using a cell-free transcription system, identified a testis-specific transcription inhibitor, TIN-1, that bound to a specific region within the PGK-1 gene and subsequently inhibited transcription (Goto et al., 1991).

LDH-X (LDH-C4) is a germ-cell specific isozyme of lactate dehydrogenase, a glycolytic enzyme. The gene is first transcribed in pachytene spermatocytes but not beyond the round spermatid. However, the protein appears mid-pachytene and increases in abundance as spermatogenesis progresses to late spermatid stage where it finally becomes a component of the mature spermatozoa. There are longer LDH-X transcripts in round and late spermatids when compared to pachytene spermatocyte, and the longer polyA tail lengths may increase the stability of the transcript. In the basal compartment of the seminiferous tubule, metabolic nutrients are supplied by lymphatic and vascular systems. Therefore, pre-meiotic germ cells (spermatogonia) and Sertoli cells utilize the LDH-B isozyme. However, anaerobic conditions prevail in the adluminal compartment, so meiotic and post-meiotic germ cells express the muscle type LDH-A isozyme, and the germ cell specific LDH-X isozyme (Thomas et al., 1990; Jen et al., 1990; Alcivar et al., 1991).

Thymosin β4 has been identified as an actin-sequestering protein. A single molecular weight species of the closely related protein, thymosin β10, is present in a number of rat tissues at the RNA level, but with an additional higher molecular weight RNA evident only in adult rat testis (Lin and Morrison-Bogorad, 1991). The testis-specific thymosin mRNA differed from the ubiquitous thymosin β10 mRNA only in it's 5'-untranslated region, beginning 14 nucleotides upstream from the translation initiation codon. Results indicated that the two thymosin β10 mRNAs are transcribed from the same gene through a combination of

differential promoter utilization and alternative splicing. Both transcripts were present in pachytene spermatocytes, but only the testis-specific transcript was expressed in post-meiotic germ cells. Immunohistochemical analysis using specific antibodies showed that the testis-specific protein was present in spermatids indicating that the mRNA is translated post-meiotically.

Two isozymes of angiotensin-converting enzyme (ACE) are present in the mouse: one produced by somatic tissues, and a smaller protein synthesized by developing spermatozoa (testis ACE). The use of transgenic mice has demonstrated a testis-specific promoter for ACE which is outlined in section 5 (i) on page 33 (Langford *et al.*, 1991; Howard *et al.*, 1993). Immunohistochemistry and *in situ* hybridization were performed to determine the cell distribution of the two isoforms and their corresponding mRNAs in various tissues of male adult humans and marmosets (Sibony *et al.*, 1993). The germinal form of ACE was expressed in a stage-specific manner, in round spermatids through to spermatozoa, while the somatic form was expressed in a variety of somatic tissues.

The proto-oncogene c-*abl*, a protein-tyrosine kinase, is expressed in most mouse cells as two distinct transcripts of 5.5 and 6.5 kb. In addition to these, the testis also expresses a 4 kb transcript at very high levels in spermatid cells. This testis-specific transcript arises as a result of 3' truncation, that is, premature termination. At present there is no obvious role for c-*abl* during spermiogenesis (Oppi *et al.*, 1987; Meijer *et al.*, 1987). Two novel testis-specific c-*kit* transcripts of 3.5 and 2.3 kb, are present in post-meiotic germ cells of the mouse (Sorrentino,V. *et al.*, 1991). This proto-oncogene, in it's somatic form encodes a transmembrane protein tyrosine kinase receptor.

A unique sized heat shock protein (*hsp*70) transcript is present in the testis of human and rat, in the absence of exogenous stress. Expression is restricted to post-meiotic germ cells. The somatic forms, 3.5 and 2.4 kb are also present but at much lower levels. As the sensitivity of mammalian germ cells to increased temperature has been observed, and the germinal compartment is most readily destroyed by external stresses, developmentally regulated *hsp* expression in germinal cells may be important (Zakeri and Wolgemuth, 1987).

There are a number of other genes that encode isoforms expressed in murine testis which exhibit a specific pattern of expression during the spermatogenic cycle, including pyruvate dehydrogenase E1 alpha subunit (Takakubo and Dahl, 1992; Iannello and Dahl, 1992), a calmodulin-dependent phosphatase (Muramatsu *et al.*, 1992), multiple mRNA isoforms of the

transcription activator protein CREB (cyclic AMP responsive element binding protein) generated by alternative splicing (Ruppert *et al.*, 1992), two isotypes of cytochrome c (Hake *et al.*, 1990; Hake and Hecht, 1993), two vasopressin transcripts (Foo *et al.*, 1994), and a truncated testis-specific transcript encoded by B94, a primary response gene inducible by tumour necrosis factor-alpha, resulting from the use of an alternative polyadenylation signal. This shorter transcript was expressed at high levels exclusively in late spermatids, and immunostaining with a specific antibody revealed B94 protein to be localized to the acrosome of mature spermatozoa (Wolf *et al.*, 1994).

2) NATURALLY OCCURRING GENETIC MUTATIONS

A variety of mutant mice with genetic defects during spermatogenesis have been identified (Shackleford and Varmus, 1987; Goldman *et al.*, 1987), but for most of these mutations, neither the genes or the proteins responsible have yet been identified (Handel, 1987). The proto-oncogene c-*kit* is encoded at the white spotting (W) locus in mice. Mutations of this gene cause depletion of germ cells, some haemopoietic cells and melanocytes. Animals homozygous for the W mutation are sterile due to developmental failure of the primordial germ cells during early embryogenesis (Koshimizu *et al.*, 1992). The gene is expressed in type A spermatogonia through to type B spermatogonia and preleptotene spermatocytes (Manova *et al.*, 1990). Later work has led to the identification of the proto-oncogene as a transmembrane tyrosine kinase receptor (Sorrentino *et al.*, 1991). Additionally, the ligand protein is encoded by the Steel gene and has been detected in Sertoli cells of adult mice (Rossi *et al.*, 1991). In order to determine a functional role for the c-*kit* protein in the testis, intravenous injection of an anti- c-kit monoclonal antibody, ACK2, into adult mice caused a depletion of differentiating type A spermatogonia, while the undifferentiated type A spermatogonia were unaffected (Yoshinaga *et al.*, 1991). The antibody also blocked mitosis of differentiating type A spermatogonia in pre-pubertal animals. These results indicated c-*kit* plays an important role in the survival and/or proliferation of the differentiating type A spermatogonia.

Various substrains of C57BL mice have poor fertilization ability, for example, KE mouse strains carry genes on the Y chromosome detrimental for fertilization (Olds-Clarke, 1988). One sperm characteristic affected is abnormal shape of the sperm. The quaking gene on chromosome 17 of mutant mice affects spermatid morphology. Male mice homozygous for

the mutation are infertile due to abnormal sperm tail development as determined by electron microscopy (Chubb, 1992). Similarly, phenotypic effects of the oligotriche gene mutation on spermatogenesis in mice have been investigated (Chubb, 1992). Animals homozygous for the mutation are also infertile due to the complete absence of a sperm tail. The identification of the protein or proteins encoded by the oligotriche and quaking genes have yet to be established.

Sex reversed (Sxr) male mice have also been used for the study of spermatogenesis, for example, XO Sxr male mice have spermatogenic cells, but spermiogenesis tends to break down, with sperm rarely being formed (Cattenach et al., 1971).

The mouse t-complex situated on chromosome 17 harbours a number of genes which have an important role during spermatogenesis. The t-complex has attracted much research interest, as heterozygous +/t males transmit their t-carrying chromosome to 95% or more of their offspring, a phenomenon known as transmission ratio distortion (Lyon, 1986; Willison, 1986; Olds-Clarke, 1988). Therefore, only sperm carrying the t-complex fertilize eggs. It has been suggested that these sperm have an advantage in fertilization over wild-type sperm from the same individual. However, it may indeed be a result of the latter being dysfunctional or non-functional. A protein specified by the t-complex TCP-1, is a non-glycosylated external membrane protein. Although the protein is expressed in somatic cells, it is most abundant in testicular germ cells. Tcp1 mRNA is up-regulated during meiosis, and is translated during spermiogenesis at the spermatid stage (Willison, 1986). The protein may be essential for acrosome formation (Dudley et al., 1991). A cDNA clone with sequences homologous to Tcp-1 has been isolated, Tcp-1x, which shows a 140 bp region of homology in the 3' portion of both genes (Dudley et al., 1991). However, Northern blot analysis reveals the Tcp-1x gene to be expressed abundantly in the liver with the detection of two transcripts, but no significant homology has been found at the nucleotide level other than the Tcp-1 sequence. In addition, the gene was found to encode a 37 kD protein (Dudley et al., 1991).

The t-complex responder (Tcr) locus has a central role in the transmission ratio distortion (TRD) phenotype characteristic of t haplotypes, and interacts with other t complex genes in order to mediate this effect. A candidate responder gene, Tcp-10b, that is expressed in both meiotic and post-meiotic male germ cells has been cloned (Cebra-Thomas et al., 1991). This report showed that a change in splicing pattern of transcripts encoded by the t-allele of this gene (Tcp-10bt) occurs during sperm differentiation, and that this specific transcript could

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encode a variant polypeptide that is essential to function as the effector of TRD. Ewulonu *et al.* (1993) used transgenic mice in an attempt to identify the transcriptional regulatory region within the Tcp-10bt gene. Results showed that a 1.6 kb DNA fragment upstream of the transcription start site contains all the regulatory signals for temporal and germ cell-specific expression of this gene.

3) SPERMATOGENIC GERM CELL ABLATION *IN VIVO*

In vivo experiments to ablate spermatogenic cell types within the seminiferous epithelium have been used in order to examine the effects of germ cell loss on spermatogenesis as a result of disruption to Sertoli cell-germ cell interaction. These include complete germ cell loss or the selective loss of a particular germ cell type. The induction of bilateral cryptorchidism in adult rats led to a rapid and massive loss of germ cells which in turn caused a significant decrease in seminiferous tubule fluid (STF) production within 48 hours (Jegou *et al.*, 1983; Sharpe, 1993). This result showed the presence of germ cells is necessary for normal STF production. The depletion of pachytene spermatocytes induced in adult rats by treatment with methoxyacetic acid (MAA), caused failure of the tubular lumen to increase in size at stages VII and VIII of the cycle at three weeks after treatment, when elongate spermatids were depleted (Sharpe, 1989; Sharpe, 1993). This result suggests that STF production by Sertoli cells is probably under germ cell control, and furthermore, that elongate spermatids are most likely to modulate STF production as one of the major functions of STF is to transport spermatozoa out of the testis. In addition, loss of elongate spermatids by maturation depletion following irradiation to induce spermatogonial germ cell loss (Pineau *et al.*, 1983; Pinon-Lataillade *et al.*, 1988), or by local testicular heating (Jegou *et al.*, 1984), treatment with busulphan (Morris *et al.*, 1987), or MAA (Bartlett *et al.*, 1988), caused reduction in the apical secretion of androgen binding protein (ABP), whereas the basal secretion increased. Depletion of late spermatids also caused an increase in peripheral blood levels of FSH. Repopulation of the seminiferous epithelium with elongate spermatids coincided with a return to normal FSH levels in the blood. Most interestingly, following MAA-induced depletion of spermatocytes, earlier germ cells still present in the testis progress normally through spermatogenesis and develop into fertile sperm (Ratnaysoori and Sharpe, 1989). Such results lead to the concept of the 'selfish' germ cell. Namely, that each germ cell type modulates

functions of the Sertoli cell essential for it's own survival and development, and that absence or depletion of any other germ cell type will have little or no effect on this interaction.

4) GENETIC ABLATION OF DEVELOPING CELLS

This involves the selective destruction of specific cell types to determine the developmental importance of intercellular interactions. One particular method uses the herpes simplex virus thymidine kinase (HSV-TK) for the reversible selective destruction of specific cell types, thus allowing the opportunity to study regenerative processes (Borrelli *et al.*, 1988). HSV-TK converts certain nucleoside analogs, such as acyclovir and gangcyclovir, into conformations where they are incorporated into replicating DNA, and thereby effect cell death. Removal of the drug allows the regeneration of these cells. In one application of this method, the growth hormone gene promoter linked to HSV-TK, was found to ablate both somatotrophs (growth hormone producing cells) and lactotrophs (prolactin producing cells) following the embryonic administration of gangcyclovir, while a prolactin gene-TK construct ablated neither (Borrelli *et al.*,1989). The results indicated that the prolactin cells arise out of growth hormone progenitor cells, which do not activate prolactin gene expression until they have become post-mitotic. As the testis is made up of numerous intercellular relations, ablating one particular spermatogenic cell type might have a profound effect on the further development of that cell type.

Although transgenesis has not been used to ablate spermatogenic cell types, other methods have in fact achieved this, which are dicussed later.

5) TRANSGENIC ANIMALS

In the years since the first transgenic mice were reported, the technique has become a widely used method for the analysis of gene expression (Palmiter and Brinster, 1986). For the study of mammalian development, it is relatively difficult to screen for developmental mutants and to identify the corresponding mutations. The approaches can be divided into those that involve change or inactivation of pre-existing genes, or those that involve addition of new genetic information (Hanahan, 1989).

i) Transgenes for characterizing cis-acting control elements

The interaction between *cis*-acting DNA regulatory elements of a gene, and *trans*-acting factors, such as transcription factors, plays a prominent role in the regulation of transcription

in eukaryotic cells (Latchman, 1990). In order to examine the specificity of expression of the regulatory elements, fusion genes have been widely used in transgenic mice to direct the expression of marker proteins to selected cell types. Fusion genes containing potential mouse protamine (P1 or P2) control sequences linked to reporter genes were introduced into mice by microinjection into fertilized eggs (Hecht, 1990). The transgenic mice that were produced were analysed to assess whether the constructs contained specific regulatory elements which could direct both tissue-specific and temporal transcription in the testis. Constructs containing a 880-nucleotide 5'-flanking sequence in a 2.4 kb DNA fragment of mouse P1 (Peschon *et al.*, 1987), or an 859-nucleotide 5'-flanking sequence for P2 linked to the proto-oncogene *myc* or the SV40 T antigen (Stewart *et al.*, 1988) were tested by injection into fertilized mouse eggs. The transgenes exhibited regulated transcription in round spermatids, indicating that the 5'-flanking regions of the mouse P1 or P2 protamine genes contain the *cis*-acting elements essential for post-meiotic transcription.

To investigate the molecular control of testis-specific isozyme, angiotensin-converting enzyme (ACE), transgenic mice were generated with a construct containing a putative testis-specific ACE promoter linked to the *E.coli* reporter gene encoding beta-galactosidase (Langford *et al.*, 1991). Histochemical analysis demonstrated the co-localization of beta-galactosidase protein and endogenous ACE within elongating spermatids. Subsequently, a strong intragenic testis-specific promoter that is contained within a 698 bp fragment immediately upstream from the transcription start site of the testis ACE gene, has been isolated. More recently, Howard *et al.* (1993) have demonstrated that the specific expression of the ACE protein in spermatids results from the activation of a 91 bp promoter which contains a cAMP-responsive element (CRE) sequence that interacts with a testis-specific transactivating factor.

Using a similar approach, one *cis*-acting control element necessary for the tissue-specific expression of the homeobox-containing gene Hox-1.4 during mouse embryogenesis and in adult male germ cells, was found to be located 2 to 4 kb 5' of the transcription start site (Behringer *et al.*, 1993). Transgenic mice were generated carrying a construct containing 4 kb of 5' flanking sequence and 1 kb of structural gene fused in frame to the lacZ gene. The construct directed expression of the Hox-1.4, β-galactosidase fusion protein which paralled the pattern of expression observed in the mouse embryo and adult testis by the endogenous gene.

Other examples for the use of transgenesis to study the regulatory elements of testis-specific genes, include the rat testis-specific promoter of the heat-shock-like (Hst70) gene (Wisniewski et al., 1993), and the somatic and germ cell-specific proenkephalin promoters of the rat (Zinn et al., 1991). Results indicated that a 0.8 kb region upstream of the endogenous rat hst70 gene was responsible for the specific expression of the gene in the testis of transgenic mice, but more specifically in pachytene spermatocytes. Also, the rat and mouse proenkephalin genes contain two distinct promoters. One of these promoters is germ cell-specific and lacks TATA sequences, is GC rich, and contains multiple initiation sites.

ii) Targeted expression of growth and differentiation factors

The basic strategy is to use hybrid genes which include the growth or differentiation factor targeted to specific cells. Nerve growth factor (NGF) expression was targeted to the pancreatic islets, which are normally innervated by sensory, sympathetic, and parasympathetic neurons, resulting in selective hyper-innervation by one subtype of sympathetic neuron (Edwards et al., 1989). The other classes of peripheral neurons were unaffected. These results demonstrated that NGF can influence developmental innervation of target tissues, as well as selectivity for the type of neurons it influences. A particular growth factor could be targeted to specific spermatogenic cells and the developmental response observed. New evidence clearly demonstrates that growth factors, such as epidermal growth factor (EGF), are likely to play a vital role in the proliferation, differentiation, and maintenance of germ cells during spermatogenesis. The abolishment of circulating plasma EGF levels in adult male mice following removal of the submandibular gland, resulted in a 45% decrease of spermatozoa in the epididymis which was fully restored after replacement therapy with the growth factor (Suarez-Quain and Wilklinski, 1990). The EGF receptor protein of 170 kD is present in Leydig and Sertoli cells, but does not appear to be in germ cells. However, there may be EGF receptor-like molecules present on germ cells that were not detected by the antibody. It was suggested from the results obtained, that EGF effects occurred directly at the level of meiotic germ cells. EGF stimulates testosterone production of human Leydig cells *in vitro*. This was demonstrated by measuring testosterone levels in the culture media by radioimmunoassay following the addition of EGF (Syed et al., 1991).

Transgenic mice were generated using a synthetic gene encoding tetanus toxin light chain such that expression was targeted to the seminiferous epithelium (Eisel et al., 1993). Tetanus

toxin is a powerful neurotoxin known to inhibit neurotransmitter release, and the light chain is a metalloprotease that cleaves some members of the synaptobrevin gene family. Expression of the tetanus toxin light chain within the seminiferous tubules resulted in severely impaired spermatogenesis with the complete absence of spermatozoa. Late spermatids exhibited abnormal morphology and acrosomal distortions, and the number of Leydig cells was greatly increased. *In situ* hybridization analysis showed the toxin to act on Sertoli cells resulting in aberrant distribution of actin filaments and the presence of large vacuoles in these cells. These results demonstrated that tetanus toxin is active in non-neuronal cells and suggests an important function of the synaptobrevin gene family during the late stages of spermatogenesis.

iii) Transgenesis using insertional mutations

Various researchers have used trangenesis to observe developmental effects caused as a result of insertional mutations. For example, transgenic mice were generated using a construct containing a hematopoietic cell kinase protooncogene which was microinjected into the pronuclei of fertilized mouse embryos (Magram and Bishop, 1991). The insertion of this transgene into the genome caused the disruption of a gene or genes important in spermatogenesis which resulted in a deletion or rearrangement of DNA. The mutation was designated *Lvs* for "lacking vigorous sperm"as it resulted in dominant male sterility. Electron microscopy showed the spermatids from sterile transgenic males to have abnormally shaped nuclei.

Another example of creating insertional mutations includes the generation of transgenic mice with a sterile male recessive mutant phenotype due to atypical sperm motility as a result of aberrant axonemal structure (Merlino *et al.*, 1991). This resulted from the integration of a human epidermal growth factor (EGF) receptor cDNA driven by the chicken β-actin gene promoter. EGF receptor RNA was detected in primary spermatocytes whereas the synthesis of the protein was restricted to elongate spermatids. At spermiation the EGF receptor was sequestered in residual bodies. These results demonstrated that the transgene was integrated into and inactivated a testis-specific autosomal gene which caused male sterility.

The disadvantage of such a technique is the random process of integration into the host genome. Any one of a number of genes may be inactivated making any affected

developmental outcome difficult to interpret. There may also be pleiotropic effects resulting from non-specific inactivation.

vi) Antisense transgenics

Antisense RNA or DNA can be used to disrupt gene expression as a result of binding to the sense strand. In a particular gene, it is one strand of the DNA, usually called the sense strand, that is transcribed in the 5' to 3' direction thus synthesizing RNA transcripts. Antisense RNA or DNA refers to the complementary sequence encoded on the opposite strand and thus running in the opposite direction. This complementary sequence has the ability to hybridize to the sense strand and thereby control gene expression by reducing or abolishing the translation of the mRNA transcripts.

Erickson *et al.* (1993) reported the first use of antisense transgenesis targeted to the testis. In this study 98% inhibition of *Wnt*-1 endogenous mRNA was achieved in the testes of antisense transgenic mice. However, there appeared to be no phenotypic effects, the mice were fertile and showed normal testicular histology. This result is supported by the recent report that *swaying*, a spontaneous mutant in *Wnt*-1 allows male fertility in the few mice that survive long enough to reproduce (Erickson, 1993). However, despite it's potential, there has been limited success of antisense transgenesis in mice.

The potential use of an alternative transgenic approach in the study of spermatogenesis includes dominant interference. It is possible to disrupt the function of some protein complexes by expressing defective subunits that associate with normal subunits to produce an inactive multimeric protein. For example, a point mutation was introduced into murine pro-α1(I) collagen gene which was later established in transgenic mice (reviewed in Hanahan, 1989). This led to neonatal death and clear aberrations in development, apparently as a consequence of distorted collagen fibrils that had incorporated the mutant protein, even though expression levels of the mutant transgene was only 10% of the endogenous collagen genes. It may be possible to introduce a mutation into a germ cell protein that has already been identified, and to direct the expression of the mutated gene from a testis-specific promoter, such as the regulatory region of the spermatid-specific protamine gene.

6) IMMUNOLOGICAL TECHNIQUES

Because spermatogenesis in mammals entails highly orchestrated cellular interactions, it is probable that specific alterations in plasma membrane proteins, glycoproteins, or glycolipids play important regulatory functions during differentiation (Bellve et al., 1977). It has indeed been demonstrated that a variety of changes in germ cell surface carbohydrate moieties do occur (Fenderson et al., 1984; Millette and Scott, 1984). In many instances these alterations take place sequentially and exhibit restricted temporal distributions during spermatogenesis (O'Brien and Millette, 1986). In addition, the secretory activity of Sertoli cells varies significantly during the spermatogenic cycle (Parvinen, 1982). These somatic cells synthesize and secrete a number of glycoproteins, which include androgen-binding protein, ceruloplasmin, testicular transferrin, plasminogen activator, and a sulphated glycoprotein (SPG2). Sertoli cells also produce inhibin, a mitogenic polypeptide and a meiosis inhibiting factor. The availability of specific antibodies raised against Sertoli or spermatogenic cell plasma membranes may allow us to identify cell surface molecules important in cell adhesion, cell-cell interaction, or sperm-egg binding during the process of fertilization.

A cell surface protein specific to pachytene spermatocytes in the rat, was isolated and subsequently used to raise specific antibodies in the rat (D'Agostino and Stefanini, 1990). It was established from in vitro experiments, that the protein allowed the adhesion of pachytene spermatocytes and Sertoli cells. Further work may determine an important function of the protein in signal transduction during spermatogenesis. Immunization of male rabbits with antibodies raised against rabbit sperm membrane protein (rSMP) expressed in spermatids of the rabbit and rat, caused arrest at the spermatid stage (Wang et al., 1990). The anti-rSMP antibodies also blocked fertilization of human sperm by zona-free hamster eggs, by interacting with a human sperm protein of 72 kD. The mRNA for retinoic acid receptor-alpha has been found in Sertoli and germ cells (Kim and Griswold, 1990). A 2.7 kb transcript was expressed only in Sertoli cells, whereas a 3.4 kb transcript was expressed in both Sertoli and germ cells. In addition, two new but minor germ cell-specific mRNAs were detected primarily in pachytene spermatocytes. It may be possible to raise anti-retinoic acid-alpha antibodies once the corresponding proteins are characterized, to evaluate (at least in vitro), the effect of blocking the receptor.

With regard to events leading up to fertilisation, much interest has been directed towards identifying the molecular mechanisms involved in sperm recognition of the egg. The concept

of receptor-mediated gamete recognition and adhesion during fertilization, is widely accepted (Metz and Monroy, 1985; Moore, 1990). In recent years, considerable progress has been made in identifying and characterising egg and sperm components that are responsible for gamete interactions during fertilization. Research on the mouse egg zona pellucida (the protective coat surrounding the egg plasma membrane), has led to the isolation and identification of the primary receptor for sperm, a glycoprotein designated as ZP3 (Bleil and Wassarman 1990; Wassarman, 1990; Wassarman, 1992), as well as the cloning of the gene. Sperm bind to the zona pellucida by the plasma membrane overlying the anterior region of the sperm head. Binding is supported by sperm receptors present in the zona pellucida, primarily ZP3, and complementary egg-binding proteins present in the sperm plasma membrane. Bound sperm then undergo the acrosome reaction, a form of signal-transduced exocytosis in order for sperm to penetrate the zona pellucida and fuse with the egg plasma membrane. As a result, sperm lose the plasma membrane that adheres to the zona pellucida. To remain bound to the egg, acrosome-reacted sperm bind by their inner acrosomal membrane (exposed as a result of the acrosome reaction) to ZP2, the secondary receptor for sperm. This interaction may be mediated by a sperm protease, perhaps acrosin, which is localized on both the inner and outer acrosomal membrane prior to the acrosome reaction (Wassarman, 1992). Having reached the perivitelline space between the zona pellucida and the egg plasma membrane, the sperm fuse with the egg plasma membrane to form a zygote. Several candidates of the putative sperm receptor for the zona pellucida have been proposed. These include, galactosyltransferase in the mouse (Macek and Shur, 1988); pro-acrosin which has been shown in the boar to be a zona pellucida-binding protein (Jones *et al.*, 1988); 95 kD protein (Leyton and Saling, 1989); and 56 kD protein, which may serve as the receptor for the zona pellucida (ZP3), as well as acting as a substrate for tyrosine kinase (Bleil and Wassarman, 1990). Anti-acrosin monoclonal antibodies (MO-AKP) to the plasma membrane overlying the acrosome of human sperm, started the acrosome reaction (Tesarik *et al.*, 1990). In addition, a monoclonal antibody, 97.25, was generated in mice immunized with hamster sperm heads, and was shown to specifically block the fertilization of zona-intact hamster spermatozoa *in vitro*, but not that of zona-free oocytes (Moore *et al.*, 1985). The antibody recognized a 95 kD antigen present on the cell membrane overlying the developing acrosome of rodent and human post-meiotic germ cells (Moore *et al.*, 1987).

7) THE DEVELOPMENT OF CULTURE SYSTEMS

Because of the complexity of the spermatogenic process, the development of culture systems that extend the life span of spermatogenic cells *in vitro* is highly desirable for the study of mechanisms involved in the initiation, progression and synchrony of spermatogenesis. Over the years, various cell culture systems have been set up and these are discussed below.

Isolation and culture of Sertoli cells has facilitated the assessment of several hormone-dependent responses *in vitro*. For example, gelatinase, a 185 kD proteinase, is induced and secreted from cultured Sertoli cells in response to follicle stimulating hormone (FSH), Sang *et al.* (1990). Sertoli cells in culture secrete paracrine factors that inhibit peritubular myoid cell (PMC) proliferation of which heparin and other heparin-like glycosaminoglycans are likely candidates (Tung and Fritz, 1991). Also, the effects of interleukin-6 (IL-6), IL-2, and tumour necrosis factor alpha, on transferrin release from Sertoli cells in culture have been investigated (Boockfor and Schwarz, 1991). Results demonstrated that these cytokines act in a stage specific manner (that is, of the seminiferous epithelial cycle), inducing the release of transferrin from Sertoli cells. These culture systems, however, are relatively free of spermatogenic cells. This is not the case *in vivo*, where cell-cell interaction with spermatogenic cells has been shown likely to play a functional role in spermatogenesis. In addition, cultured Sertoli cells released from the physiological constraints that operate *in vivo*, modify their phenotypic expression and display some functional characteristics that are newly expressed (Kierszenbaum *et al.*, 1985), or enhanced *in vitro* (Lee *et al.*, 1986). For example, the transferrin gene in cultured Sertoli cells is actively being transcribed, and the mRNA transcript translated into a secretory protein product. However, this is not the case *in vivo* where little transferrin mRNA and protein is present in whole testes and isolated seminiferous tubules (Lee *et al.*, 1986). Therefore, it is most likely that Sertoli cells *in vivo* are not actively engaged in the synthesis of a testicular transferrin but instead rely mainly on plasma transferrin contributed by the liver. In contrast, Sertoli cells *in vitro* rapidly activate the transferrin gene, abundantly synthesizing Sertoli cell transferrin protein product.

To overcome the inadequacy of a single cell type, co-culture systems using Sertoli cells and germ cells have been developed. Cocultures in serum-free medium, of neonatal Sertoli cells and gonocytes have been characterized by Orth and Boehm (1990), to study the physical and functional relationship between these cells *in vitro*. When plated on matrigel, Sertoli cells rapidly attach, and gonocytes adhere to the underlying Sertoli cells. Some of these germ cells

essentially mimic their behaviour *in vivo*, by developing cytoplasmic processes and elongating during the first day of culture. Electron microscopic examination revealed the presence of desmosome-like adhesion sites and apparent gap junctions between Sertoli cells and gonocytes. A gap junction-permeant probe (Lucifer yellow), was introduced into Sertoli cells to determine whether Sertoli cells and gonocytes are functionally coupled. Their results provided the first demonstration of gap junction-mediated communication between Sertoli cells and pre-meiotic germ cells. Spermatids co-cultured with differentiated Sertoli cells maintained on a basement membrane survive for at least 48 hours. However, spermatids alone in culture die in less than 24 hours. This suggests that Sertoli-derived survival factor(s) are synthesized and/or secreted. However, the progression of spermatids through the entire spermiogenic process has yet to be demonstrated (Cameron *et al.*, 1987).

Seminiferous tubular segments can also be isolated, incubated and analyzed by biochemical means, for example, Seidl and Holstein (1990), cultured human seminiferous tubules in serum-free medium to study the role of nerve growth factor (NGF) in the testis. The tubules were mechanically isolated, and the cut edges were sealed prior to culturing. Degeneration of germ cells occurred during the culture period accompanied by disruption of the seminiferous epithelium, morphological and functional disruption of Sertoli cells and a thickening of the lamina propria. However, in the presence of foetal calf serum (FCS) and NGF, the myoid phenotype of lamina-propria cells was maintained, thickening of the tubular wall was prevented, and Sertoli cell morphology and function was stabilized. These results indicate a role for NGF in stabilizing specific functions of seminiferous tubules.

Several reports furnish a sound basis for the concept of functional interdependence of Sertoli cells and germ cells. Evidence for Sertoli cell-germ cell exchange of newly synthesized peptides has been provided by Kumari and Duraiswami (1987), following [^{35}S] methionine pulse labelling of cultured rat seminiferous tubules, and subsequent cell separation of Sertoli cell and germ cell fractions. Two-dimensional gel electrophoretic analysis using Coomassie blue staining, and fluorography was performed on labelled proteins obtained from the separated cells, and compared by superimposition. Results indicate that various peptides synthesized in the Sertoli cell compartment are transferred to the germ cell compartment of the seminiferous tubule. Shabanowitz *et al.* (1986), identified three Sertoli cell-specific secretory proteins designated S70, S45, and S35 as [^{35}S]methionine-labelled proteins which accumulated in the incubation medium of rat seminiferous tubular segments isolated from

birth up to sexual maturity. Additionally, Cyclic protein-2 (CP-2), a stage-specific secretory product of the rat seminiferous epithelium has been isolated from seminiferous tubule fluid, and Sertoli cell culture medium. This protein, a small hydrophobic glycoprotein (MW.20,000), is most likely secreted by Sertoli cells (Wright and Luzanaga, 1986). More recently, Hakovirta *et al*. (1993) have studied the effects of activin-A, inhibin-A, and transforming growth factor-β 1 (TGF-β 1) on pre-mitotic and pre-meiotic DNA synthesis during the rat seminiferous epithelial cycle. Two-millimetre rat seminiferous tubule segments were microdissected from various stages of the cycle and incubated in the presence of activin-A, inhibin-A, or TGF-β 1, for 24, 48, and 72 hours. DNA synthetic activity of specific germ cell types at specific stages of the cycle was quantified by pulse-labelling the tubules with [^{3}H]thymidine at the end of the culture period and counting by liquid scintillation. The activated cells were detected by autoradiography. Results indicated that activin-A, inhibin-A, and TGF-β 1 are involved in the regulaton of DNA synthesis during spermatogenesis. Activin-A stimulated the synthesis of intermediate spermatogonia DNA, while inhibin-A inhibited DNA synthesis in these cells. Activin-A also stimulated DNA synthesis in preleptotene spermatocytes, while TGF-β 1 had a small but significant stimulatory effect on DNA synthetic activity at stage VII.

Bicameral chambers are dual compartment chambers which have been used to culture cells on millipore filters impregnated with a reconstituted basement membrane. Sertoli cells have been cultured in this manner for the study of the blood-testis barrier. Confluent monolayers of these cells were established and were comprised of tall, columnar and highly polarized cells. Mixing of the apical and basal media is prevented by the side wall of the culture chamber (Dym *et al.*, 1987). Rat pachytene spermatocyte protein(s) stimulate Sertoli cells grown in bicameral chambers to secrete [^{35}S]methionine-labelled ceruloplasmin,sulphated glycoprotein-1,sulphated glycoprotein-2,and transferrin in a dose-dependent manner(Onoda and Djakiew, 1991). Additionally, it was demonstrated that rat round spermatid protein (RSP) was able to stimulate total protein secretion from Sertoli cells (Onoda and Djakiew, 1990).

An automated perifusion system designed for the study of rat Sertoli-spermatogenic cell co-cultures prepared on permeable substrates, is described by Kierszenbaum and Tres (1987). This system may allow the evaluation of hormone/growth factor (GF) effects on mitotic clonal expansion of spermatogonia, and the meiotic and post-meiotic clonal differentiation of

spermatogenic cells in co-culture with Sertoli cells. It consists of modular components which are in contact with medium and cells. The integrated control/pump modules allow continuous as well as pulse delivery of growth factor/hormone(s) to Sertoli-spermatogenic cell co-cultures, to mimic a more physiological enviroment than in relatively static cell cultures. Spermatogenic cell viability, proliferation, and differentiation *in vitro*, was evaluated using [^{3}H]thymidine labelling, allowing the identification of spermatogonia and preleptotene spermatocytes actively engaged in DNA synthesis. Electrophoretic analysis of [^{3}H]arginine-lysine labelled testis-specific histone variants and *in situ* hybridization using a cDNA for a histone variant (TH2B) was observed in meiotic prophase spermatocytes (Kierszenbaum and Tres, 1987).

Thus, although various testis cell cultures have been used for some time, due to the complex organization of both somatic and germ cells progress has been limited. Whereas, complete spermatogenesis had not been achieved for a mammalian species *in vitro*, hormonal induction of all stages of spermatogenesis had been achieved in the Japanese eel (Miura *et al.*, 1991). A newly developed serum-free organ culture system containing 11-ketotestosterone, a major androgen in male eels, induced the entire process of spermatogenesis within 24 days. It was also shown that human chorionic gonadotrophin (HCG) stimulates the Leydig cells to produce 11 ketotestosterone, which in turn activates the Sertoli cells leading to the completion of spermatogenesis. More recently, a cell culture system that supports the differentiation of male germ cells of the mouse through meiosis has been established (Rassoulzadegan *et al.*, 1993). A cell line, 15P-1, was found to exhibit features characteristic of Sertoli cells such as transcription of Wilm's tumour and Steel genes. This cell line was established from testicular cells of transgenic mice that express the large T protein of polyoma virus in the seminiferous epithelium, and was shown to support the meiotic and post-meiotic differentiation in cocultures of pre-meiotic cells into spermatids. In addition, testicular cells explanted from immature 9 day old animals prior to the onset of meiosis, were co-cultured with 15P-1 cells and subsequently shown to generate haploid cells with the morphology of round spermatids and which also initiated protamine transcription.

Sertoli cell-peritubular myoid cell interactions have also been investigated using a co-culture system. These testicular cell types have an intimate association throughout spermatogenesis and there is some evidence that the mesenchymally-derived peritubular cells produce paracrine factors that may regulate the differentiation and functions of the adjacent Sertoli

cells. The peritubular cells in co-culture stimulated the production of androgen binding protein (ABP) and transferrin by Sertoli cells (Tung and Fritz, 1987). Under androgen control, the peritubular cells were also shown to produce a non-mitogenic paracrine factor, termed PModS, that stimulated transferrin and ABP gene expression (Skinner, 1993). The actions of PModS are in part mediated through the induction of early event genes involving transcription factors. These factors then influence a set of genes associated with the differentiated state of the Sertoli cells, such as transferrin gene expression. In addition, both Sertoli cells and peritubular cells produce transforming growth factor alpha (TGFα), TGFβ, and insulin growth factor 1 (IGF1), Skinner (1993). However, their role in the regulation of Sertoli cell growth and function remains to be elucidated.

The differentiative events that occur during the later stages of spermatogenesis, namely post-meiotic development, are primarily dependent upon the ordered temporal expression of groups of gene products within the spermatid. However, as mentioned previously, the expression of many of these genes is highly likely to be in response to Sertoli cell-germ cell interaction. In addition, there is increasing evidence that the presence of specific proteins in the spermatid may indeed be a result of protein transfer between Sertoli cells and spermatids. The identification and expression pattern of many post-meiotic genes has, no doubt, provided a clearer understanding of development in the mammalian testis, although the functional role of many corresponding proteins have yet to be established.

The aim of my research was, firstly, to isolate purified populations of post-meiotic germ cells (round spermatids) using an immunoselection method (panning). This rapid techique has previously been used to isolate particular cell types from various tissues to very high purities (>95%). Therefore, the immunoselection of round spermatids using a specific monoclonal antibody, might allow the rapid isolation of this germ cell stage to purities exceeding those achieved by existing methods, such as, sedimentation through a BSA gradient. Secondly, the mRNA recovered from these purified spermatids would be used to isolate stage-specific genes, that is, those specifically expressed in the post-meiotic germ cell. Of particular interest, was the isolation of genes encoding proteins that may be targeted to the acrosome. This was primarily because this sperm-specific organelle plays a fundamental role during sperm-egg interaction and subsequent penetration of the zona pellucida. Subsequently, two strategies were devised, which involved the use of antiserum raised against acrosomal membranes to 1)

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immunologically isolate polysomal mRNA from purified spermatids, and 2) to screen a human testis cDNA expression library. The characterisation of a particular clone(s) obtained, might therefore provide a potential role for this gene during post-meiotic germ cell and/or in events leading up to fertilization.

CHAPTER 2

SEPARATION OF GERM CELLS FROM RAT TESTIS AND EXTRACTION
OF mRNA

INTRODUCTION

Perhaps the most widely used method for the study of spermatogenesis is the isolation and identification of differentially expressed genes, as outlined in chapter 1. This has led to the identification of genes expressed predominantly during spermatogenesis, germ cell-specific isotypes or isozymes, and also the expression pattern of various gene products during the spermatogenic cycle (Kleene *et al.*, 1983; O'Brien and Millette, 1986; Willison and Ashworth, 1987; Thomas *et al.*, 1989; Wolgemuth *et al.*, 1992). The isolation and/or expression pattern of these gene products requires pure populations of germ cells. Type A and B spermatogonia (75% pure), preleptotene spermatocytes (85% pure), and leptotene/zygotene spermatocytes (80% pure) can be isolated from the testes of pre-pubertal animals (Bellve *et al.*, 1977; Thomas *et al.*, 1990; Alcivar *et al.*, 1991), whereas meiotic and post-meiotic germ cells (pachytene spermatocytes and spermatids, respectively) are usually isolated from adult testis. The principal cell separation techniques for testicular cells employ the differential sedimentation velocities of cells moving through a stable medium, such as a bovine serum albumin (BSA) gradient, at unit gravity or by centrifugation (Meistrich, 1977; Wolgemuth *et al.*, 1985; Willison *et al.*, 1990; Blanchard *et al.*, 1991). Under optimal conditions, such methods can produce preparations containing 90% pachytene spermatocytes and 80% early spermatids but the techniques are not always reliable. In order to achieve higher purities particularly for spermatids, a second purification step must be used, such as centrifugation through a Percoll gradient (Meistrich *et al.*, 1981). Thus, the isolation of highly purified spermatids is fairly time-consuming.

The separation of highly purified round spermatids was desirable for the isolation of actively transcribed mRNA transcripts (polysomal mRNA) encoding proteins that may be targeted to the acrosome. This chapter describes the separation of pachytene spermatocytes and round spermatids from the mature rat using sedimentation through a BSA gradient at unit gravity. Subsequently, an immunoselection (panning) procedure was developed for the isolation of round spermatids, with purities (90-95%) exceeding those achieved by other methods. The panning technique used a specific monoclonal antibody (or lectin), to immunolocalise post-meiotic germ cells and enabled the rapid purification of a relatively high yield of viable cells.

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CELL SEPARATION USING BSA GRADIENT

In any cell separation method, the preparation of the single-cell suspension from a solid tissue is of critical importance. It is necessary to maximize the number of intact cells, disperse clumps of cells, and minimize reaggregation (Meistrich, 1977; Wells, 1982). For the separation of testicular cells, it is also important to separate the syncitial arrangement of spermatogenic cells, and to control the loss of cytoplasm and flagella from elongating spermatids (Meistrich, 1977).

There are various techniques available for the preparation of testicular cell suspensions. Mechanical dissociation of the testis was the first method employed (Lam *et al.*, 1970; Meistrich, 1977). Such cell suspensions contained free nuclei, many damaged cells and other debris which contaminated separated populations of spermatogenic cells. Improved methods were achieved using trypsin, EDTA (ethylene-diamine-tetra-acetate)-trypsin, and collagenase-trypsin (reviewed in Meistrich *et al.*, 1977). Trypsin digests interstitial tissue as well as damaged cells. The cell yield and cell integrity obtained using the trypsin method was reported as 35% and 98%, respectively. However, the enzyme also caused acrosomal alterations in some round spermatids as well as loss of cytoplasm, flagella, and membranes from some elongated spermatids. The EDTA-trypsin method has the advantage of producing the highest yield of intact spermatogonia and primary spermatocytes, but the cell yield is slightly lower than the trypsin method. At present, the method most widely used is the collagenase-trypsin method, established by Romrell *et al.* (1976) and later modified by Wolgemuth *et al.* (1985). Collagenase digests connective tissue allowing the dissociation of seminiferous tubules prior to trypsin treatment.

The technique to separate cell types most widely used is differential sedimentation velocity at unit gravity using the Staput apparatus (Lam *et al.*, 1970; Meistrich, 1977; Loir and Lanneau, 1977) and later using the Celsep apparatus (Wolgemuth *et al.*, 1985; Willison *et al.*, 1990), or by centrifugal elutriation (Grabske *et al.*, 1975; Meistrich, 1977; Blanchard *et al.*, 1991). These methods are primarily dependent on cell size and have successfully been used to separate spermatogonia, spermatocytes, and spermatids. The technique relies on the differential sedimentation velocities of cells moving through a uniform stable medium under the influence of a constant gravitational, or centrifugal field. Differences in cell density and shape, and the viscosity of the medium also play a role in the separation procedure. When the

density of the medium is less than that of the cell, the latter will continuously sediment in the direction of the gravitational field, until it is slowed down by a higher density. If the medium is at rest, as in sedimentation at unit gravity, the cell will eventually sediment to the bottom of the gradient chamber. However, when the flow of the medium is equal but opposite to the sedimentation of the cell, as in centrifugal elutriation, the particle will remain stationary. Therefore, pachytene spermatocytes, the largest spermatogenic cells, will sediment at a faster rate and separate out at a relatively high density medium, when compared to other spermatogenic cells.

Centrifugal elutriation was studied by Grabske *et al.*, (1975), for the separation of mouse and hamster testis cells, and has in the past been the method of choice over unit gravity techniques. Purities of 86% pachytene spermatocytes, and 81% spermatids have been achieved. Recently, human spermatogenic cell populations have been separated using this method (Blanchard *et al.*, 1991). Purities of 74% pachytene spermatocytes, and 87% round spermatids were reported. In this method cells are loaded into one separation chamber via a peristaltic pump, eliminating the need for a more cumbersome loading chamber (Grabske *et al.*, 1975; Meistrich, 1977; Loir and Lanneau, 1977). As many as 3×10^9 cells have been separated in a single run with no problems of cell aggregation. Separation of a given cell population can be obtained in less than 30 minutes. Cells are collected in smaller volumes of media, and less BSA is consumed. However, the main disadvantage is the high initial cost of equipment compared to other methods, and it has been reported that spermatid populations are less pure than those obtained by unit gravity sedimentation (Loir and Lanneau, 1977).

METHODS

Preparation of testis cell suspension

The method I used for preparing mature rat testis cell suspensions for applying to BSA gradients, is a modification of the method outlined by Wolgemuth *et al.* (1985) and is described in the appendix. Briefly, the testes from one adult rat (3-4 g) was decapsulated by removing the tunica albuginea, and chopped into small pieces. The seminiferous tubules were allowed to dissociate by incubating the tissue in dissociation buffer containing collagenase at 33^0C for 30 minutes. In order to prevent excessive cellular digestion, the seminiferous tubules were washed with fresh buffer to remove most of the collagenase. Following this, the washed tubules were incubated in dissociation buffer for

Cell Suspension In

Separation Chamber

Gradient Maker Cylinders

low density solution   high density solution

Air Bubble
Removal
Assembly

Stirrer
Bar

Inter-
Cylinder
Clamp

50

Motor Base

Peristaltic Pump

Cell Fractions Out

Stirrer Motor

Figure 6. Diagrammatic representation of the Celsep apparatus.

15 minutes to digest interstitial and damaged cells. The trypsin reaction was stopped by adding trypsin inhibitor and foetal calf serum (FCS), and the cells were dispersed with a glass pasteur pipette. Finally, the cells were recovered by centrifugation and filtered through nylon gauze to remove cell aggregates. The cells were counted using a haemocytometer.

A cell suspension prepared from one pair of testes (3-4 g) from an adult rat contained 3-4 x 10^8 cells.

Cell Separation

Sedimentation at unit gravity was the method of choice for my own research, primarily because it was relatively inexpensive to set up in comparison to the high initial cost of an elutriator. The cell separation apparatus constructed was based on the commercially available Celsep model (DuPont; figure 6).

Cell purities that have been obtained from adult mouse testis using the Celsep apparatus are: pachytene spermatocytes 90%, and early spermatids 82% (Wolgemuth *et al.*, 1985). Similar purities have been reported by Willison *et al.* (1990). Where greater purities are required, the cells can be further enriched by centrifugation through density gradients, for example, Percoll gradients (Meistrich *et al.*, 1981). The main disadvantage of this method outlined by Wells (1982), is that streaming occurs if greater than 1-2 x 10^6 cells/ml are loaded resulting in the clumping of cells. Therefore, the maximum starting sample is less than 200 x 10^6 cells. In addition, the procedure is quite time consuming.

The cell separation apparatus I used was inexpensively constructed after the Celsep design (DuPont). The separation chamber consisted of a modified 1000 ml culture flask, with an outlet at the base of the chamber for loading and unloading of the gradient. A second outlet situated at the top of the chamber, held a syringe (without the plunger) for the loading of the cell suspension and overlay. The apparatus rested on a perspex platform which could be manually operated to orient the chamber to an angle of 30° to the horizontal. The 2-4% linear gradient was formed using a gradient maker, which consists of two 1-litre cylinders, with 450 ml of either 2% or 4% BSA in dissociation buffer. While the chamber was in the tilted position, 900 ml of the gradient was loaded via a peristaltic pump set at a speed of 50 ml/minute. Approximately 100 ml of a 10% BSA cushion was loaded via the base of the chamber, thus completely filling the latter. The cell suspension was then loaded via the syringe

onto the top of the gradient at a final concentration of approximately 5×10^6 cells/ml (in 0.5% BSA), to prevent streaming. Finally, the sample was overlaid with a minimum of 25 ml of 0.2% BSA. The chamber was then oriented to the horizontal position, and sedimentation allowed to proceed over a period of 90 minutes. Once the chamber had been reoriented to the tilted position, gradient fractions (10 ml) were collected via the peristaltic pump at approximately 30 ml/minute. Cells were pelleted following centrifugation at 500 g for 10 minutes. The supernatant was discarded and the cells were resuspended in the residual BSA.

Characterization of germ cells

Cytological examination by bright field microscopy and/or staining with various agents such as haematoxylin or toluidine blue, is the method predominantly used to identify cells in the various fractions collected from the gradient (Bellve et al., 1977; Wolgemuth et al., 1985; Willison et al., 1990). Briefly, an aliquot (500 µl) from each gradient fraction was transferred to respective wells of a 24-well plate prior to centrifugation, and examined by bright field microscopy at x40 and x240 (Willison et al., 1990). Alternatively, the cells were stained with haematoxylin. This method enabled the identification of pachytene spermatocytes as a result of the characteristic staining pattern of chromatin. Cells were collected by centifugation for 5 minutes at 500 g, the supernatant was poured off, and the cells were resuspended in the residual BSA. Glass slides were coated with the resuspended cells, air-dried, and subsequently fixed in 1% glutaraldehyde solution in phosphate buffered saline (PBS) for 5 minutes. The slides were then washed gently in tap water and subsequently immersed into Mayer's haematoxylin solution for 5 minutes. Following further gentle washes in water, the slides were mounted by adding a small drop of DPX (distrene, dibutyl phthalate, and xylene) to the specimen and carefully placing a coverslip on the drop.

The methods of cell identification outlined above were not ideal for round spermatids as the visualization of the acrosomic granule, which allows positive identification of these cells, is not always possible and often depends on the orientation of the cell. The isolation of cell-type specific markers would therefore ensure a more accurate cell identification method. The availability of such markers include various monoclonal antibodies and lectins (Moore et al., 1985; Soderstrom et al., 1984). One such monoclonal antibody (18.6), binds specifically to the acrosomal region of round and elongating spermatids, and mature spermatozoa (Moore et

Fraction Number	Pachytene Spermatocytes		Early Spermatids	
	% Purity	Total Number of Cells	% Purity	Total Number of Cells
15	40	-	0	-
18	75	-	0	-
21	85	-	0	-
24	88	1.2×10^6	4	-
27	90	0.8×10^6	6	-
30	83	-	6	-
33	63	-	15	-
36	25	-	40	-
39	15	-	45	-
42	3	-	50	-
45	0	-	58	-
48	0	-	60	-
51	0	-	75	-
54	0	-	80	2×10^7
57	0	-	65	-
60	0	-	50	-
63	0	-	20	-

Figure 7. Typical distribution of pachytene spermatocytes and early spermatids from adult rat testis suspension following sedimentation through a 2-4% BSA gradient.

The total number of cells was calculated only in the purest fractions. The symbol (-) indicates that the number of cells were not calculated.

al., 1985). Cells from gradient fractions containing spermatids were coated onto glass slides and allowed to air-dry. The cells were then fixed in methanol for 30 seconds and stained by indirect immunofluorescence with acrosome-specific antibody 18.6, using fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin as second antibody (described in the appendix). The stained cells were examined by epifluorescence microscopy.

Gradient fractions (10 ml) were collected and the cell purity (%) of pachytene spermatocytes and early spermatids in each fraction was assessed by haematoxylin staining or immunofluorescent staining with acrosome-specific antibodies, respectively.

Every third fraction is presented in the table displaying % purity of the particular cell type in that fraction, and the number of cells present in the purest fractions.

Cell yield

The yield of pachytene spermatocytes and spermatids in the purest fractions obtained following cell separation, were determined by applying an aliquot (200 μl) of solution from each of the relevant fractions to a Neubauer haemocytometer as described in the appendix.

RESULTS

Cell yield and purity

A typical distribution of pachytene spermatocytes and early spermatids following sedimentation through a 2-4% BSA gradient is presented in figure 7. Due to their large size and density (diameter ranging from 12-18 μm in the mouse), the highest proportion of pachytene spermatocytes were present in fractions 18 to 30, compared to round spermatids which sedimented to higher BSA concentrations and were thus present in fractions 51 to 54. Pachytene spermatocytes were identified by their patchy condensed chromatin following haematoxylin staining (figure 8a), with purities close to 90 ± 2% in four of the fractions (usually fractions 24-27). The remaining fractions were less pure with purities below 85 ±2%. Prior to staining, the cells were examined by bright field microscopy under low and high power (figure 8a and 8c, respectively). Fractions containing round spermatids were less pure (80 ± 2%) than those containing pachytene spermatocytes, most likely contaminated with cells of comparable size. Usually only two fractions contained 80 ± 2 % spermatids (usually between fractions 51 to 54), the remaining fractions with purities below 75 ± 2%. Spermatids

were much smaller than pachytene spermatocytes as examined by bright field microscopy under low and high power (figure 8b and 8d, respectively), or following haematoxylin staining (figure 8f). These cells were identified by the staining of the acrosomic granule following immunofluorescent localization of this organelle with specific antibody 18.6 (figure 8h). However, the acrosome was not stained in a number of cells, indicating that these cells were not round spermatids. To ensure that the antibody bound specifically to the acrosome, rat testis cell suspensions were also stained with antibody 18.6. Intense staining of the acrosomes on mature spermatozoa was evident (figure 8g) but there was no evidence of staining in other cell types.

The cell yields obtained for the purest pachytene spermatocyte or spermatid fractions are presented in figure 7. The number of cells present in each of the purest pachytene spermatocyte fractions (24-27) was approximately 1×10^6. Therefore pooling the purest fractions (usually four) gave a cell yield of approximately 4×10^6, with a purity of 90%. The number of cells present in each of the purest spermatid fractions (53 and 54) was approximately 2×10^7. Usually only two of these fractions contained the highest purity of spermatids (80%), and therefore a cell yield of approximately 4×10^7.

Figure 8. Separation of spermatogenic cells from adult rat testis cell suspension by sedimentation through a BSA gradient.

Cells present in a typical pachytene spermatocyte fraction are shown at x40 (a), x200 (c), or following haematoxylin staining (e).

Cells present in a typical spermatid fraction are shown at x40 (b), x200 (d), of following haematoxylin staining (f). Positive identification of these cells was achieved by immunofluorescent staining with acrosome-specific monoclonal antibody 18.6 at x500 (h). Arrows indicate the acrosomic granule.

Immunofluorescent staining of adult rat testis cell suspension with 18.6 antibody (x500) shows specific staining of the acrosome present on spermatozoa (g).
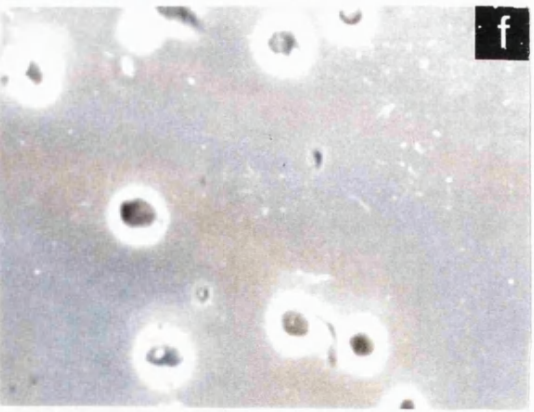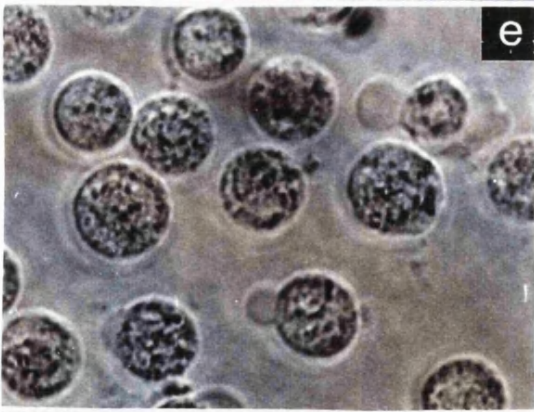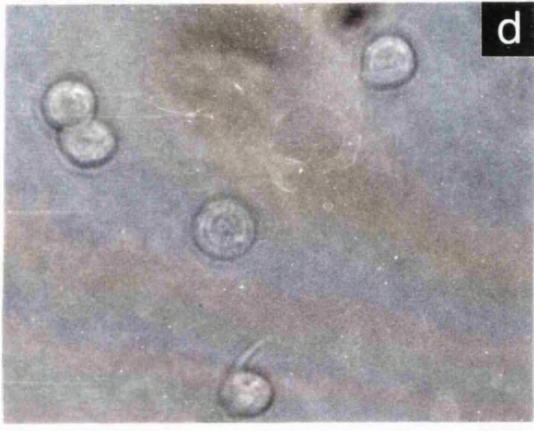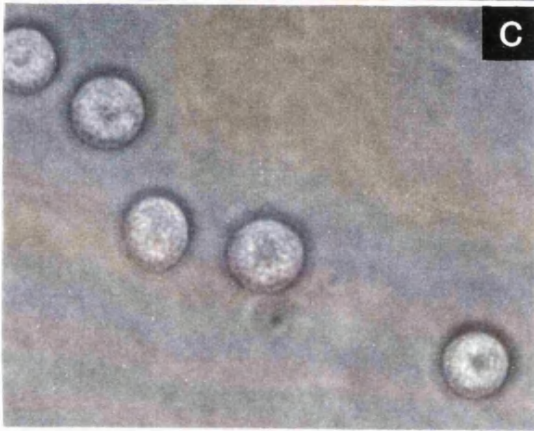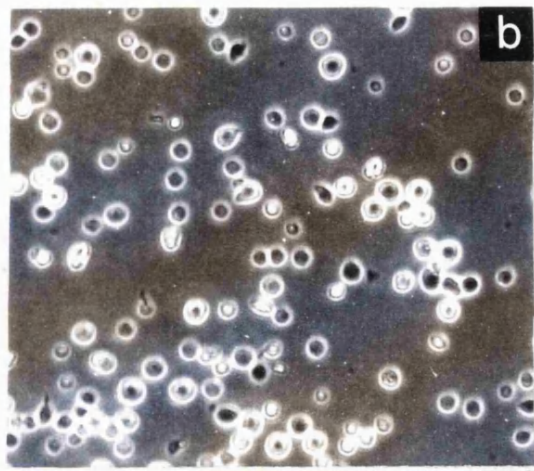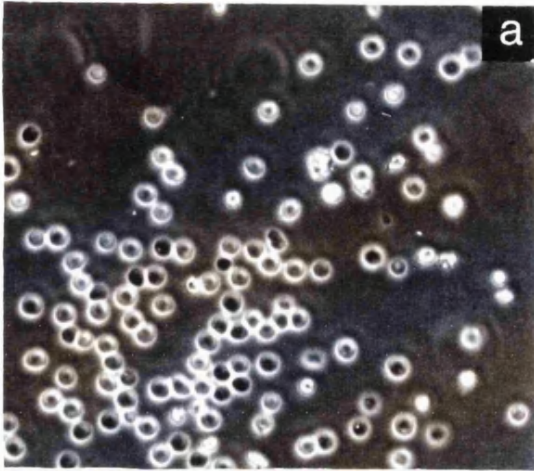
Coat Petri-dish with first
antibody (rabbit anti-mouse
immunoglobulins)

Add second antibody
(hybridoma supernatant 97.25)
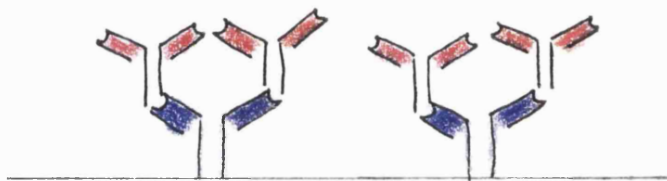
Pour on testis cell suspension
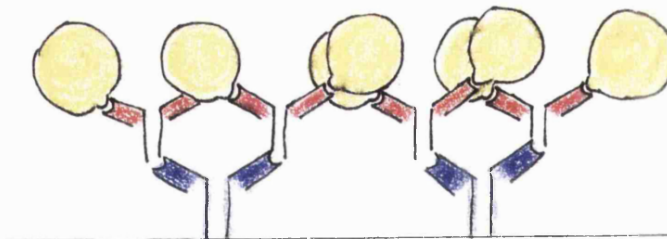
Wash off unbound cells

Figure 9. Diagrammatic representation of the immunoselection method (panning) using
monoclonal antibody as a ligand.

ISOLATION OF ROUND SPERMATIDS USING A PANNING TECHNIQUE

The isolation of purified spermatids from rodent testis is most frequently achieved by sedimentation through a BSA gradient. However, as outlined above, the purity obtained for this cell type is approximately 80%. Therefore, the presence of other cell types may compromise the accuracy of results obtained when using the spermatid population in molecular and cellular biological techniques designed for the study of sperm development (see Introduction). Moreover, the lifespan of separated germ cells in suspension is short, and separation times must be kept to a minimum. Consequently, a novel method for the isolation of round spermatids from the rat was devised in order to obtain purities which exceeded those achieved by other methods (95%), and which was less time consuming. This technique positively immunoselects spermatids from testis cell suspensions by using a specific monoclonal antibody that binds to antigens on the cell surface of spermatids (panning).

The panning technique was first established by Barker *et al.* (1975) for the isolation of cells in the immune system, but has since been adopted by others for a variety of cell types including fibroblasts (Assouline *et al.*, 1983), retinal ganglion cells (Barres *et al.*, 1988), macrophages and Schwann cells (Khan and Mudge, University College, London, personal communication). This method allows for the rapid selection of cells expressing a specific antigenic phenotype. Thus, cells are purified by positive selection using a substratum coated with an antibody which binds specifically to the cell membrane. A simplified diagram illustrating the panning method is presented in figure 9.

METHODS

Preparation of testis cell suspension

The preparation of rat testis cell suspensions initially used for panning was identical to the protocol described previously for cell separations using BSA gradients. Later, a modification of the protocol was used. This was primarily to minimize germ cell loss from enzymatic or mechanical damage, as a crucial factor in obtaining satisfactory yields of cells by panning was the initial digestion of the seminiferous tubules and the preparation of the cell suspension.

Preparation of panning dishes and panning procedure

For initial panning experiments, a specific monoclonal antibody (97.25) that recognizes a 95 kD plasma membrane protein overlying the acrosome on all stages of rodent and human post-meiotic germ cells (Moore *et al.*, 1987), or the lectin *Dilochus biflouris* (DBL) which binds specifically to cell membranes of rat early spermatids (Soderstrom *et al.*, 1987) was used, to positively select round spermatids.

Petri dishes (140 x 15 mm, Sterilin, UK) were first coated with 15 mM Tris buffer (pH 9.5) containing 10 µg/ml rabbit anti-mouse immunoglobulins for 1-2 hours at room temperature, and subsequently with antibody 97.25 hybridoma supernatant (1-2 µg/ml IgG, produced and collected as described in Appendix) for 45 minutes at 37^0C in 5% CO_2 in air, to allow the Fc portion of the monoclonal antibody to bind to the secondary antibody Fab portion. Alternatively, Petri dishes were coated solely with DBA lectin at a concentration of 4 µg/ml in Tris buffer for 1-2 hours at room temperature. The testis cell suspension (3-4 x 10^8 cells) was then poured onto the dishes (approximately 6 x 10^7 cells per dish) and incubated for 10 minutes at 37^0C to allow specific binding to take place with brief agitation after 5 minutes. The cell suspension was then removed by aspiration and the dishes washed four times with dissociation buffer (33^0C) to remove unbound cells.

Quantitation and characterization of panned cells bound to dishes

To quantify the yield of bound cells, a 1 cm^2 eyepiece graticule was used to determine the area of the field of view. Cells in 5 random fields were counted and the mean value was used to determine the total number of cells bound to the dish. This was achieved by multiplying the mean value by x100 (x10 eye-piece magnification, and x10 objective lens) to obtain the number of cells in 1 cm^2, and then by 154 cm^2 (area of a 140 mm dish).

Cells were examined by bright field microscopy while still bound to the dish, or by immunohistochemical labelling. The latter method provided confirmation of the cell type. Immunolabelling of cells was achieved following removal of cells from the dish by adding trypsin (1.25 mg/ml in dissociation buffer) for 5-10 minutes at 37^0C, as monitored by microscopy. FCS was added at a final concentration of 10% to inhibit the enzyme reaction, and the cells were pelleted for 10 minutes at 500 g and gently resuspended in a

small volume of dissociation buffer (approximately 1 ml). The cells were finally applied to poly-L-lysine (1 mg/ml) coated glass slides, fixed in methanol for 30 seconds, and stained with an acrosome-specific monoclonal antibody supernatant (18.6) by indirect immunofluorescence (Moore *et al.*, 1987), as described in the appendix.

RESULTS

Cell yield and purity

The average cell yields per 140 mm dish obtained using 97.25 monoclonal antibody and DBA lectin were 1.5×10^5 cells and 3×10^5 cells, respectively. Therefore, if 10 dishes were set up cell yields would be approximately 1.5×10^6 and 3×10^6 respectively. These cell yields were low compared to those obtained using unit gravity sedimentation (approximately 1×10^7 spermatids per gradient run). Cells immunoselected using 97.25 (figure 10a and 10c), or DBL (figure 10b and 10d) were examined by bright field microscopy while still bound to the dish, under low and high power, respectively. These cells were positively identified by immunohistochemical labelling with acrosome-specific antibody 18.6 (figure 10e and 10f, respectively).

All cells present on the slides were round spermatids as determined by positive acrosome staining, indicating that spermatid purities were approaching 100%.

Figure 10. Immunoselection of round spermatids using monoclonal antibody 97.25 or lectin DBL as the ligand.

Immunoselection of round spermatids following panning with rat testis suspension using monoclonal antibody 97.25 (a), or lectin DBL (b) as the ligand; x60. Higher magnification (x240) of these panned cells is shown in (c) and (d), respectively.

Positive identification of these cells was achieved by immunofluorescent staining with acrosome-specific monoclonal antibody 18.6 at x500 (e), or with DBL at x400 (f), following their removal by trypsinization. Immunofluorescent staining of total testis cell suspension with lectin DBL (g) shows specific staining of the cell membrane and developing acrosome of a round spermatid (x500).
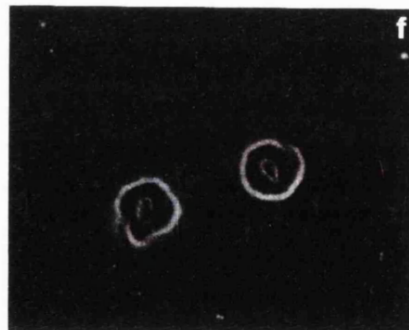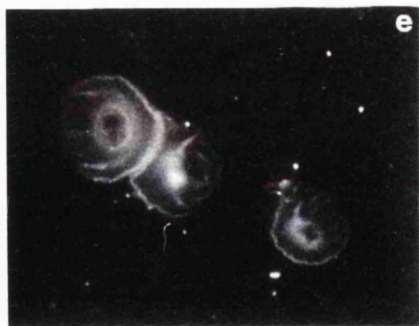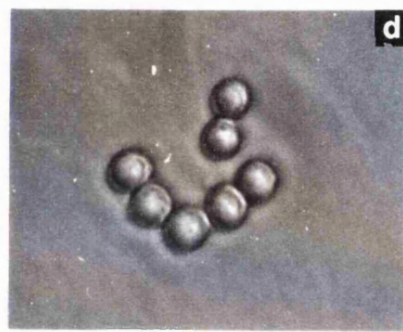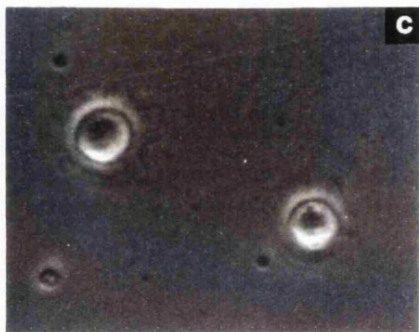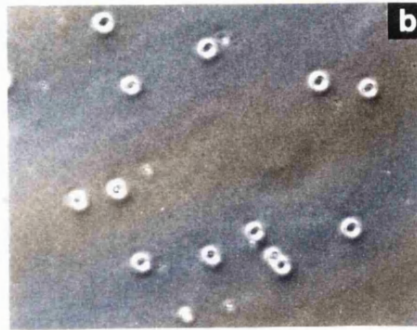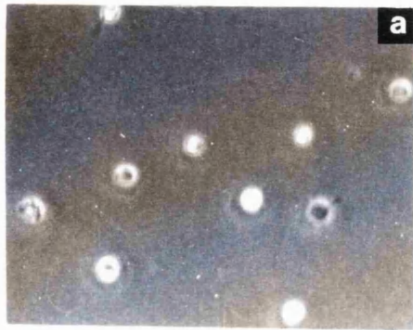
REVISED METHOD

The panning procedure was later modified to improve cell yields. This was achieved by 1) improving the testis cell suspension as outlined previously, and 2) increasing the concentration of both primary and secondary antibodies.

Cell suspension

Briefly, the testes (3-4 g) were decapsulated by removing the tunica albuginea and chopped into small pieces. The seminiferous tubules were dispersed by incubating the tissue in modified phosphate buffered saline (PBS-GL) supplemented with 5.4 mM sodium lactate and 5.6 mM glucose containing 0.5 mg/ml collagenase and 0.1 mg/ml DNase for 10 minutes at 33^0C in a shaking water bath. Trypsin was not used as it did not appear to improve the cell suspension, and in fact could lead to a reduced yield of panned cells. This may be a result of the removal of important epitopes from the cell membrane necessary for the immunoselection process. FCS was then added to a concentration of 10% to inhibit the enzymatic reaction. The suspension was centrifuged at 500 g for 5 minutes to gently pellet the tubules and resuspended by gently mixing in a final volume of 60 ml of PBS-GL containing 0.01 mg/ml DNase. A suspension of single cells was achieved by very gently pipetting using a 10 ml pipette, as it was noted that vigorous pipetting with a glass Pasteur pipette damaged spermatids and significantly reduced cell yields. The suspension was finally filtered through 80 μm nylon mesh to remove cell aggregates.

Preparation of panning dishes and panning procedure

This procedure was essentially the same as previously, except higher concentrations of primary and secondary antibodies were used. Petri dishes were first coated with 10 ml of rabbit anti-mouse immunoglobulin at a concentration of 25 μg/ml for 1-2 hours at room temperature. The dishes were then incubated with 10 mls of 97.25 supernatant collected from hybridoma cells that were grown till cell death in order to obtain concentrated monoclonal antibody (high-titre hybridoma supernatant, produced and collected as described in the appendix) for 45 minutes at 37^0C in 5% CO_2 in air. A series of dilutions of 97.25 hybridoma supernatant was also set up to assess the specificity of this monoclonal antibody for spermatids.

Control dishes were also indirectly coated with macrophage mouse monoclonal antibody (ED2), or directly with the lectin *Datura stramonium* (DSL) at a concentration of 10 µg/ml or 4 µg/ml, respectively, in 15 mM Tris buffer, pH 9.5 (10 ml). DSL has high affinity for N-acetyllactosamine residues present on many cell types (Crowley *et al.*, 1984).

The testis cell suspension (3-4 x 10^8 cells, in a final volume of 60 ml PBS-G) was poured onto 6 dishes (10 ml per dish, approximately 6.6 x 10^6 cells/ml) and incubated for 10 minutes at 37^0C with a brief agitation of each dish after 5 minutes. The unbound cells were removed as described previously.

Quantitation and characterization of panned cells bound to dishes

Once the preparation of the cell suspension had been optimized, the cell yields from at least 10 panning experiments were quantified as before. Positive identification of spermatid binding was achieved by staining cells with Periodic acid-Schiff reagent (PAS) while still bound to the Petri dish using the protocol outlined by Pearse (1968). PAS is known to stain substances containing 1-2 glycol or α-amino alcohol groups (polysaccharides), which are present in the head caps and acrosomes of spermatids, allowing the precise staining of these structures (Leblond and Clemont, 1952). Material reacting with PAS is not present in spermatogonia but gives a uniform, weakly stained spherical mass in the cytoplasm of spermatocytes. Also, the Sertoli cell contains granules throughout the cytoplasm that react with PAS. Briefly, bound cells were fixed by adding 4% paraformaldehyde in PBS to the dish for 30 minutes at room temperature. The cells were then washed four times with PBS, and 1% periodic acid in water was added for 10 minutes. Periodic acid was washed off with PBS, and Schiff reagent was added for 10 minutes and finally washed extensively with PBS. The cells were viewed using bright field microscopy using low and high power. Alternatively, cells from 97.25 antibody dishes were either trypsined off the dish and stained with acrosomal monoclonal antibody 18.6 on poly-L-lysine coated slides as described previously, or stained directly on the dish with the acrosome-specific polyclonal antibody R10, as described in the appendix.

Ligand (titre)	Mean number of cells (x 10^6)	Proportion of round spermatids
Antibody 97.25		
1/1	13.8 ± 0.15	95 ± 5
1/5	10.0 ± 0.20	95 ± 5
1/10	4.8 ± 0.20	90 ± 5
1/50	1.5 ± 0.40	50 ± 10
1/100	1.5 ± 0.50	10 ± 10
Antibody ED2		
1/1	1.5 ± 0.50	10 ± 5
Lectin DSL		
1/1	10 ± 0.50	30 ± 5
Tris buffer		
0	0	0

Figure 11. Cell yield and proportion of round spermatids bound following the panning of testicular cells with various ligands.

The results obtained from 10 panning experiments are presented in the table above. Dishes coated with monoclonal antibody 97.25 bound 95% (± SD) round spermatids. Dilution of the hybridoma supernatant to greater than 1/5 caused a decline in yield and purity.

Control dishes coated with macrophage monoclonal antibody (ED2), lectin DSL or Tris buffer bound a small proportion of round spermatids in comparison.

RESULTS

When dishes were coated with monoclonal antibody 97.25, most (90-95%) of the bound cells were spermatids (figure 11) and a relatively high yield of $1.38\pm0.15 \times 10^7$ cells per dish was obtained. Dilution of the hybridoma supernatant to 1/5 with PBS was equally effective but further dilutions caused a decline in the yield and/or purity (figure 11) indicating high specificity of 97.25 antibody for round spermatids.

Most of the cells (90-95%) panned with 97.25 antibody showed striking acrosomal staining with PAS (figure 12a and 12b), or immunofluorescent staining with acrosome-specific antibodies 18.6 (figure 13e) and R10 (figure 13a-d). Dilution of the hybridoma supernatant exceeding 1/10 caused a decline in the purity (figure 11) with an increasing proportion of cells not exhibiting acrosomal staining with PAS.

Poor cell suspensions leading to cell damage from over digestion with collagenase and/or mechanical damage during pipetting of suspension to obtain single cells, resulted in very low cell yields on the panning dishes, or the binding of spermatids of smaller size in very high yields (1×10^8 cells per dish), figure 12a (inset).

Control dishes coated with monoclonal antibody ED2 bound less than 10% spermatids (figure 11) with the majority of cells (95%) being macrophages as determined by their granular appearance and weak cytoplasmic staining with PAS (figure 12c). *Datura* lectin (DSL) bound 30% spermatids (figure 11), the remaining cells being a variety of testicular cell types including spermatocytes (figure 12d). Uncoated dishes failed to bind cells indicating the absence of non-specific binding to the Petri dish.

Cell viability

To determine cell viability, propidium iodide and carboxyfluorescein diacetate staining was performed according to the method of Harrison and Vickers (1990) for spermatozoa. Cells were removed by adding trypsin (1.25 mg/ml in PBS-GL) for 5-10 minutes at 37^0C. The trypsin was inactivated by the addition of FCS to a final concentration of 10% and the cells were gently dislodged from the bottom of the dish by pipetting the solution around the dish. The cells were then centrifuged for 10 minutes at 500 g and resuspended in the solution of fluorescent probes (Harrison and Vickers, 1990). Under epifluorescence microscopy, viable cells stained green while dead cells were red. Nearly all the cells panned with 97.25 antibody were viable (>90%).
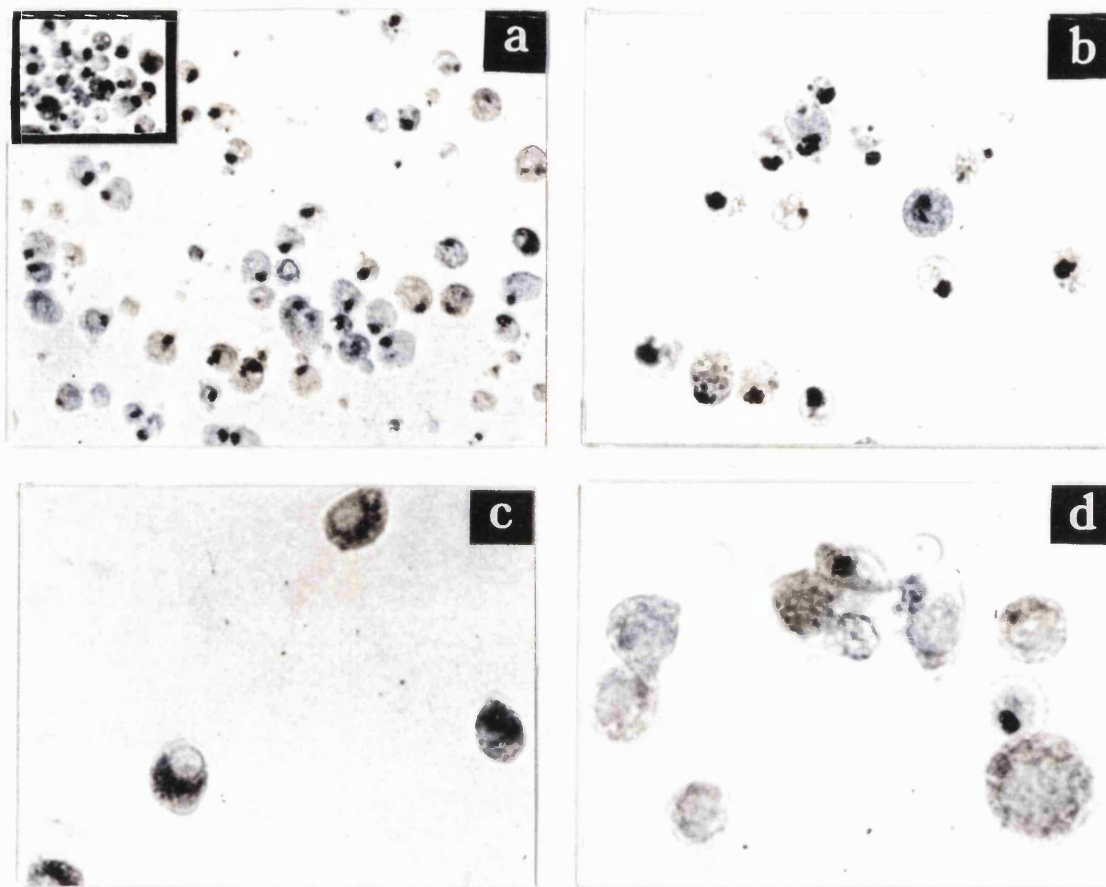
Figure 12. Immunoselection of round spermatids using monoclonal antibody 97.25 as the ligand (revised method).

Immunoselection of round spermatids following panning with rat testis suspension using monoclonal antibody 97.25 as the ligand; x120 (a) and x240 (b). Cells were stained with PAS while bound to the dish to display a dark acrosomic granule. The inset in (a) shows PAS-stained spermatids (x120) which are smaller in size as a result of overdigestion with collagenase or mechanical damage.

Control dishes showing immunoselection of testicular macrophages (c), and various testicular cell types (d) using monoclonal antibody ED2 and *Datura stramonium*, respectively (x240). Cells were also stained with PAS.
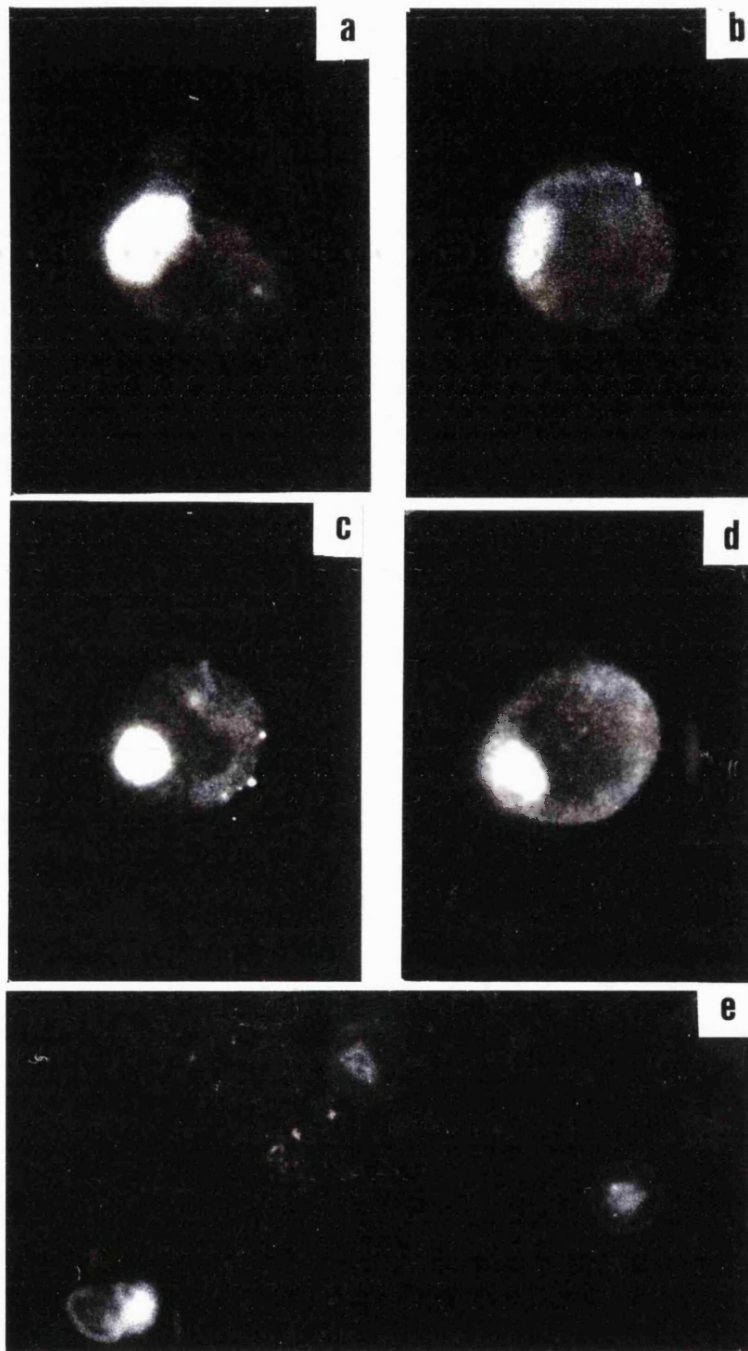
Figure 13. Immunofluorescent staining of the developing acrosome in cells immunoselected with monoclonal antibody 97.25.

Cells were either stained directly on the dish with acrosome-specific antiserum R10 (a-d) x650, or with acrosome-specific monoclonal antibody 18.6 (e) x500, following their removal by trypsinization. Second antibody was fluorescein-labelled anti-rabbit or anti-mouse IgG, respectively.

RNA EXTRACTION AND NORTHERN BLOT ANALYSIS

RNA Extraction

In order to determine if intact RNA could be obtained from panned spermatids for use in Northern blot analysis, total RNA was extracted directly from the dishes using a modification of the method by Chomczynski and Sacchi (1987).

Denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-Lauroylsarcosine, 0.1 M β-mercaptoethanol) was added to one dish (2 ml) and allowed to spread across all cells. The homogenate was scraped to the side of the dish, drawn in and out of a 23-gauge needle several times to shear the DNA, and then transferred to subsequent dishes. The final homogenate was transferred to five microfuge tubes (400 µl per tube), and the following reagents were sequentially added to each tube, mixing thoroughly following each addition: 40 µl of 2 M sodium acetate (pH 4), 400 µl phenol, and 80 µl of chloroform. Following a 15 minute incubation on ice, the tubes were centrifuged for 20 minutes at 12,000 g. The upper aqueous phase containing RNA was transferred to fresh tubes and 400 µl of isopropanol was added and incubated at -20^{0}C for at least 1 hour to precipitate the RNA. The RNA was pelleted following centrifugation for 20 minutes at 12,000 g, resuspended in 200 µl each of denaturing solution and isopropanol, and precipitated as before. The final RNA pellets were washed in 70% ethanol, air-dried, and then resuspended in a small volume (usually 4 µl) of RNase-free water (described in the appendix).

The quantity and purity of RNA was obtained by spectrophotometric analysis at 260 and 280 nm wavelengths. The 260 nm readings allows the quantitation of RNA or DNA (1 absorbance unit at 260 nm is equivalent to 40 µg RNA, or 50 µg of DNA). The total RNA yield obtained from 10^7 cells was 15-20 µg which is comparable to spermatid RNA yields obtained previously (Kleene et al., 1983). The 260/280 nm ratio of RNA samples was 1.9-2.2 indicating a high degree of purity.

Northern Blot Analysis

To measure RNA integrity, total RNA from panned spermatids (5 and 10 µg) and from whole rat testis (10 µg) was loaded onto a 1% agarose gel containing formaldehyde, and transferred to Hybond-N membrane, as described in the appendix. The RNA was fixed
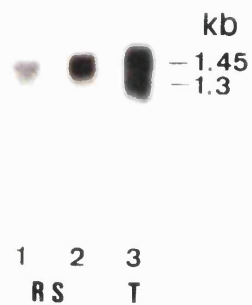
Figure 14. Expression of GAPDH mRNA in rat testis and round spermatids.

Expression of GAPDH mRNA in adult rat testis (T), and round spermatids (RS) purified by panning. Total RNA: 5 µg (1), and 10 µg (2,3).

One band of 1.45 kb is present in spermatid RNA compared to whole testis which displays two bands, the somatic GAPDH transcript of 1.3 kb and the spermatid-specific transcript of 1.45 kb.

by UV irradiation for 2 minutes and subsequently probed with ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rat cDNA, using the protocol outlined in the appendix. Hybridization took place over 16 hours at 42^{0}C, and the blot was then washed at 54^{0}C under high stringent conditions (3x 10 minutes in 2x SSC/0.1% SDS, and 30 minutes in 0.1x SSC/0.1% SDS) to prevent non-specific binding. The blot was finally exposed to X-ray film for 24 hours.

RESULTS

Northern blot analysis indicated that intact poly (A+) RNA was obtained from panned spermatids. Spermatid RNA probed with GAPDH cDNA displayed one band at approximately 1.45 kb, compared to whole testis RNA where two bands of 1.3 and 1.45 kb were detected (figure 14). Previous studies have shown that the 1.45 kb transcript is specifically confined to round and condensing spermatids (Welch et al., 1982; Mori et al., 1992).

DISCUSSION

The separation of purified populations of round spermatids was initially set up to isolate specific mRNA transcripts that were being actively translated in the post-meiotic germ cell. The ability to increase the purity of this germ cell stage using a panning technique was highly desirable to prevent non-specific transcripts from being isolated. Subsequently, Northern blot analysis using RNA extracted from purified pachytene spermatocytes and round spermatids, was performed to investigate the expression pattern of a specific mRNA transcript expressed during spermatogenesis in the pre- and post-pubertal rat, described in chapter 4.

The isolation of purified germ cells (spermatids and pachytene spermatocytes) from adult rodent testis is most frequently achieved by sedimentation at unit gravity using the Celsep apparatus (Wolgemuth et al., 1985; Willison et al, 1990), or by sedimentation using an elutriator (Meistrich, 1977; Blanchard et al., 1991). I used the former method to isolate round spermatids and pachytene spermatocytes, using an inexpensive cell separation chamber made from a one litre tissue culture flask. Germ cell purities of 90% pachytene spermatocytes, and 80% round spermatids were achieved, with cell yields of approximately 5 x 10^6, and 1-2 x 10^7, respectively, for the most pure fractions. These

results are comparable to those obtained by other researchers (Wolgemuth *et al.*, 1985; Willison *et al.*, 1990). However, to obtain higher purities of spermatids a second purification step must be used, such as centrifugation through a Percoll gradient (Meistrich *et al.*, 1981). In order to isolate round spermatids with a higher degree of purity, I devised a positive immunoselection technique (panning), a procedure which is rapid and relatively inexpensive. This panning technique immunoselects viable round spermatids from adult rat testis suspension to purities of 95%. Moreover, intact and pure RNA was extracted from these cells. The specific isolation of spermatids was due to their binding to monoclonal antibody 97.25. This antibody recognizes a 95 kD antigen on all stages of rodent and human post-meiotic germ cells (Moore *et al.*, 1987). Recent studies indicate that this antigen is the primary receptor for the zona pellucida (Burks *et al.* submitted). Usually round (rather than elongating) spermatids bound to the dishes, even though the 95 kD antigen would be present on all post-meiotic cell types. The binding capacity of round cells may be greater because there is a high density of antigen on the plasma membrane overlying the developing acrosome. In the elongated cell, antigen is more widely distributed and immunostaining less intense, suggesting that the overall antigen density is lower (Moore *et al.*, 1987). Thus, elongated cells may have been dislodged from the dish during the washing steps. Initial panning experiments using 97.25 antibody or DBA lectin, isolated low yields of round spermatids. Subsequent modifications to the preparation of the testis cell suspension, and coating dishes with higher concentrations of primary and secondary antibodies, resulted in the isolation of significantly higher cell yields, comparable to those obtained using the BSA gradient. However, it was found that different batches of DBA lectin resulted consistently in lower spermatids yields.

Northern blot analysis using GAPDH cDNA probe displayed a 1.45 kb transcript from panned round spermatids, previously shown to be expressed specifically in round and condensing spermatids (Welch *et al.*, 1992; Mori *et al.*, 1992).

A crucial factor in obtaining satisfactory yields of cells was the initial digestion of the seminiferous tubules and the preparation of the cell suspension. The main factors to be considered are 1) the batch of collagenase and the concentration of the enzyme; 2) the incubation time; and 3) the very gentle pipetting of the suspension to obtain single cells. A poor cell suspension resulting from over digestion with collagenase and/or trypsin, or

through mechanical damage, can lead to very low yields on the panning dish. It was noted that collagenase batches varied enormously with regard to their enzymatic properties, such that particular batches would cause cell damage over a relatively short incubation time of 10 minutes. The subsequent release of DNA from lysed cells caused the suspension to become sticky contributing to cell aggregation. Subsequently, cell binding on 97.25 antibody coated dishes was either significantly reduced to very few cells, or bound a very high yield of round spermatids (up to 1×10^8 cells per dish) that appeared to be smaller in size when compared to previous pannings. In the former case, the loss of epitopes from the cell membranes of spermatids, or loss of spermatids through cell lysis, was confirmed by indirect immunofluorescence staining of the same testis cell suspension with 97.25 antibody. Very few positively stained spermatids or spermatozoa were found. The binding of the smaller spermatids at high yield may be the result of loss of cytoplasmic material following collagenase digestion and/or through pipetting the suspension too vigorously with a glass Pasteur pipette. Similar observations were described by Meistrich (1977), who reported the susceptibility of elongating spermatids to loss of a discrete quantity of their cytoplasm during preparation of cell suspensions. Several observations indicated that following cytoplasmic detachment, the plasma membranes must be resealed. My observations showed the cell membranes of these smaller spermatids to be intact, following propidium iodide and carboxyfluorescein diacetate staining. Also, the integrity of RNA extracted was always high with no signs of degradation. In addition, the acrosomic granules of these cells were clearly stained with PAS.

It should be possible to extend this panning technique to other spermatogenic cells with the identification of specific cell surface antibodies. For example, specific antibodies to a cell surface protein on rat pachytene spermatocytes have been obtained by D'Agostino and Stefanini (1990). Furthermore, two-step panning procedures could be performed where antibodies are used in succession, first to deplete one particular cell type and then select for another. Therefore, if an antibody is specific for two cell types such as pachytene spermatocytes and spermatids, a first panning step using 97.25 antibody could deplete the suspension of spermatids, with the subsequent panning step using the second antibody to isolate only pachytene spermatocytes. A similar approach has been used successfully by Barres *et al.* (1988) to purify retinal ganglion cells.

CHAPTER 3

ISOLATION AND PRELIMINARY CHARACTERIZATION OF A POTENTIAL

ACROSOMAL cDNA CLONE

INTRODUCTION

The aim of this study was to isolate and characterize post-meiotic mRNA transcripts, but more specifically, those encoding proteins targeted to the acrosome and the plasma membrane overlying it. As the acrosomic granule first appears in the round spermatid, it is highly likely that a number of mRNA transcripts encoding acrosomal proteins, and the proteins themselves, are expressed at this stage, although it is clear that some acrosomal proteins are present prior to the spermatid stage (Escalier *et al.*, 1991; Anakwe and Gerton, 1990).

The isolation of differentially expressed genes may be accomplished by a number of methods, all of which are capable of detecting differences in gene expression between closely related cell types. These include plus-minus screening, subtractive hybridization, and subtracted libraries. Plus-minus screening is the simplest form of differential screening and in practice works well only for genes expressed in abundance in one of the two cell types, whereas the use of subtractive hybridization or subtracted libraries allow the isolation of cDNAs corresponding to less abundant mRNAs. Plus-minus screening has been used by a number of workers to isolate genes expressed exclusively or more abundantly in meiotic or post-meiotic male germ cells. For example, Thomas *et al.* (1989) differentially screened mouse testis and pachytene spermatocyte cDNA libraries with radiolabelled cDNA synthesized from poly (A+) RNA isolated from pachytene spermatocytes or round spermatids. Although the characterization of these genes was not described, these clones provided molecular markers for stage-specific transcription during germ cell differentiation. A similar approach used earlier by Kleene *et al.* (1983) led to the isolation of 17 cDNA clones (2.2% of clones) from a mouse testis library, which were shown to hybridize much more intensely to cDNA from round spermatids. The putative post-meiotic cDNAs were subsequently shown by DNA sequence analysis, to code for transition protein 1 (Kleene *et al.*, 1988) and two protamines (Kleene *et al.*, 1985; Yelick *et al.*, 1987), both present in high abundance. Using a similar approach, a large number of additional genes have been identified, including transition protein-2 (Kleene and Flynn, 1987) and t-complex polypeptide 1 (TCP-1: Willison *et al.*, 1986, 1989) that are temporally expressed in post-meiotic germ cells.

The acrosomal organelle is established in step 1-8 spermatids. Consequently, the genes coding for components of the organelle might be expected to be transcribed and then translated over a relatively short period in development, making it unlikely that the mRNA is stored prior to translation. As a result, the mRNA transcripts would be expected to be of lower abundance in comparison to those mentioned above. Therefore, the plus-minus screening method was not considered appropriate for this study. Another disadvantage is that a number of genes are expressed during both meiotic and post-meiotic phases of sperm development (Gold *et al.*, 1983; Wolgemuth *et al.*, 1987; Anakwe and Gerton, 1990; Escalier *et al.*, 1991). Consequently, these gene products may not be detected. An important factor was that only 80% pure round spermatids were available at this stage, by unit gravity sedimentation through BSA gradients. Therefore, 20% of the cell population were contaminants, increasing the likelihood of isolating non-specific gene products that are not expressed in the spermatid.

To avoid the potential problems outlined above, two alternative strategies were pursued in an attempt to isolate mRNA transcripts encoding proteins targeted to the acrosome:

1) the immunological isolation of specific polysomes from rat testis or round spermatids using polyclonal antibodies specific to the mammalian acrosome (Moore *et al.*, 1985). The mRNA isolated from these polysomes would be used to subsequently screen a testis cDNA library. Polysomal mRNA is being actively translated rather than stored between transcription and translation as a ribonucleoprotein. Therefore, by employing the differential screening methods previously outlined, polysomal mRNA extracted from pachytene spermatocytes and spermatids could be used to isolate genes that are translated in only one of the cell types. This would circumvent the problem of those genes that are transcribed during and following meiosis but translated only during the post-meiotic stage of spermatogenesis.

2) The second strategy used was to directly screen a human testis cDNA λgt11 expression library with antiserum raised against hamster acrosomal membranes (Moore *et al.*, 1985).

Purify round spermatids

Isolate polysomes

Incubate polysomes with
acrosomal antibodies

Collect polysome-antibody
complexes by passing the
mixture through a protein A
-Sepharose column

Elute bound polysomes from
column and purify
poly (A+) RNA

Reverse transcribe
poly (A+) RNA to cDNA

Probe cDNA testis library
and select positive clones
for characterization
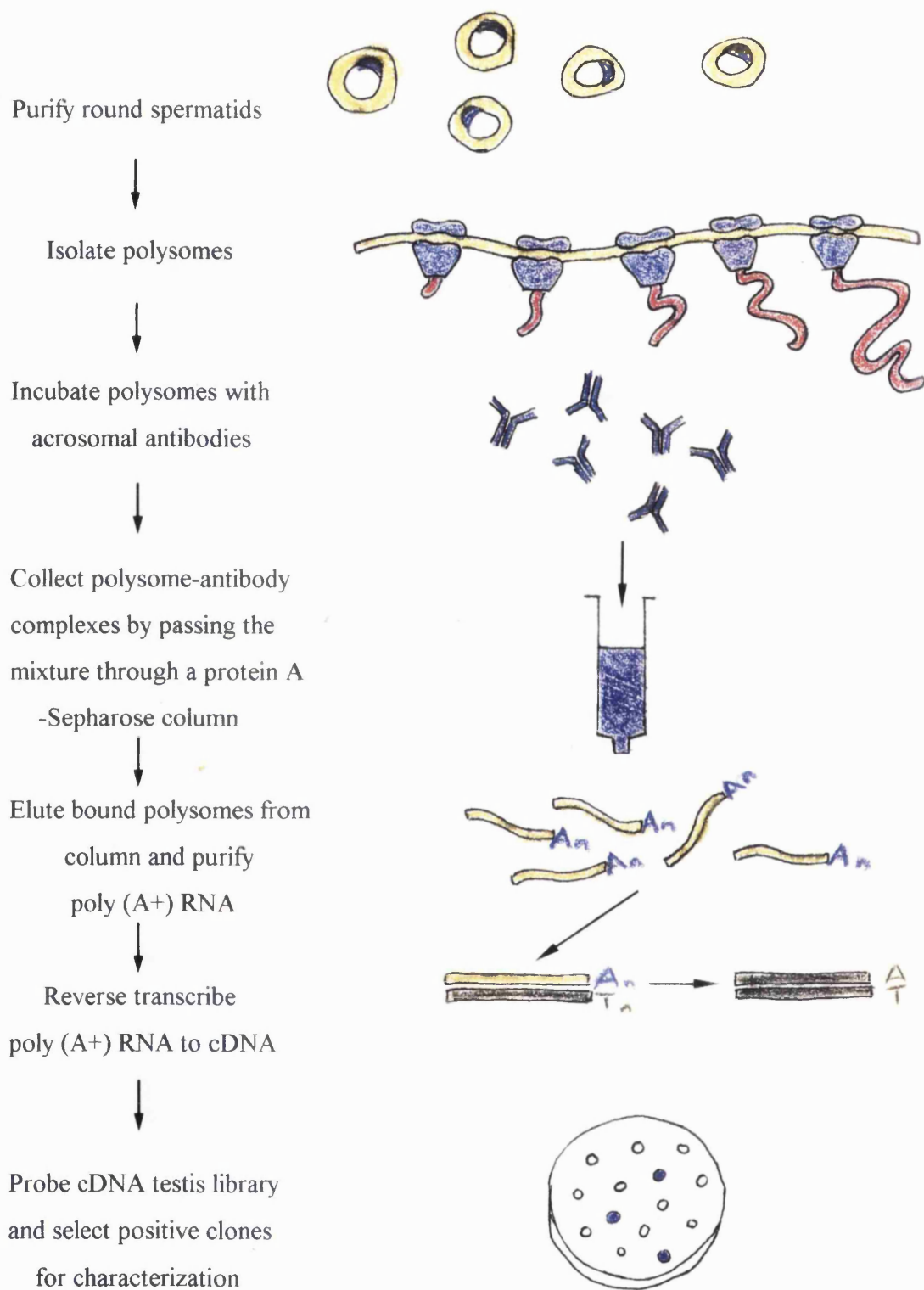
Figure 15. Diagrammatic representation of the polysomal strategy used for the isolation
of cDNA clones encoding acrosomal proteins.

IMMUNOLOGICAL ISOLATION OF SPECIFIC POLYSOMES USING ANTIBODIES SPECIFIC TO THE MAMMALIAN ACROSOME

The isolation of polysomal mRNA has been employed by various workers to investigate the possibility of translational regulation of mRNA transcripts in various tissues or cell types. For example, Kleene *et al*. (1984) measured the relative abundance of mouse protamine 1 (MP-1) mRNA in the polysomal and nonpolysomal RNA fractions of purified populations of round and elongating spermatids by Northern blot analysis. Their results demonstrated that MP-1 mRNA is stored as an untranslated ribonucleoprotein particle (RNP) in round spermatids, and that it is translated in elongating spermatids. Similar approaches have been used by Gold *et al*. (1983) and Moss *et al*. (1989) to investigate possible translational regulation of PGK-2 mRNA, and the histone 2b mRNA, respectively, during spermatogenesis. In addition, polysomes have been used to isolate specific mRNA using antibodies which bind to nascent polypeptide chains. For example, polysomes that bound to anti-ovalbumin antibodies were isolated from hen oviduct (Palacios *et al*., 1972).

The principle for using polysomes extracted from rat testis or round spermatids to isolate specific mRNA transcripts, was to allow the nascent polypeptides on polysomes to react with polyclonal antibodies specific to components of the mammalian acrosome, and subsequently collect the polysome-antibody complexes by protein A-Sepharose. The specific polysomal mRNA would be purified to remove polysomal peptides and ribosomal proteins, reversed transcribed into cDNA, and subsequently used to screen a testis λgt10 library. Corresponding cDNA clones obtained from the library may then be characterized. The polysome strategy is digrammatically represented in figure 15.

PRODUCTION OF SPERM ACROSOMAL ANTIBODIES

Antiserum (designated R1-10) against acrosomal membrane components was produced by injecting rabbits with purified acrosomal / peri-acrosomal membranes isolated from hamster epididymal spermatozoa. The membranes were solubilized by treating with 0.1% Nonidet-P40 and 8 M urea, and subsequently subjected to heat denaturation at 100^0C, as described in the appendix. Western blot analysis was performed using acrosomal preparations (100 µg) treated with endoglycosidase F or H, to remove complex and high

mannose, N-linked carbohydrate chains, respectively, or with trifluoromethanesulphonic acid (TFMS) which removes O-linked carbohydrate (outlined in the appendix). The protein preparations were denatured on an SDS-polyacrylamide gel and transferred to Hybond-C nitrocellulose membrane, as described in the appendix. The membrane was subsequently probed with antiserum which displayed binding to a variety of proteins ranging in size from 30 to 90 kD (Moore *et al.*, 1993). The antibodies bound to both glycosylated and deglycosylated proteins suggesting that a proportion recognized primary sequence epitopes rather than carbohydrate secondary modifications. Antiserum was shown to specifically localize to the acrosomal region of the sperm head on all methanol fixed hamster, mouse, and rat epididymal spermatozoa, and human ejaculated sperm (see figure 27).

ISOLATION OF POLYSOMES FROM RAT TESTIS

A number of methods have been published for the isolation of polysomes. These include:

I) separation of polysomes on a discontinuous gradient, where the polysomal band is present at the boundary between layers of 40 and 85% sucrose (Palacios *et al.*, 1972)

II) fractionation of polysomal and non-polysomal components on a 10-40% continuous sucrose gradient (Kleene *et al.*, 1984; Hake *et al.*, 1990)

III) collecting polysomes by ultracentrifugation through a layer of 65% sucrose (Shapiro and Young, 1981), and,

IV) precipitation of polysomes in the presence of high $MgCl_2$ concentration (100mM), (Palmiter, 1974).

In order to determine which of these methods would give the highest recovery of polysomes, I performed all the above methods. The precipitation method (IV) not only gave higher yields of polysomes, but was less time consuming. Rat testis cell suspensions were used initially in the setting up of the polysomal technique. It was anticipated that once the conditions were

optimized, round spermatids purified by panning would be used. Before describing the precipitation method (IV), techniques I-III are briefly outlined in order to compare polysome yields.
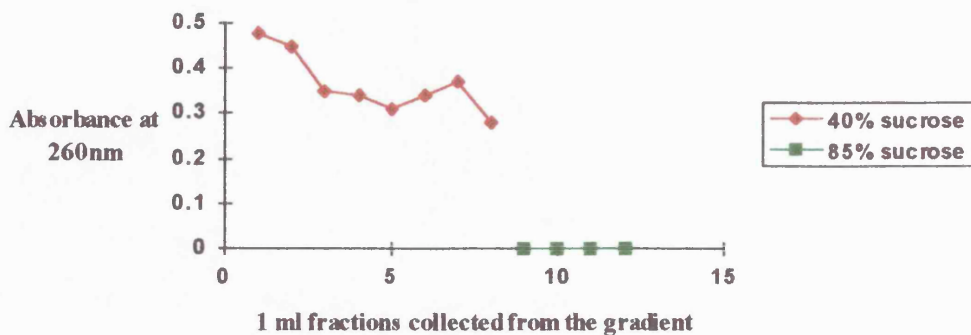
Figure 16. Sedimentation profile of testis post-mitochondrial supernatant separated on a discontinuous sucrose gradient.

Polysomes from 10^8 rat testis cells were separated on a 40% (w/v) and 85% (w/v) discontinuous sucrose gradient, for 90 minutes at 40,000 rpm in an SW40 rotor. Fractions (1 ml) were collected from the top of the gradient and absorbance readings at 260 nm were measured.
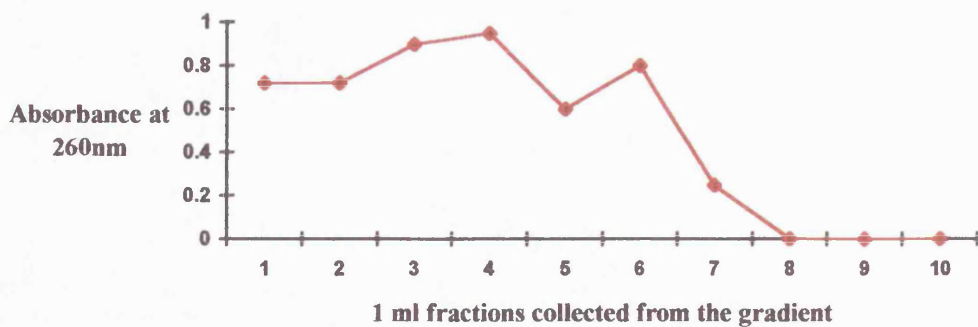
Figure 17. Sedimentation profile of testis post-mitochondrial supernatant separated on a continuous sucrose gradient.

Polysomes from 10^8 testis cells were separated on a 10-40% (w/v) continuous sucrose gradient for 90 minutes at 40,000 rpm in an SW40 rotor. Fractions (1 ml) were collected from the top of the gradient and absorbance readings at 260 nm were recorded.

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I) Discontinuous sucrose gradient

A modification of the protocol described by Palacios (1972) was used to extract polysomes from 1×10^8 testis cells (outlined in the appendix).

The sedimentation profile of a testis post-mitochondrial supernatant from 1×10^8 cells separated on a discontinuous gradient, is presented in figure 16. The majority of polysomes (60%) are expected to lie at the boundary between the 40% and 85% (w/v) sucrose (Palacios *et al.*, 1972; Kraus and Rosenberg, 1982), with the non-polysomal RNA distributed in the lighter sucrose layer. The 85% (w/v) sucrose layer contained no polysomal RNA as indicated from the absorbance readings.

As shown in figure 21, the recovery of polysomes collected from the boundary (corresponding to fraction 8), was low. It is unclear whether the peak of absorbance observed in fraction 7 might also contain polysomes. The protocol which was followed suggested that only the boundary fraction was collected.

II) Continuous sucrose gradient

This was performed according to the protocol outlined by Kleene *et al* (1984), and Hake *et al* (1990), and is described in the appendix.

A sedimentation profile of the post-mitochondrial supernatant from 1×10^8 testis cells separated on a 10-40% (w/v) continuous sucrose gradient, is illustrated in figure 17. Nonpolysomal RNA is present in the upper part of the gradient (fractions 1 to 4), with the polysomal RNA peaking in fractions 5 to 8.

Polysome recovery from the pooled polysomal fractions (5-8) collected from the gradient is shown in figure 21 where it is compared with other methods.

III) Ultracentrifugation

This was performed according to the protocol outlined by Shapiro and Young (1981), and is described in the appendix.

The polysome recovery determined by absorbance at 260 nm was low (0.2 OD units) and is shown in figure 21 in comparison to other methods. In addition, this method also leads to aggregation of polysomes following the centrifugation.

IV) Precipitation of polysomes in the presence of high MgCl₂ concentration

The protocol used was a modification of that outlined by Palmiter (1974) and involved a reduction in the volumes of solutions used during polysome extraction (described in the appendix). This was necessary to maximize polysome recovery as I was attempting to isolate polysomes from smaller amounts of starting material (1×10^6 to 1×10^8 cells) as compared to at least 1 g of tissue (Palmiter, 1974), or 10 g of tissue (Korman *et al.*, 1982).

Briefly, polysomes were allowed to precipitate in the presence of 200 mM $MgCl_2$ and subsequently collected by centrifugation through a layer of 0.2 M sucrose. The polysomal pellet was resuspended in 1 ml of 20 mM Hepes buffer (pH 7.4), and centrifuged for 5 minutes at 12000 g in a microfuge to pellet any polysomal aggregates which might have otherwise compromised the preparation. Absorbance readings at 260 and 280 nm of the resuspended polysomes were recorded to estimate polysome recovery, and a sedimentation profile of the resuspended polysomes was obtained by separating the polysomes on a 10-65% (w/v) continuous sucrose gradient centrifuged for 90 minutes at 40,000 rpm using an SW40 rotor.

An EDTA control was used to demonstrate that polysomal RNA was indeed being precipitated with high concentrations of $MgCl_2$. Polysomes were allowed to precipitate in the presence of 50 mM EDTA (see appendix) instead of $MgCl_2$, which released the mRNA thereby causing disassembly of the polysomal unit.

RESULTS

Absorbance of polysomes extracted from: 1×10^6, 5×10^7, and 1×10^8 testis cells using the precipitation method above, was measured at 260 nm and 280 nm. The table presented in figure 18 shows absorbance readings before and after centrifugation. Polysomes extracted from 1×10^6 cells, were resuspended in 200 µl. Absorbances for EDTA controls was also measured. The obvious reduction in absorbance confirms that magnesium is important for the precipitation of polysomes (Palmiter, 1974). These results are presented graphically in figure 19, and show a greater recovery of polysomes with increasing number of testis cells. The absorbance values presented for polysomes precipitated with $MgCl_2$ and EDTA controls, correspond to readings taken prior to

Number of testis cells	Absorbance before centrifugation		Absorbance after centrifugation		Absorbance of EDTA controls	
	260 nm	280 nm	260 nm	280 nm	260 nm	280 nm
1 x 10^6 (200 µl) †1.	0.18	0.20	0.14	0.17	0.08	0.13
2.	0.20	0.20	0.20	0.23	*	
3.	0.27	0.22	0.22	0.17	0.11	0.11
5 x 10^7 (1 ml) 1.	1.60	1.70	0.86	0.66	*	
†2.	1.32	1.13	1.16	0.92	0.35	0.40
3.	1.80	1.60	1.50	1.10	*	
1 x 10^8 (1 ml) 1.	3.80	2.10	1.69	1.45	0.90	1.20
2.	2.20	1.44	1.32	0.89	*	
1.2 x 10^8 (1 ml) 1.	*		1.36	1.04	*	
2.	*		1.55	1.40	*	

Figure 18. Recovery of polysomes from testis cells using the Precipitation method.

Absorbance values (A_{260} and A_{280}/ ml) of polysomes extracted from testis cells, before, and after centrifugation (to pellet, and discard aggregated polysomes). An A_{260} of 1 unit is equivalent to 40 µg RNA/ml. Polysomes extracted from 1 x 10^6 testis cells were resuspended in 200 µl, making the final amount of polysomal RNA 1/5th of the value presented in the table.

* Not done
† RNA was extracted from these polysomal suspensions, and analysed by gel electrophoresis.
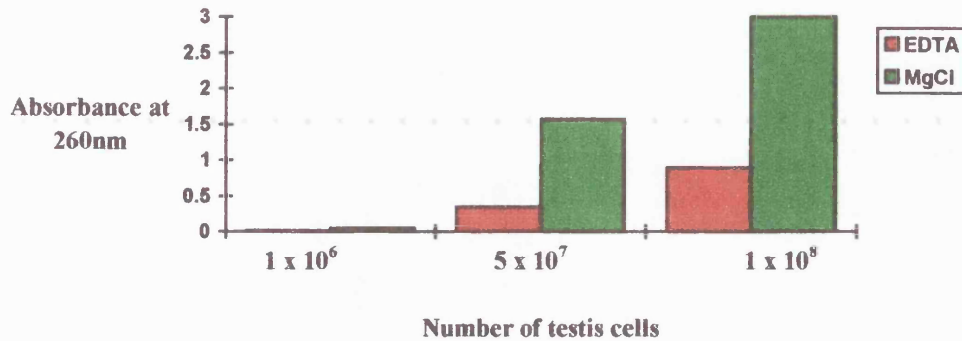
Figure 19. Histogram showing absorbance readings (260 nm) of polysomes extracted from rat testis cells using the Precipitation method.

The presence of high concentrations of $MgCl_2$ (100 mM) show an increase in polysome recovery when compared to replacement with EDTA.
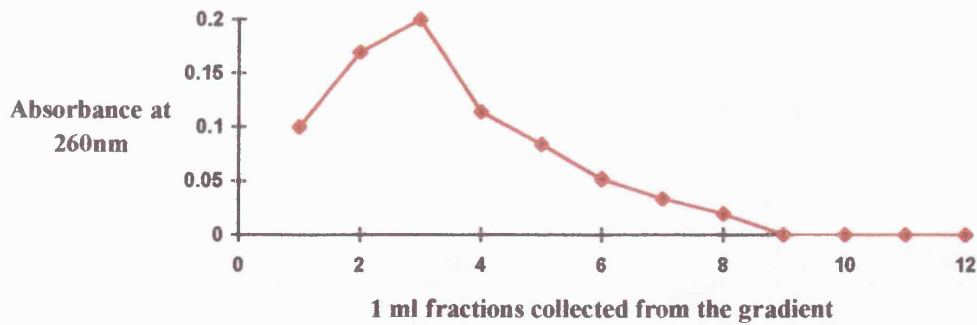
Figure 20. Sedimentation profile of polysomes separated on a continuous sucrose gradient.

centrifugation for the removal of polysomal aggregates. In addition, the sedimentation profile of a resuspended polysomal pellet from 5×10^7 testis cells separated on a 10-65% (w/v) continuous sucrose gradient is illustrated in figure 20. Fractions (1ml) collected from the top of the gradient (1 to 4) contain a high proportion of polysomes, with the polysomal concentration decreasing with progressive gradient fractions. This indicates that smaller polysomes make up the majority of polysomes recovered using the precipitation method. A resuspended polysomal pellet isolated from 5×10^7 rat testis cells were separated on a 10-65% (w/v) continuous sucrose gradient for 90 minutes at 40,000 rpm in an SW40 rotor. Fractions (1 ml) were collected from the top of the gradient and absorbance readings at 260nm were measured.

COMPARISON OF THE VARIOUS METHODS USED FOR POLYSOME ISOLATION

The precipitation method (IV) gave the highest polysomal recovery when compared to the other methods (I-III). Four experiments were carried out for each method using 10^8 testis cells, and the average absorbance measurement at 260 nm is presented in figure 21. Using the precipitation method (IV), absorbance at 260 nm was 3 OD units, but following centrifugation to remove polysomal aggregates this figure was reduced to 2.1.

The absorbance at 260 nm of polysomes collected following fractionation on a continuous sucrose gradient (method I), was 1.65 OD units. Although this method also resulted in a high recovery of polysomes, it was much more time-consuming when compared to method (IV). The remaining two methods resulted in much lower polysomal recoveries; separation of polysomes on a discontinuous sucrose gradient (method II) resulted in low absorbance readings of 0.28 OD units, while ultracentrifugation (method III) gave readings of 0.2 OD units.
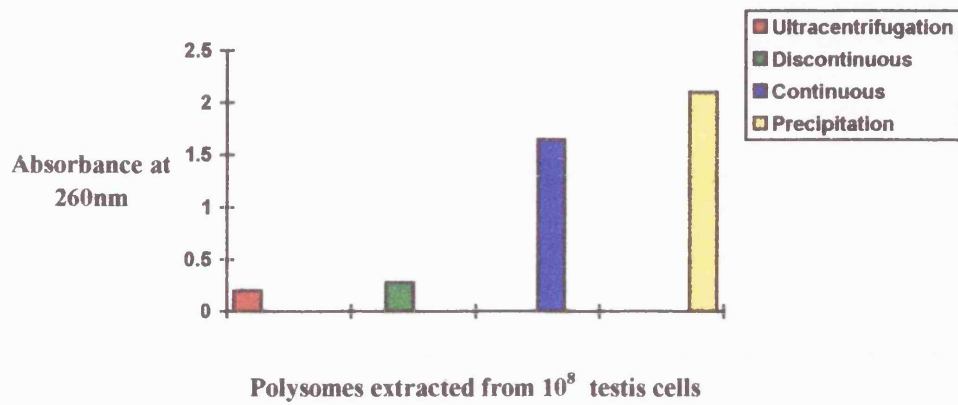
Figure 21. Comparison of available methods used for polysome extraction.

Polysomes were extracted from 10^8 rat testis cells and absorbance readings at 260 nm were measured. Four experiments were set up for each method, and average absorbance readings are presented in the histogram.

ISOLATION OF POLYSOMAL RNA

Once it was established that polysomes could be recovered, it was important to confirm that intact RNA could be extracted from the testis polysomal suspensions prior to incubating with acrosome-specific antibodies. A modification of the method by Palmiter (1974) was used and is outlined in the appendix.

The RNA samples were submitted to agarose gel electrophoresis and transferred to nylon membrane (Hybond-N) as described in the appendix. The blot was subsequently stained with methylene blue (outlined in the appendix), to assess the integrity of the RNA. Total RNA extracted from rat testis was also present to act as a positive control.

RESULTS

RNA Yield

Total RNA was extracted from selected polysomal preparations (figure 18), using the method previously described. The concentration and purity are presented in figure 22. The amount of total RNA extracted from polysomes isolated from 10^6 and 5×10^7 testis cells was 1.1 µg and 32-36 µg, respectively. The ratios of absorbance at 260/280 nm were 1.75-2.25, indicating that samples contained little or no contaminating or ribosomal proteins. Therefore, although absorbance readings at 260 nm almost remained unchanged following RNA extraction (figure 22), indicating minimal loss of RNA, the RNA extraction process removed ribosomal protein. EDTA controls show an obvious drop in RNA yield, when compared to samples containing $MgCl_2$, indicating that polysomes were indeed recovered by the precipitation method.

RNA Integrity

The stained blot (figure 23) showed intense staining of RNA in lane 1 (20 µg of rat testis total RNA), with the 28S and 18S ribosomal bands being clearly visible. Lane 2 (12 µg of total RNA from polysomes extracted from 5×10^7 rat testis cells) showed a less intense stain with 28S and 18S ribosomal bands just visible. Lanes 3 and 4 (16 µg of total RNA from polysomes extracted from 2.5×10^7 rat testis cells) exhibited faint staining with visible 28S and 18S ribosomal bands.

Number of testis cells	Absorbance at:		Absorbance of RNA extracted		260/280 ratio of RNA	Recovery of RNA (µg)
	260nm	280nm	260nm	280nm		
1×10^6 (200µl)						
MgCl$_2$	0.14	0.17	0.14	0.08	1.75	1.10
EDTA	0.08	0.13	0.08	0.04	2.0	0.64
5×10^7 (1 ml)						
MgCl$_2$ 1.	1.16	0.92	0.90	0.40	2.25	36
2.	0.86	0.66	0.80	0.40	2.0	32
EDTA	0.30	0.40	0.30	0.14	2.14	12

Figure 22. Total RNA extraction from rat testis polysomes.

Absorbances at 260 and 280 nm, of RNA extracted from polysomes, using MgCl$_2$ precipitation method, and EDTA controls. Purity was measured by 260/280 ratios, and the concentration of RNA (µg) was estimated using A$_{260}$ of 1 unit = 40 µg of RNA/ml.
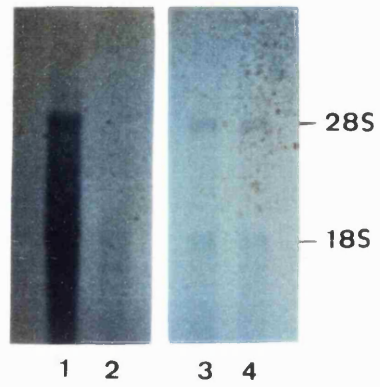
Figure 23. Methylene blue stained Northern blot of RNA isolated from rat testis polysomes.

1) Rat testis total RNA 20 μg

Total RNA from polysomes extracted from:

2) 5 x 10^7 testis cells, using MgCl$_2$; 12 μg loaded

3 and 4) 2.5 x 10^7 testis cells, using MgCl$_2$; 16 μg loaded

These results indicated that the RNA extracted from polysomes was intact, as both ribosomal bands were visible. Replacing $MgCl_2$ with EDTA resulted in a significant drop in polysome recovery, suggesting that polysomes were indeed recovered by precipitating with $MgCl_2$. Following the confirmation that intact RNA could be extracted from testis polysomes, the next step was to isolate specific polysomal mRNA by reacting this preparation with acrosome-specific antibodies. The sequence of steps followed were:

1) Incubating testis polysomes with polyclonal rabbit antibodies (R10) specific for components of the mammalian acrosome,

2) Collecting the polysome-antibody complexes by protein A-Sepharose,

3) Purification of mRNA from eluted polysomal RNA, and

4) Assessing the purity and integrity of unbound and eluted mRNA fractions.

1) POLYSOME-ANTIBODY PREPARATION

Two successive experiments were set up to allow binding of antibodies (R10) to specific epitopes present on nascent polypeptides being synthesized on rat testis polysomes:

Polysomes from 5×10^7 cells (A_{260} of 0.86, figure 18), and from two preparations of 5×10^7 cells (A_{260} of $1.16 + 1.50 = 2.66$, figure 18) were each incubated with 0.5 mg (250 μl) of purified R10 polyclonal antibodies, specific to components of the mammalian acrosome, for 1-2 hours at 4^0C in a sterile 30 ml universal tube with continuous stirring.

2) COLLECTION OF SPECIFIC POLYSOMES BY PROTEIN A-SEPHAROSE

Polysome-antibody complexes were isolated by applying the mixture to a protein A-Sepharose column, and the bound polysomes were subsequently collected following an elution step.

Preparation of the protein A-Sepharose

A protein A-Sepharose column of 1 ml bed volume, was prepared in 20 mM Hepes buffer (pH 7.4), as described in the appendix.

Collection of bound and unbound polysomes

The polysome-antibody mixture (2.25 ml) was passed through the protein A-Sepharose column twice. The eluate (1.8 ml) collected from experiment 2 (figure 24) was reserved for isolation of total RNA from unbound polysomes by phenol/chloroform extraction as

Eluted Polysomal Fractions	Experiment 1		Experiment 2	
	A_{260}	A_{280}	A_{260}	A_{280}
1	0.060	0.190	1.00	0.87
2	0.054	0.180	0.68	0.64
3	0.045	0.170	0.28	0.32
4	0.040	0.170	0.20	0.25
5	0.033	0.160	0.10	0.14
6	0.000	0.100	0.07	0.10
7	0.000	0.080	0.03	0.05
8	0.000	0.067	0.00	0.04
9	0.000	0.050	0.00	0.04
10	0.000	0.050	0.00	0.03

Figure 24. Collection of specific rat testis polysomes by protein A-Sepharose.

Polysomes extracted from 5×10^7 rat testis cells (experiment 1), and from 1×10^8 rat testis cells (experiment 2) were incubated with R10 acrosomal antibodies, and subsequently applied to a protein A-Sepharose column to isolate specific polysome-antibody complexes. Unbound polysomes were collected as the eluate, and bound polysomes were eluted from the column.

Ten 100 μl or 200 μl fractions (experiments 1 and 2, respectively) were eluted and absorbance readings at 260 and 280 nm (A_{260} and A_{280}) of each were recorded.

(An A_{260} of 1 unit is equivalent to 40 μg RNA/ml).

described previously. Absorbance readings of the eluate at 260 nm and 280 nm were recorded before and after phenol/chloroform extraction to estimate RNA recovery.

Bound polysomes from experiments 1 and 2 were eluted from the column by ten applications of 100-200 μl of elution buffer (25 mM Tris-HCl (pH 7.6), 20 mM EDTA, and 20 U/ml Heparin). Each fraction was collected in separate Eppendorf tubes, and absorbance readings at 260 and 280 nm were taken in RNase-free 50 μl cuvettes treated (as described in the appendix), to assess recovery and purity of RNA.

RESULTS

Since the absorbance readings at 260 nm in experiment 1 did not indicate adequate recovery of polysomes (figure 24), only the fractions collected in experiment 2 were used for the subsequent isolation of poly (A+) RNA.

Unbound polysomal fraction

Prior to phenol/chloroform extraction to remove protein, the estimated RNA recovery was 88 μg as deduced from absorbance at 260 nm (figure 25). However, as the 260/280 ratio was only 1.4, it was likely that this value was compromised due to the presence of protein. Following removal of proteins, the 260/280 ratio increased to 2.2 which indicated that the sample was now free from protein. The RNA concentration was estimated to be 80 μg (figure 25).

Bound polysomal fractions

Eluted fractions (1 to 6) collected from experiment 2 (figure 24) were pooled and estimated to contain 18.6 μg of total RNA in a final volume of 1.2 ml (figure 25). However, as the 260/280 ratio was only 1.03 due to the presence of polysomal proteins, the RNA figure estimated was not reliable. Subsequently, poly(A+) RNA was isolated from the eluted fractions which is described in the next section.

	Polysomes incubated with R10 A_{260} A_{280}	Unbound polysomal fraction A_{260} A_{280}	Phenol/ chloroform extraction A_{260} A_{280}	Eluted polysomal fractions A_{260} A_{280}	mRNA from eluted polysomes A_{260} A_{280}
	2.91 2.44	1.23 0.88	2.00 0.90	0.39 0.38	0.68 0.37
260/280	1.19	1.40	2.20	1.03	1.80
RNA (μg)	*116	*88	80	*18.6	5.4

* Inaccurate RNA concentration due to low 260/280 ratios.

Figure 25. Assessment of RNA isolated from unbound and eluted polysomes.

Rat testis polysomes (A_{260} 2.66) were incubated with R10 acrosomal antibodies, and applied to a protein A-Sepharose column (experiment 2, table 3). The unbound polysomal fraction (1.8 ml) was measured at 260 and 280 nm (A_{260} and A_{280}) to estimate total RNA concentration. Following purification by phenol/chloroform extraction, unbound polysomal RNA was quantified, and 260/280 readings indicated excellent purity. Bound polysomes were eluted from the column, and poly(A+) RNA was isolated from the pooled fractions (1.2 ml) using the Micro-FastTrack kit.

(An A_{260} of 1 unit is equivalent to 40 μg/ml).

3) mRNA ISOLATION FROM ELUTED POLYSOMAL RNA

The eluted polysomal fractions (1 to 6) collected from experiment 2 (figure 24) were pooled and then applied to oligo (dT) cellulose using Micro-FastTrack mRNA isolation kit in order to isolate poly(A+) RNA present in the polysomal unit. The advantage of using this kit was primarily that it allowed the rapid isolation of poly(A+) RNA (approximately 60 minutes) from small amounts of cells (as few as 100 cells).

The polysomal RNA solution was adjusted to 0.3 M NaCl final concentration, and then bound to oligo (dT) cellulose. Non-polyadenylated RNA and ribosomal proteins were released from the resin with high salt buffer (Binding buffer), and tRNA and rRNA with low salt buffer. The polyadenylated RNA was eluted in the absence of salt (Elution buffer). For quantitation of RNA yield, the eluted sample was measured by spectrophotometry at 260 nm in RNase-free cuvettes. The mRNA was then precipitated overnight at -20^0C as described in the appendix, and the RNA pellet finally resuspended in a small volume (4 µl) of RNase-free water (see appendix).

A total of 5.4 µg mRNA was isolated from pooled eluted polysomal fractions collected from experiment 2 (figure 24), as determined by absorbance readings at 260 nm (figure 25). The 260/280 ratio was 1.8 which indicated good purity largely free from protein.

(4) ANALYSIS OF UNBOUND AND ELUTED POLYSOMAL RNA

To check the integrity of total RNA and mRNA isolated from unbound and eluted polysomes, respectively, the relevant samples were subjected to agarose gel electrophoresis and transferred to nylon membrane (Hybond-N) by capillary action (described in the appendix). The blot was then stained with methylene blue, as outlined in the appendix.

The RNA isolated from bound and eluted polysomes appeared intact as assessed by methylene blue staining (figure 26). Both 28S and 18S ribosomal bands were present in the unbound total RNA samples which indicated that minimal RNA degradation had occurred. The mRNA isolated from the eluted polysomes appeared as a smear ranging from approximately 4.4 kb to 0.24 kb. This indicated that minimal degradation had occurred, and that ribosomal RNA was not present since 28S and 18S ribosomal bands were absent.
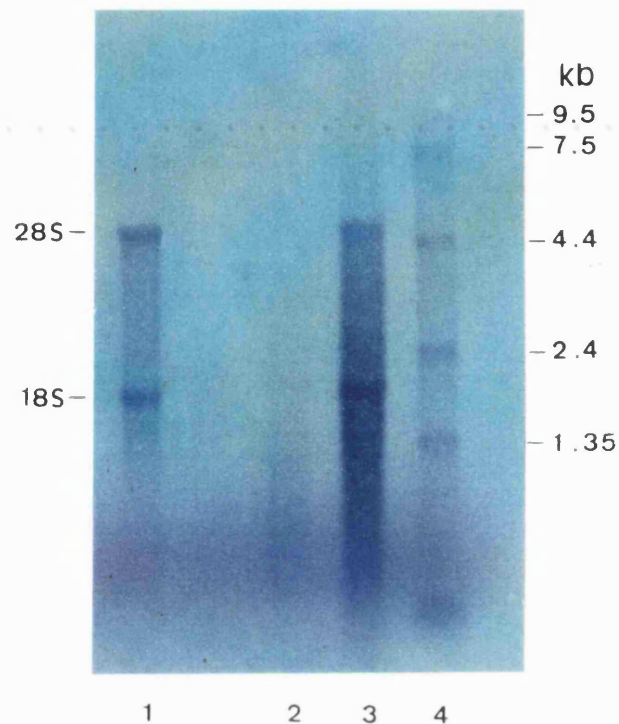
Figure 26. Methylene blue stained blot containing RNA isolated from unbound and eluted polysomes.

Rat testis polysomes were incubated with R10 polyclonal antibodies raised against acrosomal membrane, and subsequently passed through a protein A-Sepharose column. RNA was then purified from the unbound polysomal fraction and from bound polysomes which were eluted from the column. The 28S and 18S ribosomal bands are indicated.

(1) Total RNA (20 μg) from unbound polysomes, (2) mRNA (5.4 μg) isolated from eluted polysomes, (3) total RNA (20 μg) from adult rat testis, (4) RNA ladder (3 μg).

DISCUSSION

Antibodies generated against acrosomal membranes were used in an attempt to isolate a specific polysomal fraction rich in mRNA transcripts encoding acrosomal proteins. Initially polysomes were extracted from rat testis suspensions using relatively low amounts of cells (10^6 to 10^8) to ascertain whether it would be eventually feasible to use round spermatids recovered by panning (see chapter 2). These cells were highly purified but in low abundance. Although a number of methods were available for the extraction of polysomes, the $MgCl_2$ precipitation method described by Palmiter (1974) resulted in a higher polysomal recovery. Subsequently, the protocol was modified for use with the amount of starting material available. Testis polysomes were isolated from a lower number of cells (1×10^8) compared to published data (Shapiro and Young, 1981; Kraus and Rosenberg, 1981; Korman et al., 1982), and pure, intact mRNA (5.4 µg) could be recovered from testis polysomes specifically bound to acrosomal antibodies. However, a number of difficulties resulted in this approach being abandoned in favour of direct screening of a testis cDNA expression library. Firstly, the yields of spermatids that could be obtained by the panning method had not been optimized at this stage of the study, and in single preparations, at least, insufficient for isolating enough polysomes to react with acrosomal antibodies. As discussed earlier, the reason for wanting to use purified spermatids rather than whole testis, was primarily to reduce potential non-specific binding of polysomes extracted from the testis. A high proportion of testis polysomes would be expected to come from a variety of cell types, whereas polysomes extracted from round spermatids would contain those translating mRNA encoding acrosomal proteins and others important in post-meiotic development. One possibility (although time consuming) was to pool stored polysomes extracted from spermatids on different days. But stored polysomes aggregated (Sala-Trepat et al., 1978) and these complexes had to be removed by pelleting. This resulted in considerable polysomal losses from a relatively low concentration of polysomes. Secondly, although the antibodies had been shown to specifically stain the acrosomal region on slides of spermatozoa or testis cell suspensions, the antibodies may also recognize epitopes on proteins present in other cell types. For example, diffuse antigens, even when present at high concentration, can be difficult to detect or distinguish from background signals (Harlow and Lane, 1988). Therefore, diffuse staining may not be easily identified when compared to more intense

localized staining seen for the acrosome. Thirdly, although R10 antiserum was raised against denatured and deglycosylated acrosomal membrane proteins, it is always possible that the antibodies now recognize particular polysomal epitopes that were not exposed in the acrosomal preparation used to raise the antiserum.

Nevertheless, the polysomal strategy remains a viable method and was adapted here for the isolation of specific mRNA transcripts encoding acrosomal proteins from relatively low numbers of cells. Apart from being a novel approach for the isolation of mRNA transcripts expressed during mammalian sperm differentiation, it has the advantage over differential screening methods in that the specific transcripts are being actively transcribed rather than stored as untranslated ribonucleoprotein particles.
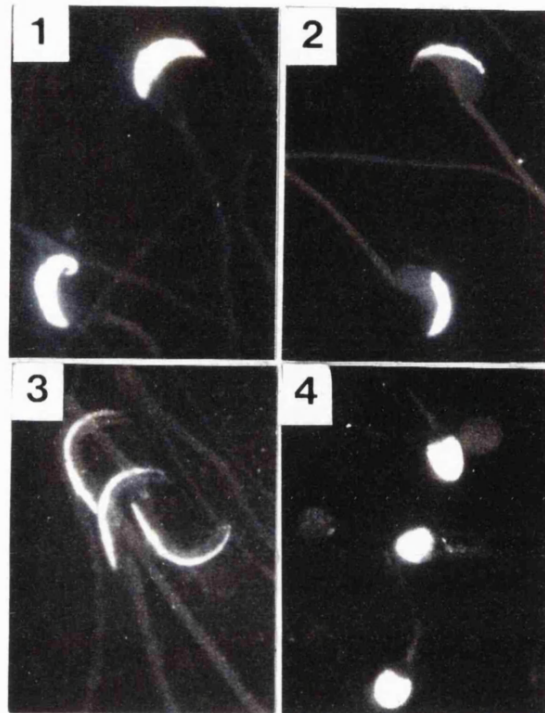
Figure 27. Immunofluorescent localization of antiserum R4 (from Moore *et al.*, 1993)

Spermatozoa were stained with R4 antibodies and fluorescein-labelled anti-rabbit IgG.
(1) Hamster epididymal spermatozoa, (2) mouse epididymal spermatozoa, (3) rat epididymal spermatozoa, and (4) human ejaculated spermatozoa.

SCREENING A HUMAN cDNA TESTIS LIBRARY WITH ANTISERA RAISED AGAINST ACROSOMAL MEMBRANE

The second strategy used to identify post-meiotically expressed genes encoding proteins targeted to the acrosome, was the screening of a human testis cDNA λgt11 expression library with antiserum raised against acrosomal membranes. Despite the difficulties encountered using the polysomal method, the library screening approach was expected to give rise to a number of positive clones corresponding to mRNA transcripts encoding acrosomal proteins.

LIBRARY SCREENING

Rabbit antiserum that specifically recognised acrosomal components was generated as described on page 79. Immunofluorescent localisation of antibody (R4) binding to spermatozoa revealed specific binding to the acrosomal region (figure 27). With testis preparations, antiserum bound only to post-meiotic germ cells (Moore *et al.*, 1993). A human testis cDNA library constructed in theλgt11 phage expression vector (Clontech) was plated out at a density of 1.6×10^6 plaque forming units (pfu's), 4×10^5 per 20 cm plate, on *E.coli* Y1090 and overlayed with Hybond-C filters impregnated with 10 mM Isopropyl-β-D-thio-galactoside (IPTG) to induce protein expression from the λgt11 clones according to standard techniques (see appendix). The filters were then removed and processed as for a Western blot using R4 antiserum and peroxidase-conjugated secondary antibody, as described in the appendix. A number (21) of positive clones were selected, the phage titre for each was checked, and then plated out at a density of 1-2 x 10^2 pfu's per plate (see appendix). As before, the plates were overlayed with filters, and positive plaques (30) were picked, titred, and replated for tertiary screening to ensure they were plaque-pure, that is, each positive deriving from a single phage. On the basis of intense immunostaining and preliminary novel nucleotide sequence data, a single clone (352) was selected for further characterisation.

INITIAL CHARACTERIZATION OF 352 CDNA CLONE

Size of 352 cDNA

Phage DNA was prepared from the 352 λgt11 cDNA clone using standard protocols (Maniatis *et al.*, 1982), and subsequently digested with *Eco*R I restriction enzyme to excise the insert DNA, as described in the appendix. The size of 352 cDNA insert was estimated by agarose gel electrophoresis and visualized under UV illumination in the presence of ethidium bromide (see appendix). The cDNA was shown to be approximately 2 kb in size, and to contain no internal *Eco*R I sites.

In order to obtain sufficient amounts of 352 cDNA for further characterization, polymerase chain reaction (PCR) was used to amplify the DNA using oligonucleotide primers corresponding to the λgt11 sequences flanking the *Eco*R I restriction enzyme site (see appendix). Agarose gel electrophoresis was used to confirm that the PCR product was equivalent in size to the *Eco*R I digest of the λDNA.

SUBCLONING OF 352 CDNA

The 352 PCR (2 kb) product was digested with *Eco*R I to remove primer sequences, and then subcloned into Bluescript plasmid vector (SK+) for large scale preparation of 352 DNA, to generate DNA probes for Northern and Southern blot analyses, construction of a restriction endonuclease map, and for double-stranded sequencing.

The sequence of steps used in subcloning are outlined below:

1) Digestion of 352 cDNA and vector DNA with restriction endonucleases,

2) Purification of 352 insert,

3) Phosphatase treatment of the vector,

4) Ligation of insert and vector,

5) Transformation of competent *E. coli* host cells, and

6) Recovery of recombinant DNA by plasmid preparation.

These steps are briefly described next.

1) Digestion of 352 cDNA and vector DNA

Standard protocols were used for digestion reactions and are described in the appendix. For the subcloning of 352 insert into Bluescript plasmid vector, the 2 kb 352 PCR product was digested with *EcoR* I to remove the primers. Bluescript plasmid was digested with the same restriction endonuclease in order to generate the compatible termini necessary for subsequent ligation of insert and vector DNA.

All restriction digests were subjected to agarose gel electrophoresis and visualized under UV illumination in the presence of ethidium bromide (see appendix) to ensure the reaction had gone to completion.

2) Purification of 352 cDNA insert

If the 352 cDNA digest reaction was complete, as established from agarose gel electrophoresis, the remaining digest was electrophoresed on a 1% agarose gel prepared using low melting point (LMP) agarose, and the band of interest excised and purified using Geneclean™ kit according to manufacturer's instructions (see appendix). An aliquot of the purified 352 cDNA insert was subjected to electrophoresis and visualized under UV illumination in the presence of ethidium bromide, to assess recovery and estimate concentration.

3) Phosphatase treatment of vectors

Prior to the ligation of vector DNA to purified 352 insert DNA, the cut vector (Bluescript plasmid) was treated with calf intestinal alkaline phosphatase to prevent re-ligation of the DNA (see appendix).

4) Ligations

Ligation reactions were performed to ligate 352 cDNA insert to cut Bluescript using standard protocols (see appendix).

5) Transformation

The bacterial cells (*E. coli* JM101) were made competent by calcium chloride treatment outlined in the appendix, in order to take up the plasmid DNA, a process known as transformation. Uncut vector (1 ng) was used to assess the transformation efficiency.

Subsequently, transformations using ligated samples (352 insert ligated to vector) were carried out using standard protocols (see appendix).

Plates containing successful ligations: vector + insert, would give rise to clear colonies, due to the interruption of the Lac Z gene. The ligation control would give rise to blue colonies, as the religated vector has restored the function of the Lac Z gene.

6) Plasmid Preparation

The white colonies formed on the ligated sample plates, would indicate there were a number of putative recombinants, that is 352 DNA insert cloned into Bluescript plasmid vector. In order to recover the 352 DNA, the protocol for plasmid preparation was followed, as described in the appendix.

To assess recovery of recombinant DNA, an aliquot of each sample was subjected to electrophoresis and visualized under UV illumination in the presence of ethidium bromide (see appendix). Where necessary, Southern blot analysis was performed to confirm the presence of 352 insert.

FURTHER CHARACTERISATION OF 352 cDNA

Abundance and size of 352 mRNA transcript in human and rat testis

Northern blot analysis was used to determine the size of the mRNA corresponding to 352 cDNA in human testis and it's homologue in the rat testis. As the clone was isolated from a human testis library, it was essential to use RNA from human testis to establish the size of the mRNA transcript and thus what proportion of the sequence 352 cDNA represented. In addition, it was important to see whether a homologue could be detected in rat testis, as apart from the limited availability of human testis samples, spermatogenesis is much better characterized in the rat and mouse.

Northern blots containing either 1) total RNA from human and rat testis, or 2) mRNA from human and rat testis, were probed with ^{32}P-labelled 352 (2 kb) insert.

1) Northern blots containing total RNA

Total RNA (20 µg) extracted from adult human and rat testis, as described in the appendix, was denatured and run on a 1% agarose gel containing formaldehyde and
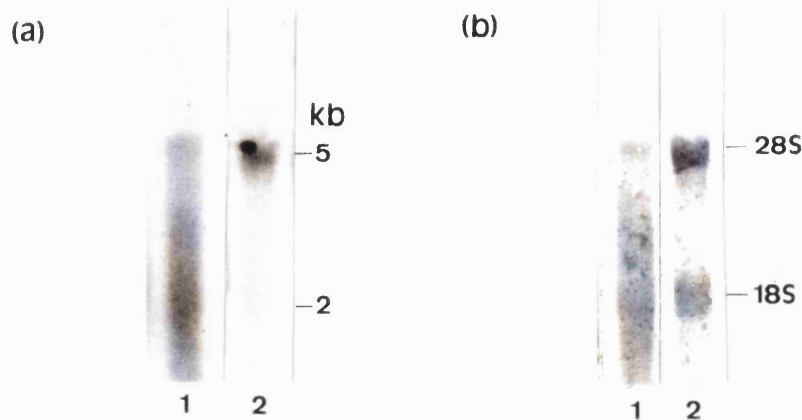
Figure 28. Expression of 352 mRNA in adult human and rat testis.

(a) Northern blot analysis of total RNA (20 µg) from human testis (1) and rat testis (2) probed with ^{32}P-labelled 352 cDNA insert. The blot was washed under high stringent conditions and shows a band of approximately 5 kb in human and rat testis. There is also evidence of a faint signal of approximately 2 kb in both lanes; (b) Methylene blue staining shows 28S and 18S ribosomal bands in human (1) and rat testis (2) indicating the presence of intact RNA in the testis. The less distinct ribosomal bands present in human testis may be the result of partial degradation.
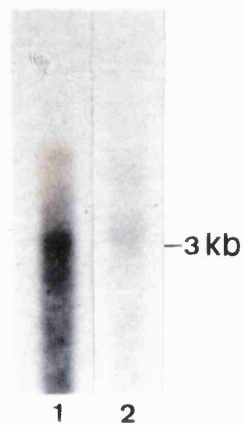
Figure 29. Expression of 241 mRNA in adult human and rat testis.

The blot was reprobed with radiolabelled 241 cDNA insert and washed at high stringency. A band of approximately 3 kb is evident in human testis (1), with a less intense band present in rat testis (2). There is very little evidence of 28S and 18S ribosomal RNA in either lane, indicating minimal non-specific binding.

subsequently transferred to nylon membrane (Hybond-N) by capillary action (see appendix). After fixing the RNA by UV illumination for 2 minutes, the blot was stained with methylene blue (see appendix) to check the integrity of the RNA, and was subsequently probed with ^{32}P-labelled 352 cDNA insert, as described in the appendix. The blot was then washed under low and high stringent conditions (see appendix) to establish whether there was a 352 homologue in rat testis, and if so, the transcript size and the degree of homology that existed between the human and rat mRNA transcripts.

RESULTS

After low or high stringency washes, the blot was exposed to X-ray film with intensifying screens, for 3 and 9 days, respectively. Following low stringent washes, there was heavy background staining making it difficult to detect any distinct bands. After high stringent washes, the autoradiograph showed a distinct band of approximately 5 kb present in rat testis (figure 28a, lane 2) and a less intense band of approximately the same size in human testis (figure 28a, lane 1). A very faint band of approximately 2 kb was also visualized in both lanes. Methylene blue staining shows distinct 28S and 18S ribosomal RNA bands in rat testis (figure 28b, lane 2) indicating the presence of intact RNA. Human testis (figure 28b, lane 1) contained less distinct ribosomal RNA bands, possibly a result of partial RNA degradation which is reflected in figure 28a (lane 1) where the 5 kb band is much less intense when compared to rat testis (lane 2).

Subsequently, the same blot was probed with a radiolabelled cDNA (241) whose transcript size was already known to be approximately 3 kb in both human and rat testis. The autoradiograph revealed a 3 kb band, as well as a faint band situated at the position of the 28S ribosomal in human testis (figure 29, lane 1). In rat testis, a faint 3 kb band was evident (figure 29, lane 2).

From these results, it was concluded that:

i) the size of the mRNA transcript from which 352 cDNA derived was approximately 5 kb in human testis with the rat homologue of equivalent size, ii) the degree of homology between human and rat 352 mRNA was expected to be high, and iii) the 5 kb band (which was situated at a similar position to the 28S ribosomal band) was likely to be specific, and not the result of non-specific binding to ribosomal RNA.
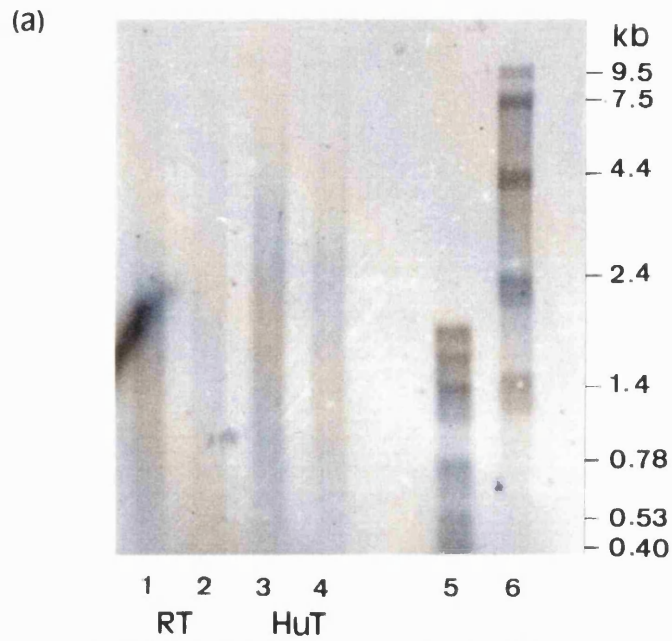
(a) Methylene blue stained blot containing poly (A+) RNA from rat testis (RT) and human testis (HuT). Poly (A+) RNA: 3 μg (1,3) and 1 μg (2,4). RNA ladders: low molecular weight (5) and high molecular weight (6).
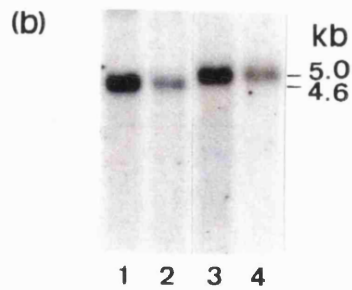
(b) Blot probed with ^{32}P-labelled 352 cDNA insert and washed at high stringency. A 4.6 kb and 5 kb band is present in rat testis (1,2) and human testis (3,4), respectively.

Figure 30. Expression of 352 mRNA in adult human and rat testis.

2) Northern blots containing mRNA

Northern blot analysis using mRNA from human and rat testis was used in order to confirm the mRNA transcript size corresponding to 352 cDNA. The use of mRNA would exclude the possibility of any non-specific binding to ribosomal RNA, and would establish the abundance of the mRNA.

Poly(A+) RNA (1 and 3 μg) isolated using oligo (dT) cellulose columns (see appendix), was denatured and run on a 1% agarose gel containing formaldehyde and transferred to nylon membrane, as outlined in the appendix. The blot was stained with methylene blue to assess the integrity of RNA, and subsequently probed with ^{32}P-labelled 352 cDNA (see appendix), washed at high stringency, and exposed to X-ray film for 10 days.

RESULTS

Methylene blue staining showed a smear consisting of mRNA transcripts ranging from 9.5 kb to 0.4 kb in all lanes (figure 30a), indicating that the mRNA was intact

A single distinct band of approximately 5 kb was evident in human testis (figure 30b, lanes 3 and 4), with a 4.6 kb band present in rat testis (figure 30b, lanes 1 and 2).

These results:

i) confirmed the presence of 352 mRNA in human and rat testis,

ii) confirmed the size of the 352 mRNA transcript, and

iii) indicated that 352 mRNA was of low abundance.

Phylogenetic conservation of the 352

Southern blot analysis of genomic DNA from a variety of eukaryotic species was used to investigate the conservation of the 352 gene across species.

A ZOO-BLOT (Clontech) was probed with ^{32}P-labelled 352 cDNA (2 kb) following the manufacturer's instructions (see appendix).

Briefly, the blot was prehybridized for 3-6 hours at 65°C, and subsequently hybridized with 352 radiolabelled probe at a concentration of 1-2 x 10^6 cpm/ml, for 18-24 hours at the same temperature. The blot was washed at 60°C under low or high stringent conditions as described in the appendix, and exposed to X-ray film for 7 days with intensifying screens.
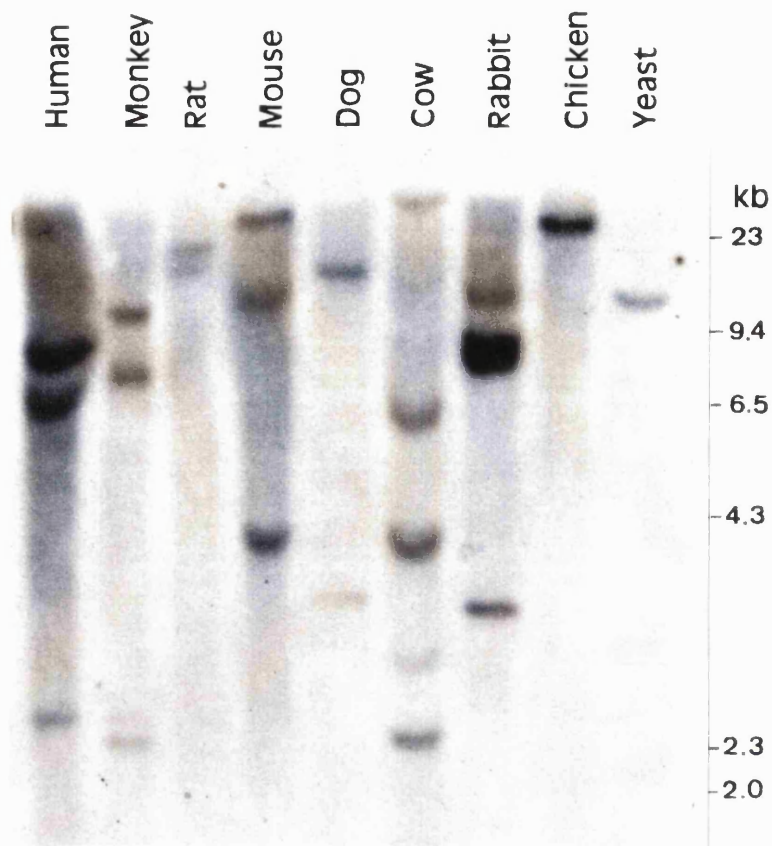
Figure 31. Phylogenetic conservation of the 352 gene

Southern blot analysis of genomic DNA from a variety of eukaryotic species probed with
^{32}P-labelled 352 cDNA insert and washed under high stringent conditions.

RESULTS

The ZOO blot showed a simple band pattern present in all species following high stringent washes (figure 31). The pattern indicated that the 352 cDNA was derived from a highly conserved gene, present in a variety of species from humans to yeast.

Tissue Expression Pattern of 352 mRNA

Northern blot analysis was used to establish the pattern of expression of 352 mRNA in tissues other than the testis. Human multiple tissue Northern (MTN) blots (Clontech) containing mRNA (a minimum of 2 µg) from a variety of human tissues, were probed with ^{32}P-labelled 352 cDNA, following the manufacturer's instructions (see appendix). Briefly, the blots were prehybridized for 3-6 hours at 42^{0}C, and subsequently hybridized with the radiolabelled 352 cDNA at a concentration of 1-2 x 10^6 cpm/ml for 18-24 hours at the same temperature. The blots were washed at high stringency, and exposed to X-ray film at -70^{0}C with intensifying screens. MTN blots were subsequently probed with radiolabelled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, and mouse β-actin cDNA to assess even loading of mRNA in all lanes.

RESULTS •

The human MTN blots (I and II) showed a prominent band of 5 kb in all tissues presented (figure 32a), but significantly more abundant in the testis (blot I, lane 4). In contrast to previous blots containing human and rat mRNA (figure 30b), 352 cDNA hybridized to three additional bands of approximately 3.2 kb, 8.5 kb, and greater than 9.5 kb in a variety of tissues, but these bands were much less intense. The 3.2 kb band was present at low levels in the testis (blot I, lane 4) but was not visible in any other tissues. The 8.5 kb band appeared in all tissues at low levels. The 9.5 kb (or greater) band was evident (although very faint) in those tissues with increased expression of the 5 kb transcript, namely placenta (blot II, lane 3), spleen, thymus, testis, and peripheral blood leucocytes (blot II, lanes 1, 2, 4, and 8, respectively).

MTN blots probed with radiolabelled GAPDH cDNA showed a single band of 1.3 kb present in all lanes (figures 32b). The testis also contained a faint spermatid-specific band of 1.45 kb (blot I, lane 4). It appeared that GAPDH mRNA was present at
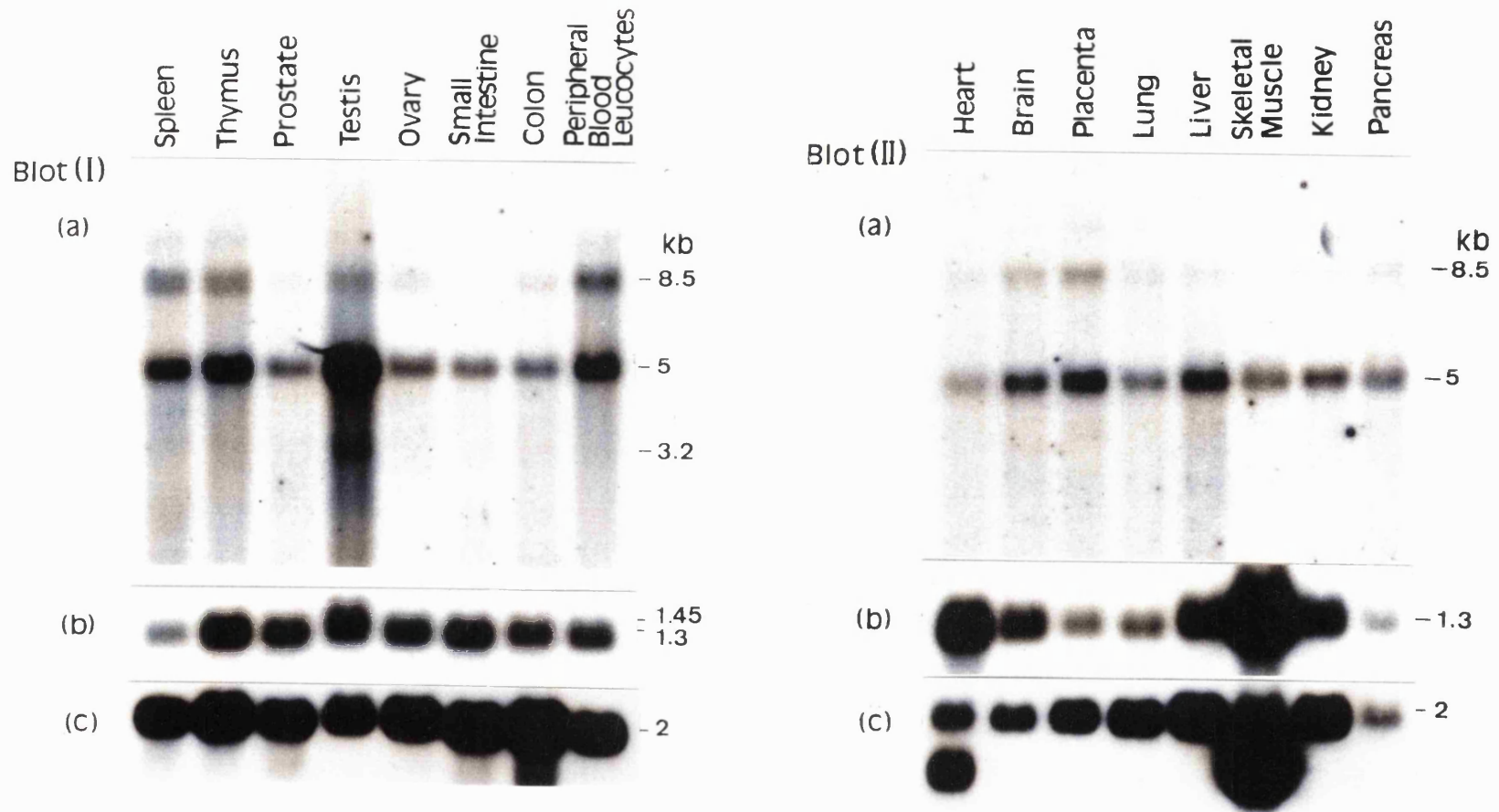
Figure 32. Expression of 352 mRNA in a variety of human tissues

Human multiple tissue Northern blots (I and II) were probed with 32P-labelled 352 cDNA insert (a), and subsequently with GAPDH cDNA (b), or β-actin cDNA insert (c).

relatively equal levels in all tissues, indicating even loading of mRNA and confirming the more abundant expression of 352 mRNA in the testis compared to other tissues. Although the GAPDH band was significantly more intense in heart and skeletal muscle (blot II, lanes 1 and 6, respectively), this glycolytic enzyme has previously been shown to be expressed at significantly higher levels in these tissues (Fort *et al.*, 1985).

Reprobing the blot with radiolabelled β-actin cDNA showed a 2 kb band of relatively equal intensity in all lanes (figure 32c). Again, heart and skeletal muscle exhibited higher levels of expression with the presence of a second transcript of lower molecular weight (1.8 kb), consistent with previous findings, Cleveland *et al.* (1980).

DISCUSSION

The present chapter outlines two strategies that were used to isolate mRNA transcripts encoding acrosomal proteins, which include: 1) the isolation of specific polysomal mRNA from rat testis and round spermatids using acrosome-specific polyclonal antibodies, and 2) screening a human testis cDNA expression library with acrosome-specific antiserum. The first strategy was devised to isolate specific polysomal mRNA initially from rat testis, but with the intention of using a more specific approach with polysomes from purified round spermatids once the technique had been optimized. The use of specific antibodies to isolate polysomal mRNA from testis or spermatogenic cell populations is a novel approach to the study of sperm differentiation, and has the advantage of isolating specific mRNA transcripts that are being actively translated, rather than stored as untranslated ribonucleoprotein particles. The reasons for wanting to use pure (95%) round spermatids have previously been discussed (page 97). However, the yield of round spermatids obtained using the immunoselection method (panning), was not sufficient, at this stage, to isolate enough polysomes necessary for reacting with acrosomal antibodies.

While successfully optimizing panning yields, a number of cDNA clones were isolated from a human testis cDNA expression library using specific antiserum raised against acrosomal membranes. Antibody recognised components of the plasma and outer acrosomal membrane as well as some matrix material underlying these membranes. Therefore, it was expected that a number of specific mRNA transcripts isolated using

either strategy, would encode proteins targeted to the outer acrosomal membrane, the plasma membrane or perhaps acrosomal contents. One particular clone, designated 352, was selected for further study as initial sequencing data suggested that it derived from a novel gene. Given the time available, it was not possible to return to the polysomal strategy although specific mRNA was isolated with this technique.

Northern blot analysis using human and rat RNA, showed that the 2 kb cDNA clone 352 derived from a 5 kb mRNA transcript in human testis, with a rat homologue of 4.6 kb. This established the proportion of the nucleotide sequence that 352 represented, and also the presence of a homologue in rat testis which was essential for further characterization of the clone. The homology between the human and rat 352 mRNA transcripts was expected to be high, as a distinct 4.6 kb band was still present in rat testis following washes at high stringency. The results obtained using mRNA indicated that 352 mRNA was present at low levels, as the blot had to be exposed to X-ray film for 10 days in order to visualize a distinct band. Previously, the use of total RNA from rat and human testis showed that 352 mRNA was very close in size to 28S ribosomal RNA. This indicated the possibility of specific 352 hybridization being obscured by non-specific binding to the 28S ribosomal RNA. However, reprobing the blot with a radiolabelled cDNA insert (241) previously shown to hybridize to a 3 kb mRNA transcript in testis, displayed a 3 kb band in both rat and human testis with minimal detection of hybridization to 28S ribosomal RNA. Subsequently, confirmation of the mRNA tanscript size corresponding to 352 cDNA was achieved by Northern blot analysis using mRNA.

Southern blot analysis showed that 352 derives from a highly conserved gene present in a variety of species ranging from human to yeast, suggesting it's function might also be conserved. Finally, Northern blot analysis demonstrated that the 5 kb mRNA was expressed at low levels in a range of human tissues but was significantly more abundant in the testis. Additional bands (3.2 kb, 8.5 kb, and greater than 9.5 kb) of much lower intensity were also visible in some tissues including testis, that had not been detected previously on blots containing human and rat testis mRNA. The most obvious explanation as to why these additional bands were not evident previously is that a higher concentration of mRNA was present on the multiple tissue Northern blots making it possible to visualize these less abundant transcripts. The significance of these bands is

not clear, but they may represent the products of closely related members of a multigene family, or may result from alternative splicing of RNA transcripts.

From these results, it appeared that the 352 gene product was unlikely to be acrosome-specific, as the mRNA was present in a variety of human tissues. However, significantly lower levels of 352 mRNA were present in all tissues compared to the testis. This might suggest that the corresponding protein (assuming 352 protein is synthesized) is likely to be present at much lower levels in these tissues and may not be localized. In contrast, 352 mRNA was present at much higher levels in the testis. This may be necessary as higher levels of the corresponding protein may be targeted to the acrosome.

The remaining parts of this study investigated the developmental expression pattern of 352 mRNA in the testes of pre and post-pubertal rats, the expression of the transcript in the post-meiotic germ cell, and sequence analysis to obtain information about a potential translation product.

CHAPTER 4

DEVELOPMENTAL EXPRESSION OF 352 mRNA TRANSCRIPT IN THE RAT TESTIS

INTRODUCTION

Postnatal development of the seminiferous epithelium is characterized by the sequential appearance of germ cells at progressively more advanced stages of differentiation, as discussed in chapter 1 (Bellve *et al.*, 1977; Bellve *et al.*, 1979; Shabanowitz *et al.*, 1986; Thomas *et al.*, 1989). By undertaking Northern blot analysis with mRNA isolated from different stages of testis development it is possible to correlate the presence of specific messages with the initial appearance of stage-specific cell types. Moreover, to confirm whether the temporal accumulation of a particular mRNA transcript is a result of it's expression in the more advanced germ cell stage, Northern blots prepared from RNA isolated from the equivalent populations of purified germ cells can be undertaken. This approach has been used by a number of workers to establish the developmental expression pattern of a variety of genes in rodent testis, including: the lactate dehydrogenase (LDH) multigene family (Thomas *et al.*, 1990; Jen *et al.*, 1990; Alcivar *et al.*, 1991); choline acetyltransferase (Ibanez *et al.*, 1991); zinc finger proteins, *Zfp-35* (Cunliffe *et al.*, 1990), and *Zfp-29* (Denny and Ashworth, 1991); isotypes of cytochrome, c_T and c_S, (Hake *et al.*, 1990); a novel G-protein coupled receptor (Meyerhof *et al.*, 1991); and proacrosin (Kashiwabara *et al.*, 1990). In some cases, confirmation of the cell type(s) expressing a particular transcript also included *in situ* hybridization studies with seminiferous tubules of pre- and post-pubertal animals using antisense RNA. This was necessary to confirm the presence of the mRNA in a particular germ cell stage, as some purified germ cell preparations used for Northern blot analysis were contaminated by other cell types.

It was established from the previous chapter, that the 5 kb mRNA transcript corresponding to 352 cDNA was present at significantly higher levels in adult human testis when compared to other tissues. This up-regulation might suggest a specific function in the testis such as a role in the post-meiotic differentiation of spermatozoa. Therefore, the expression pattern of 352 mRNA during postnatal development of the testis, was investigated in order to correlate the presence of 352 transcripts with the appearance of a specific germ cell type, such as the spermatid. Subsequently, purified populations of meiotic and post-meiotic germ cells were used to investigate the presence of 352 mRNA in these cell types.

Age (days)

kb 7 10 15 22 27 27 31 34 45 55 90

(a)

9.5—
7.5—
4.4—
2.4—
1.4—
0.78—
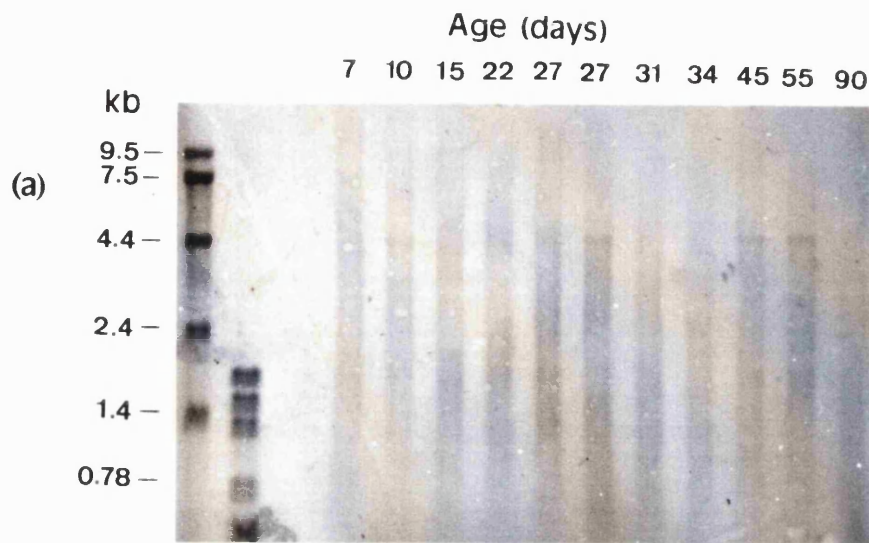
(a) Methylene blue stained blot containing poly (A+) RNA (1 µg) from the testes of pre- and post-pubertal rats shows the presence of intact mRNA in all lanes. The age (in days) of the rats from which RNA was isolated is presented above each lane. Stained RNA molecular weight markers are shown on the left of the blot.

Age (days)

7 10 15 22 27 27 31 34 45 55 90

(b)

kb
— 4.6

(c)

—1.45
—1.3
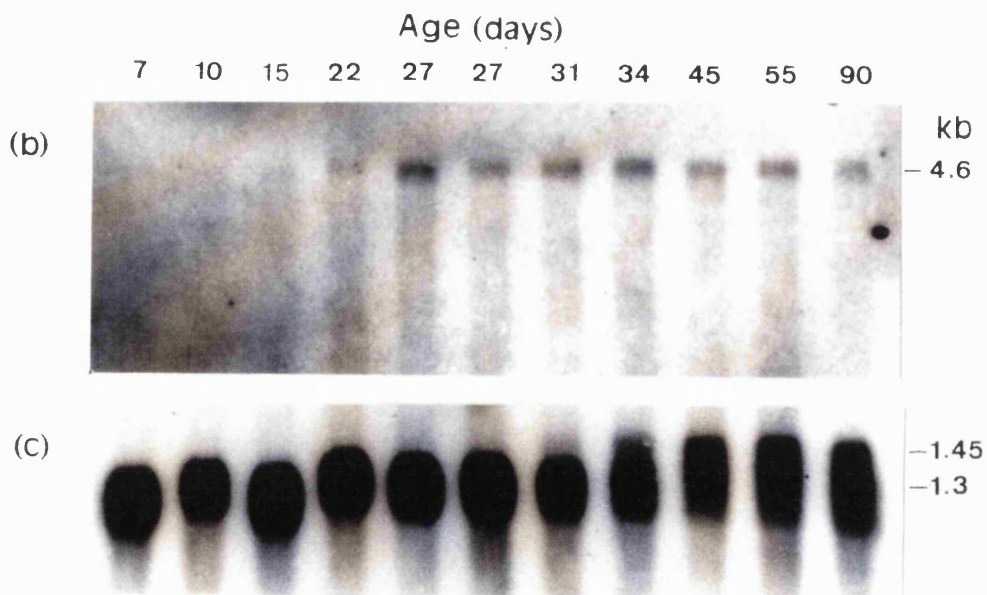
The Northern blot was probed with 32P-labelled 352 cDNA insert (b), and subsequently reprobed with GAPDH (c). The blot was washed under high stringent conditions.

Figure 33. Expression pattern of 352 mRNA during the postnatal development of rat testis.

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DEVELOPMENTAL EXPRESSION PATTERN OF 352 mRNA IN RAT TESTIS

Northern blot analysis was used to investigate the expression pattern of 352 mRNA during the postnatal development of the rat testis. The presence of 352 mRNA transcripts at a particular stage of seminiferous epithelial development, may coincide with the appearance of a particular germ cell at a more advanced stage of differentiation. This might therefore suggest a specific functional role for 352 protein in the differentiation of this germ cell stage.

Total RNA was extracted from the testes of 7, 10, 15, 22, 27, 31, 34, 45, 55, and 90 day old rats using the method of Chomsckynski and Sacchi (see appendix), and poly(A+) containing RNA isolated by oligo (dT) cellulose, outlined in the appendix. Messenger RNA (1 µg) from each age point was run on a 1% agarose gel containing formaldehyde, and subsequently transferred to Hybond-N nylon membrane by capillary action as described in the appendix. The Northern blot was stained with methylene blue (see appendix) to assess the integrity of the RNA. Following destaining, the blot was probed with ^{32}P-labelled 352 cDNA (see appendix), washed at high stringency (see appendix) and then exposed to X-ray film with intensifying screens for 10 days in order to allow the detection of a signal. To normalise loading of mRNA in all lanes, the blot was subsequently probed with ^{32}P-labelled rat GAPDH cDNA, washed at high stringency, and exposed to X-ray film for 24 hours.

RESULTS

The methylene blue stained blot showed a smear stretching from over 9.5 kb to less than 1 kb in all lanes, indicating the high integrity of mRNA samples (figure 33a). Northern blot analysis showed a very faint band of 4.6 kb present in the testes of 15 day old rats (figure 33b, lane 3), increasing in intensity at 22 days (lane 4), and becoming most intense from 27 days through to adulthood (lanes 5 to 11). Therefore, 352 mRNA is expressed at low levels coinciding with the accumulation of pachytene spermatocytes within the seminiferous epithelium, and becomes more abundant at 27 days concomitant with the appearance of round spermatids. The 352 mRNA levels remain high throughout post-meiotic differentiation. Subsequent hybridization with ^{32}P-labelled GAPDH cDNA showed a discrete band of 1.3 kb in all lanes (figure 33c), indicating that the integrity of RNA species had been maintained, and that all RNA samples had been loaded evenly. A

faint band of 1.45 kb was evident at 31 days (lane 7), increasing in intensity by 34 days (lane 8), and becoming most intense from 45 onwards (lanes 9 to 11). This higher molecular weight band represents a spermatid-specific GAPDH transcript (Welch *et al.*, 1992; Mori *et al.*, 1992).

GERM CELL EXPRESSION OF 352 mRNA

The expression pattern of 352 mRNA in the testis of pre- and post-pubertal rats, indicated the developmental regulation of the 352 gene within the rat testis. An increase in the level of expression of the 4.6 kb transcript, was evident at 22 days coinciding with the presence of pachytene spermatocytes, with highest expression occurring at 27 days concomitant with the appearance of round spermatids. The high levels of expression remained constant throughout post-meiotic development up to maturity (90 days).

Northern blot analysis

Northern blot analysis using RNA from adult rat testis versus round spermatids was performed in order to establish whether the spermatid was the cell type responsible for the elevated levels of 352 mRNA expression at 27 days. If this was the case, a 4.6 kb band of higher intensity would be expected in the lane containing RNA from round spermatids when compared to adult testis or pachytene spermatocytes.

Total RNA was extracted from round spermatids (95%) isolated by panning (chapter 2), using the method outlined previously (page 70). Total RNA was extracted from adult rat testis with guanidinium thiocyanate and cesium chloride solutions described in the appendix. Northern blots were prepared with total RNA (20 µg) from adult rat testis and round spermatids. Total RNA was run on 1% agarose gels containing formaldehyde, and transferred to Hybond-N nylon membrane as described in the appendix. The blot was prehybridized and subsequently hybridized with radiolabelled 352 cDNA, and then exposed to X-ray film for 10 days.

The blot was probed with radiolabelled GAPDH cDNA to ensure RNA integrity had been maintained, and to normalize loading of RNA in both lanes.
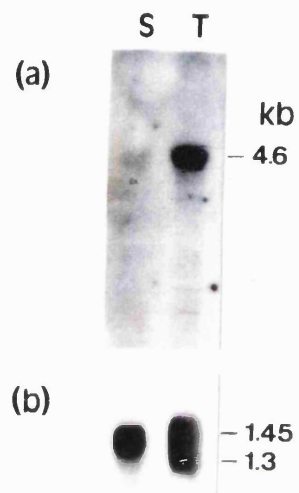
Figure 34. Germ cell expression of 352 mRNA.

Northern blot analysis of total RNA (20 μg) from adult rat testis (T) versus 95% round spermatids (S), probed with 32P-labelled 352 cDNA insert (a), and subsequently with GAPDH cDNA insert (b).

RESULTS

The Northern blot (figure 34a) showed a distinct band of approximately 4.6 kb present in testis (T), and a much less intense band present in spermatids (S). Following hybridization with radiolabelled GAPDH cDNA (figure 34b), a 1.3 kb somatic transcript and a 1.45 kb spermatid-specific transcript was evident in testis, with a single more intense band of 1.45 kb present in spermatids.

DISCUSSION

The previous chapter showed that the 5 kb mRNA transcript from which 352 cDNA derived, is expressed at a higher level in human testis when compared to other tissues. This increase in expression might suggest a specific role of the corresponding protein during sperm differentiation. During postnatal development, the seminiferous epithelium is characterized by the appearance of spermatogenic cells at progressive stages of development (Bellve *at al.*, 1977; Shabanowitz *et al.*, 1986; Thomas *et al.*, 1989). Therefore, Northern blot analysis of RNA from the testes of pre- and post-pubertal rats can be used to correlate the temporal expression of a specific mRNA transcript with the appearance of a particular germ cell at a more advanced stage of development. This approach was used in order to establish whether the gene encoding 352 was developmentally regulated within the testes of juvenile rats, and if so, to correlate the presence of 352 mRNA with the appearance of a specific germ cell stage.

Northern blot analysis of mRNA from the testes of juvenile rats, showed a 4.6 kb transcript expressed at low levels in 15 and 22 day old animals, with an increase in expression evident by 27 days. The low levels of expression at 15 and 22 days coincide with the accumulation of pachytene spermatocytes within the seminiferous epithelium, such that, this cell type is present in 20-30% of seminiferous tubules by day 15, and in 80% of tubules by day 22 (Shabanowitz *et al.*, 1986). However, the increase in 352 expression in 27 day old animals correlates with the appearance of stage 1-5 spermatids, which occurs at approximately 25 days of age (Bellve *et al.*, 1979). The levels remain elevated throughout spermiogenesis. These results indicate that the 352 gene is developmentally regulated within the testes of juvenile rats, and that the increase in expression of 352 mRNA in 27 day old animals concomitant with the appearance of

spermatids, might be related to the formation of round spermatids and the subsequent post-meiotic differentiation events that take place to form spermatozoa.

The next step was to investigate the cell type(s) responsible for the elevated level of 352 mRNA expression at 27 days. Published data have shown the appearance of specific transcripts or an increase in their expression, to be a result of predominant expression in the more advanced germ cell type formed at that particular stage of testicular development (Thomas *et al.*, 1989; Thomas *et al.*, 1990; Jen *et al.*, 1990; Meyerhof *et al.*, 1991). This suggested that spermatids might be the major source of 352 mRNA expression, as these germ cells are not present in the seminiferous epithelium of 22 day old rats when levels of 352 mRNA were low, but are present by 27 days when levels had increased significantly. A similar pattern of expression was observed in the testes of pre- and post-pubertal mice for PGK-2 mRNA (Thomas *et al.*, 1989), and subsequently found to be present at low levels in pachytene spermatocytes but at much higher levels in round and elongating spermatids.

In the present study, Northern blots were prepared with total RNA from adult rat testis, and round spermatids (95%) purified by panning. Autoradiographs showed a distinct 4.6 kb band present in testis but much lower levels in spermatids. These results indicated that spermatids were unlikely to be the cell type responsible for the increase in 352 mRNA expression seen at 27 days. As low levels of 352 mRNA were present with the accummulation of pachytene spermatocytes (at 15 and 22 days), it was intended to establish whether 352 is expressed in this germ cell stage. However, the RNA isolated from these cells was degraded, most likely a result of insufficient freezing of cells during their transportation. Despite this, it was reasoned that even if pachytene spermatocytes do express 352 mRNA, it is unlikely to be the cell type responsible for the elevated levels of expression that occur at 27 days. This is primarily because the highest proportion of pachytene spermatocytes are present by 22 days, so the increased levels of 352 mRNA would be expected to be evident at this stage of testicular development. Also, the levels remain elevated throughout spermiogenesis, which would not be expected if predominant expression occurred in pachytene spermatocytes, since expression decreases once spermatids have appeared.

From these results it was suggested that the elevated levels of 352 mRNA present in the testes of 27 day old rats and throughout spermiogenesis, may be a result of predominant

expression in Sertoli cells, although other cell types cannot be ruled out. This hypothesis, as well as future work necessary to further characterize 352, is discussed in Chapter 6.

CHAPTER 5

SEQUENCING OF THE 352 CLONE
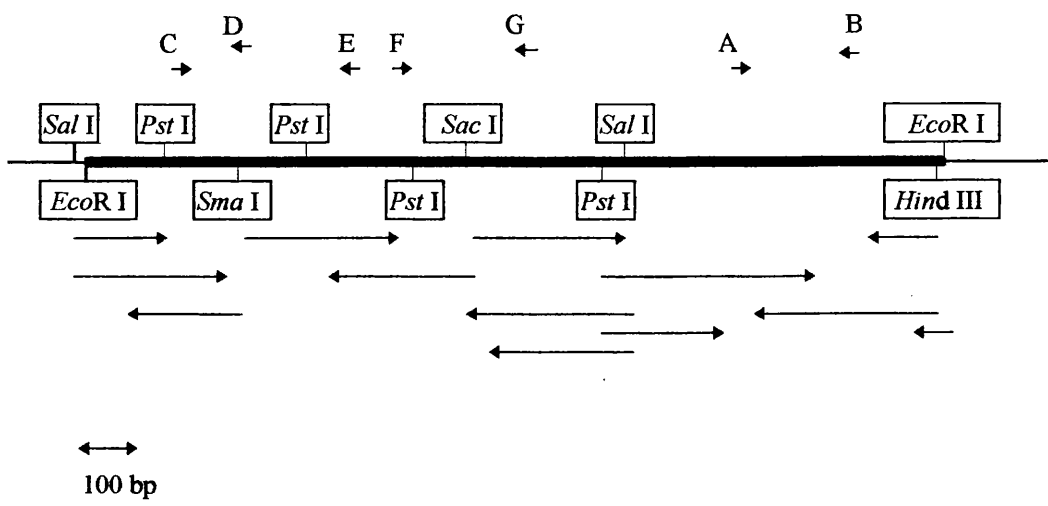
Figure 35. Diagrammatic representation of a partial restriction map of 352 cDNA and the sequence strategy used.

Various restriction digests of 352 cDNA in Bluescript plasmid vector were subcloned into M13mp18 and M13mp19 phage vectors for sequencing. Arrows indicate the location and extent of each sequence. A, B, C, D, E, F, and G correspond to primers used to confirm sequence, particularly areas across restriction sites of adjacent subclones.

INTRODUCTION

It was established that 352 derived from a 5 kb mRNA transcript in human testis (rat homologue of 4.6 kb), indicating that 352 represented a partial clone which was lacking 3 kb of sequence. Furthermore, it appeared the corresponding mRNA transcript is developmentally regulated within the testes of juvenile rats with increased expression correlating with the appearance of spermatids, suggesting that 352 protein may indeed have a role during spermiogenesis.

This chapter outlines the strategy used to obtain the nucleotide sequence of the 352 clone, and subsequent analysis of this sequence data in order to investigate homologies at the nucleotide and protein levels to previous published sequences.

SEQUENCE STRATEGY

The 2 kb 352 cDNA insert cloned into Bluescript plasmid vector (as outlined in chapter 3), was digested with various restriction enzymes in order to obtain overlapping DNA fragments. From this information, a partial restriction map was constructed which defined the location of these restriction enzyme sites along the length of the 352 clone (figure35). Each fragment was subcloned into M13mp18 or M13mp19 following standard procedures described in the appendix (phosphatase treatment of the vector, ligation of insert and vector, transformation of recombinant M13 into E.coli, and template preparation), and single-strand sequenced in both directions using the Sanger dideoxy chain termination method with M13 primers (see appendix). The sequence across the restriction enzyme sites of adjacent subclones was confirmed by double-stranded sequencing in Bluescript vector using primer pairs flanking these regions which were synthesized according to previously defined sequences from the 352 clone. A diagrammatic representation of the sequence strategy as well as the location of the primers, is shown in figure 35.

```
    1   gggcgcacgagttcgtgacactcatctctactaccatggatgcaatcacacctctaatca    60
    1    A  H  E  F  V  T  L  I  S  T  T  M  D  A  I  T  P  L  I  S   20
   61   gcaccaaggtccaagacaagctgctgctatctgcgtgccacttactggtctcactggcca   120
   21    T  K  V  Q  D  K  L  L  L  S  A  C  H  L  L  V  S  L  A  T   40
  121   ccaccgtgcggcccgtctttctgatcagcatccctgcagtgcagaaagtattcaacagaa   180
   41    T  V  R  P  V  F  L  I  S  I  P  A  V  Q  K  V  F  N  R  I   60
  181   tcactgatgcctctgccctgcgacttgtcgataaggcccaggtgttggtgtgccgagccc   240
   61    T  D  A  S  A  L  R  L  V  D  K  A  Q  V  L  V  C  R  A  L   80
  241   tctctaacatcttgctgcttccgtggccaaaccttccagagaatgagcagcagtggcccg   300
   81    S  N  I  L  L  L  P  W  P  N  L  P  E  N  E  Q  Q  W  P  V  100
  301   tgcgctccatcaaccatgccagcctcatctctgcactctcccgggactatcgcaacctga   360
  101    R  S  I  N  H  A  S  L  I  S  A  L  S  R  D  Y  R  N  L  K  120
  361   agcccagtgctgttgccccacagagaaagatgccactggatgacaccaaactgattatcc   420
  121    P  S  A  V  A  P  Q  R  K  M  P  L  D  D  T  K  L  I  I  H  140
  421   accagacactcagcgtcttagaagatattgtggagaatatctcgggggagtccaccaagt   480
  141    Q  T  L  S  V  L  E  D  I  V  E  N  I  S  G  E  S  T  K  S  160
  481   ctcgacagatttgctaccagtcgctgcaggaatctgttcaggtctccctggccctctttc   540
  161    R  Q  I  C  Y  Q  S  L  Q  E  S  V  Q  V  S  L  A  L  F  P  180
  541   cagcttttatccatcagtcagatgtgactgatgagatgctgagcttcttcctcactctgt   600
  181    A  F  I  H  Q  S  D  V  T  D  E  M  L  S  F  F  L  T  L  F  200
  601   ttcgaggccttagagtacagatgggtgtgcctttcactgagcaaatcatacagactttcc   660
  201    R  G  L  R  V  Q  M  G  V  P  F  T  E  Q  I  I  Q  T  F  L  220
  661   tcaacatgtttaccagagagcagttagccgagagcatcctccacgacagcacaggctgcc   720
  221    N  M  F  T  R  E  Q  L  A  E  S  I  L  H  D  S  T  G  C  R  240
  721   gggtggtggagaagtttctgaagatcctgcaggtggtggtccaggagccaggccaggtgt   780
  241    V  V  E  K  F  L  K  I  L  Q  V  V  V  Q  E  P  G  Q  V  F  260
  781   tcaagcccttcctccccagcatcatcgccctgtgcatggagcaagtgtatcccatcattg   840
  261    K  P  F  L  P  S  I  I  A  L  C  M  E  Q  V  Y  P  I  I  A  280
  841   ccgagcgtccctcccctgatgtgaaggccgagctgtttgagctccatcacaactggaggt   900
  281    E  R  P  S  P  D  V  K  A  E  L  F  E  L  H  H  N  W  R  Y  300
  901   acttcttcaagtccaccgtgctggccagtgtccagaggggggatcgctgaggagcagatgg   960
  301    F  F  K  S  T  V  L  A  S  V  Q  R  G  I  A  E  E  Q  M  E  320
  961   agaatgagccccagttcagtgccatcatgcaggctttcggacagtcctttctccagcccg   1020
  321    N  E  P  Q  F  S  A  I  M  Q  A  F  G  Q  S  F  L  Q  P  D  340
 1021   acatccacctttttaaacaaaatctcttctacttggagactctcaacaccaagcagaagc   1080
  341    I  H  L  F  K  Q  N  L  F  Y  L  E  T  L  N  T  K  Q  K  L  360
 1081   tgtaccacaagaagatcttccggactgccatgctgttccagtttgtgaacgtgctgctcc   1140
  361    Y  H  K  K  I  F  R  T  A  M  L  F  Q  F  V  N  V  L  L  Q  380
 1141   aggtcctggtccacaagtcccatgatcttctgcaggaggagattggcatcgcatctacaa   1200
  381    V  L  V  H  K  S  H  D  L  L  Q  E  E  I  G  I  A  S  T  T  400
 1201   catggcctcagtcgactttgatggcttctttgccgccttcctcccagagttcctgaccag   1260
  401    W  P  Q  S  T  L  M  A  S  L  P  P  S  S  Q  S  S  *        420
 1261   ctgtgatggtgtggatgccaaccagaaaagtgtgctggggcggaatttcaagatggatcg   1320
 1321   ggacctgccctcattcacccagaatgtgcacaggctggtcaacgactgcgctactacaga   1380
 1381   ctctgcaacgacagcctgcccctggcactgtgaagctctaggcctgctactgcctgggg   1440
 1441   caaccggacttctgctgctgccacctgcgccagccctaccttccaccacagatgtctcca   1500
 1501   gatgggccttggtcacactccttggcttctcccaccgcaagcaacgctgcctgcctctgc   1560
 1561   cgctcctccacatcttgccgctgccagcagagctggcttctgggtccacctgagcactgg   1620
 1621   acggtgctcccagggcgttgggagcaggagggggtgtgtggccaggtactaggaggcac   1680
 1681   caggaaatccgcgggggtggcccatgcagaccaggcgcacgtggctcatggggcagaattg   1740
 1741   ccaaggacagctcacgacagtgccaccttctcaccattccagccaaggagagatgtgacg   1800
 1801   ttggaactgctctggcacttctgtcaagcctcccccgccccaattgccttgagatctctg   1860
 1861   ctctttgtcagagatttgcaaagactcacgtttttgttgttttctcatcattccattgtg   1920
 1921   atactaagaaactaagaagcttaatgaaaagcccgaattcctg   1963
```

Figure 36. Complete nucleotide sequence of 352 clone showing the computer predicted ORF.

Computer translation of the nucleotide sequence in the three possible reading frames on both DNA strands, revealed a potential open reading frame (ORF) of 1255 bp representing 417 amino acids. The translational stop codon and the polyadenylation site, designated TGA and AATGAA respectively, are indicated in boxes. Amino acid 152 (N, asparagine) was identified as a potential glycosylation site, and is underlined.

The underlined sequence situated between nucleotides 1955 and 1963 represents Bluescript vector sequence which includes the EcoR I cloning site (GAATTC).

RESULTS

Analysis of DNA sequence data

The 1963 bp nucleotide sequence of the 352 clone is presented in figure 36, and reveals a putative polyadenylation signal (sequence AATGAA) found between nucleotides 1943 and 1948, but no poly A tail. Computer translation of the nucleotide sequence in the three possible reading frames on both DNA strands revealed a potential open reading frame (ORF) of 1255 bp representing 417 amino acids, with a putative translational stop codon, TGA, at nucleotide 1254 (figure 36). Amino acid 152 (N, asparagine) was identified as a glycosylation site.

Although a comparison of the 352 sequence to those of the Genbank/EMBL nucleotide database revealed no significant homology to previously published sequences, one particular region of the clone included a 53% match in a 256 bp overlap (between nucleotides 758 and 1003) with *brt*, a mouse gene encoding a novel receptor-type protein-tyrosine kinase (Fujimoto and Yamamoto, 1994). This homology lay within the extracellular IgL domain, an immunoglobulin-like repeat conserved among extracellular matrix proteins, neural cell adhesion molecules (N-CAM), and cell surface receptors with tyrosine kinase or phosphatase activities. However, comparison of the polypeptide encoded by the 352 cDNA sequence to the Owl non-redundant protein database, did not reveal significant homology to the IgL domain, or to any other protein.

Structural Motifs in the Peptide Sequence

Computer analysis was used to determine potential secondary structure, such as α-helices, β-sheets, turns and coils, within the 352 peptide sequence, which is presented as a two dimensional plot in figure 37. The antigenicity of the protein sequence according to Chou and Fasman (1978) revealed a length of 411 amino acids at residues 4 to 414 as antigenic.

In addition, computer predicted hydrophobicity plots (Kyte and Doolittle, 1982) revealed the presence of both hydrophilic and hydrophobic areas within the 352 protein sequence (figure 38).

Figure 37. Computer predicted 2-dimensional plot representing the secondary structure of the 352 peptide sequence (Chou and Fasman, 1978).

The α-helices are shown as a sine wave, β-sheets with sharp saw-tooth waves, turns with 180^0 turns, and coils with a dull saw tooth wave.

PLOTSTRUCTURE of: 352.pep3 ck: 7375

TRANSLATE of: 352.rev chord: 5492 from: 3 to: 1963

Chou-Fasman Prediction
January 6, 1995 18:26

KD Hydrophilicity >=1.3

KD Hydrophobicity >=1.3

NH2

50

100

150

200

250

300

350

400

HOOC
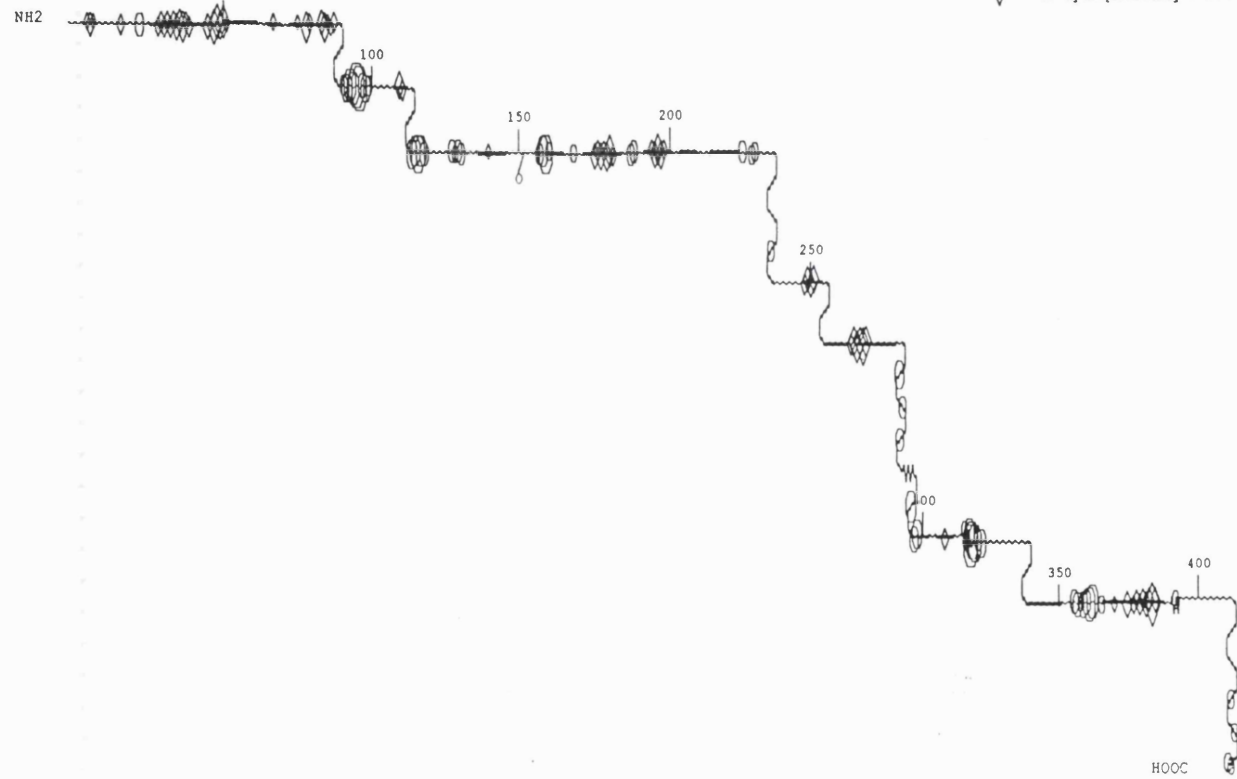
Figure 38. Computer predicted one-dimensional plot of 352 hydrophobicity (Kyte and Doolittle, 1982).

The amino acid residues are numbered below the plot, and the curve is plotted as the average of a residue specific hydrophobicity index. Hydrophobic regions are indicated when the line is situated in the upper half of the frame (above 0), while hydrophilic regions are indicated when the line is situated in the lower half (below 0).

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Kyte-Doolittle
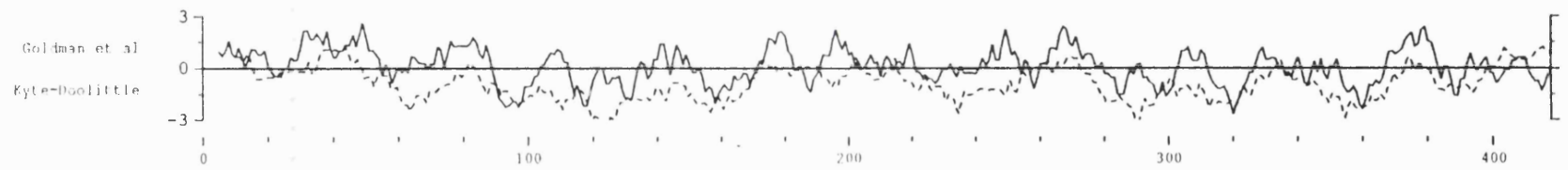
HPhobic

HPhilic

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DISCUSSION

Although this data suggests that 352 represents a novel gene, the possibility that the presently unsequenced region from the 5' end of the clone may be homologous to DNA or protein motifs present in the databases, cannot be excluded. In order to obtain additional sequence data, the human testis cDNA library could be rescreened with ^{32}P-labelled 352 cDNA probe. Positive clones might include a longer 352 cDNA from which 5' sequence could be obtained. Alternatively, RACE (rapid amplification of cDNA ends)-PCR could be persued (Frohman, 1990).

Although there was little evidence of any significant homology to published sequences at either the nucleotide or protein level, analysis of the nucleotide sequence revealed 53% homology to the IgL domain of the *brt* gene (a novel receptor-type protein-tyrosine kinase). The IgL domain is an immunoglobulin-like repeat conserved among extracellular matrix proteins, neural cell adhesion molecules (N-CAM), and cell surface receptors with tyrosine kinase or phosphatase activities. The acrosomal antiserum used to screen the testis expression library was raised against acrosomal membranes which might also contain the overlying plasma membrane and some matrix molecules. Therefore, it is feasible that the 352 clone might contain such domains that play a part in mediating cell adhesion. This is an important feature during spermatogenesis as the maturing germ cell moves up through the seminiferous epitheium. However, translation of 352 nucleotide sequence revealed no significant homology at the protein level, and consequently gave little indication of the possible function of 352.

A potential glycosylation site is situated at amino acid residue 152 (N, asparagine) of the 352 translated sequence. Glycosylation of various amino acids is common in membrane proteins and extracellular domains. However, the relevance of this single glycosylation site is not clear at present. Computer predicted secondary structure of 352 protein shows the first level of folding to form α-helices, β-sheets, turns and coils. However, due to the absence of the entire amino acid sequence, the tertiary protein structure which results from second and third levels of folding to form particular protein domains, is not known.

Although conventional polyadenylation of RNA polymerase II transcripts requires an AATAAA hexanucleotide 10 to 29 nucleotides 5' of the poly(A) addition site, this sequence was not present in 352 cDNA. Instead, an AATGAA hexanucleotide was

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situated between nucleotides 1943 and 1948, 15 bp upstream from the 3' end of the cDNA. This polyadenylation signal sequence has also been found within the 3' untranslated region of various spermatid-specific histone H2b transcripts (Challoner *et al.*, 1989). Furthermore, it was shown that the AATGAA element was located within the H2b purine-rich box, CAATGAAAGA. This consensus sequence, as well as an upstream hairpin structure, is required for the U7 small nuclear ribonucleoprotein-mediated cleavage reaction that generates the 3' ends of poly(A)- H2b mRNAs in somatic cells, during the S phase of the cell cycle. However, although these elements are present in the poly(A)+ H2b spermatid-specific transcripts, they do not function as signals for endonucleolytic cleavage. Instead, these spermatid mRNAs retain both these sequences and are polyadenylated at a more distal site (Challoner *et al.*, 1989). The hairpin structure and purine-rich elements were not found in the 3' UTR of the 352 cDNA.

Most of the peptide sequence is potentially antigenic over a length of 411 amino acids at residues 4 to 414. Therefore, antibodies could be raised for immunohistochemical analysis of seminiferous tubules in order to identify the cell type(s) that expresses 352 protein.

In conclusion, the sequence data presented in this chapter suggests that 352 cDNA might derive from a novel gene. However, sequence analysis of the 5' terminus might reveal significant homologies to DNA or protein motifs such as a membrane protein or secretory protein.

CHAPTER 6
DISCUSSION

Spermatogenesis is a complex and fascinating differentiation process which involves gross morphological changes to convert the spermatogonial stem cell into a spermatozoa. These unique events are largely a result of cell-cell communication that exists within the complex organization of the mammalian seminiferous epithelium, and have been outlined in chapter 1. However, it is this complex developmental system that has at times made it difficult to study, and thus limited the progress of our understanding of male germ cell development. A variety of experimental approaches have been used over the years (reviewed in chapter 1), many of which have provided us with further insight into the mechanisms of mammalian sperm development and it's regulation. It is, however, also important to remember that some of these approaches, such as the use of various culture systems, have shortcomings which have made the significance of various data confusing and inconclusive. With this knowledge in mind, more research is required to provide us with a clearer understanding of these complex cellular events.

A much widely used method is the isolation and subsequent characterization of genes that are expressed at specific stages of germ cell development (chapter 1). Furthermore, many of these genes have been found to be expressed exclusively or almost exclusively in the testis. The identification of such developmentally regulated genes, which are most likely switched on as a result of cell-cell interactions with Sertoli cells, is important for our understanding of this differentiation process. For example, the protein products encoded by a number of genes expressed at meiotic and post-meiotic spermatogenic stages, may contribute to the biogenesis of particular organelles present in spermatozoa, such as the acrosome, or the formation of structures like the flagellum, which contribute to the later function of the spermatozoon.

The aim of my research was to identify genes expressed in the post-meiotic germ cell, but more specifically, those encoding proteins that may be targeted to the acrosome or the plasma membrane overlying it.

During spermiogenesis, the post-meiotic germ cell (round spermatid) undergoes perhaps one of the most dramatic morphological changes of all cells in the body to form the spermatozoon. This transformation includes the formation of the acrosome from the Golgi apparatus vesicle situated at the anterior pole of the spermatid nucleus. As the acrosome plays a fundamental role in events leading up to fertilization, the identification of acrosomal proteins is essential in order to investigate the functional roles these

proteins may have in the biogenesis of this organelle and in the events preceding fertilization. With this in mind, two experimental strategies were devised in order to isolate mRNA transcripts that may encode acrosomal proteins.

The first strategy was designed to immunologically isolate polysomal mRNA encoding proteins targeted to the acrosomal region, by reacting them with antiserum raised against mammalian acrosomal membranes. This method is a novel approach for identifying genes expressed during mammalian sperm differentiation, and has the advantage over other methods of isolating specific transcripts that are actively synthesizing polypeptides. As a number of mRNAs are stored as ribonucleoprotein particles during spermatogenesis prior to translation at a later stage of development, the isolation of stage-specific cDNA clones using conventional library screening methods, does not necessarily establish that the corresponding mRNA is also being translated.

The polysomal strategy did prove a viable method but further modification to the methodology was required to achieve optimal practical results. These points are discussed in detail in chapter 3. Briefly, the more desirable option to use polysomes extracted from highly purified round spermatid in comparison to adult rat testis is still required. Because extracted polysomes can aggregate, it is probably important that sub-fractionation of germ cell types is undertaken prior to polysome extraction with specific antiserum.

In order to increase the purity of round spermatids and consequently the isolation of stage-specific polysomal mRNA, an immunoselection method (panning) was adopted. This rapid and relatively inexpensive technique was developed that used a specific monoclonal antibody, 97.25, which binds to a 95 kD antigen present on the plasma membrane of spermatids overlying the developing acrosome. Moreover, in addition to extracting polysomes, intact and pure RNA was extracted from these viable cells and subsequently used for Northern blot analysis. Although initial panning experiments were successful in that purities of 95% were achieved, at this stage of the study, spermatid yields were not high enough to provide sufficient polysomal mRNA for reacting with acrosomal antisera. Spermatid yields were significantly increased by modifying the primary preparation of testis cell suspensions so that cell lysis was minimized. In addition, the coating of the panning dishes with higher concentrations of primary and secondary antibodies led to greater recovery of spermatids.

At the same time the modifications to the panning technique were being investigated, the second strategy devised to isolate mRNA transcripts that may encode acrosomal proteins, was pursued. A human testis cDNA expression library was screened with specific rabbit antiserum raised against hamster acrosomal membranes which cross reacted solely with acrosomal components of germ cells in the rat testis and no other cell type (Moore *et al.*, 1993). A number of cDNA clones were isolated, and based on immunospecificity of expression protein, and initial sequence data, a 2 kb clone designated 352 was selected for further characterisation. Furthermore, due to the method of isolation, it was considered quite possible that this potentially novel cDNA clone might encode an acrosomal protein. Given the limited time available the polysomal strategy was not pursued further. Nevertheless it remains a viable method for use in future research.

Northern blot analysis revealed that the human testis cDNA clone 352 was derived from a 5 kb mRNA transcript in human testis, with a rat homologue of 4.6 kb. The homology between the human and rat 352 transcripts was high as there was a distinct band present in rat tissue following high stringent washes. Southern blot analysis showed the gene from which 352 derives to be highly conserved across species, and that the 5 kb mRNA transcript was expressed at low levels in a range of human tissues but present at much higher levels in the testis. Assuming the corresponding protein is synthesized, it appeared that 352 gene product would be restricted to a particular cell type in the testis. At this stage, however, there was no direct evidence as to the identity of this cell type.

In order to investigate which cell type(s) within the testis expressed 352 mRNA, Northern blot analysis using mRNA isolated from the testes of pre- and post-pubertal rats was employed in order to correlate the temporal expression of 352 mRNA with the appearance of a particular stage of germ cell development (chapter 4). My results demonstrated that a 4.6 kb transcript is first expressed at low levels in 15 and 22 day old rats coinciding with the initial development of pachytene spermatocytes within the seminiferous epithelium. However, significantly increased levels of expression were present in 27 day old animals concomitant with the appearance of stage 1-5 spermatids, which remained elevated throughout spermiogenesis. From these results it was concluded that the gene encoding 352 appeared to be developmentally regulated during postnatal testicular development with increased transcription correlating with the

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appearance of round spermatids. Further Northern blot analysis indicated that round spermatids were not responsible however for elevated expression of 352 mRNA in 27 day old rats. In addition, despite the inability to establish whether 352 expression occurs in pachytene spermatocytes (due to degraded RNA resulting from insufficient freezing during transportation), it is unlikely that this cell type is responsible for the elevated levels of expression that occur in the testes of 27 day old rats. This is primarily because the highest proportion of pachytene spermatocytes are present by 22 days, so the increased levels of 352 mRNA would be expected to be evident at this stage of testicular development. Also, the levels remain elevated throughout spermiogenesis, which would not be expected if predominant expression occurred in pachytene spermatocytes, but a decrease in expression once spermatids have appeared. A similar pattern of expression was observed in the testes of pre- and post-pubertal mice for PGK-2 mRNA (Thomas *et al.*, 1989), and subsequently found to be present at low levels in pachytene spermatocytes but at much higher levels in round and elongating spermatids.

It is hypothesized that the elevated levels of 352 mRNA present in the testes of 27 day old rats and throughout spermiogenesis, may be a result of predominant expression in Sertoli cells but further research is required to confirm this premise. Spermatogenic cells remain in close association with Sertoli cells and there is much evidence to indicate a functional interdependence of Sertoli cells and germ cells essential for sperm development (Parvinen *et al.*, 1986; Jegou, 1991; Sharpe, 1993; Parvinen, 1993). Therefore, a number of gene products synthesized in Sertoli cells are likely to play important roles during particular stages of sperm differentiation as a result of cell-cell interaction. It is reasoned that 352 mRNA might be up-regulated in Sertoli cells at 27 days, at which time the gene product has a specific function during the post-meiotic differentiation of spermatids. Each spermatogenic cycle within the seminiferous epithelium consists of 14 stages of germ cell development. Within each stage there is a particular set of germ cell associations each at different stages of development. It has been established for some time that the structure and function of Sertoli cells changes according to the spermatogenic cycle, and therefore the germ cell complement with which they are associated. These functional changes are in accordance with the requirements of each germ cell stage and are thought to be the result of the direct influence of germ cells on Sertoli cells (chapter 1). For example, testosterone has been

shown to have a major effect on cells within stage VI-VII seminiferous tubules of adult rats, such that overall protein secretion is increased two-fold, and more specifically, androgen-regulated proteins (ARPs) are produced (Sharpe *et al.*, 1992). The cellular source of these proteins are likely to be Sertoli cells, peritubular cells, and round spermatids (Sharpe *et al.*, 1992; McKinnell and Sharpe, 1992). Depletion of either pachytene spermatocytes, round spermatids or elongate spermatids at stages VI-VIII largely prevents the normal stage-dependent increase in total protein secretion as well as affecting secretion of ARPs (McKinnell and Sharpe, 1992). If both pachytene spermatocytes and round spermatids are depleted during these stages, then most of the androgen-dependent changes are abolished completely (Sharpe, 1994) demonstrating that a normal germ cell complement is essential for testosterone to exert it's effects on protein secretion by cells of the seminiferous epithelium. Therefore, it is quite feasible that the appearance of spermatids in the testes of 27 day old rats could induce Sertoli cells to synthesize 352 gene products required for spermiogenesis, as a result of Sertoli cell-germ cell interaction. For example, step 1-8 spermatids influence Sertoli cells to secrete androgen-binding protein (ABP), transferrin, inhibin, gamma glutmyl-transpeptidase, ceruloplasmin, sulphated glycoproteins (SGP1 and SGP2); Onoda and Djakiew, 1990. Some of the proteins secreted by Sertoli cells or germ cells may play a role in the differentiation of particular germ cell stages. Recent data suggests that nerve growth factor (NGF) production by germ cells of the rat and mouse (Ayer-Le Lievre *et al.*, 1988) may modulate levels of mRNA for ABP in the Sertoli cell by binding to receptors on Sertoli cells at stages VII-VIII of the spermatogenic cycle (Lonnerberg *et al.*, 1992). It has therefore been proposed that NGF has a role in the onset of meiosis, that is, DNA synthesis in preleptotene spermatocytes (Parvinen *et al.*, 1992). Also, phagocytosis of residual bodies from elongate spermatids induces secretion of IL-1 by rat Sertoli cells *in vitro*, which in turn stimulates spermatogonial stem cell proliferation (Jegou 1991; Jegou *et al.*, 1992; Gerard *et al.*, 1992).

As the 352 clone was isolated from a testis expression library screened with specific antiserum raised against acrosomal components, it might be anticipated that 352 cDNA derived from a transcript encoding an acrosomal membrane protein (discussed in chapter 3). During spermiogenesis, the Sertoli-germ cell relation is intensified with appearance of ectoplasmic specializations and tubulobulbar complexes. The gap junctions, Sertoli cell

processes, tubulobulbar complexes, and residual bodies, are involved in the transfer of material between spermatids and Sertoli cells (Jegou *et al.*, 1992). In addition, Sertoli cell processes which penetrate spermatids may allow the intimate transport of molecules between Sertoli cells and spermatids. There is also *in vitro* evidence for the transfer of Sertoli cell proteins to the germ cell compartment of the seminiferous tubule (Kumari and Duraiswami, 1987). Apart from energy metabolites such as lactate and pyruvate necessary to support metabolism of early spermatids (Jutte *et al.*, 1981), and the transport of amino acids to germ cells, Sertoli cell-derived proteins have been shown *in vitro* to be endocytosed by various germ cells. For example, pachytene spermatocytes and round spermatids are able to bind and internalize transferrin by a receptor-mediated endocytotic process, whereas late spermatids have lost their ability (Toebosh *et al.*,1987). It is therefore feasible that 352 is transcribed and translated in Sertoli cells, and the synthesized protein subsequently transferred to spermatids. Other examples of the transfer of Sertoli cell products to spermatids include SGP2, TGF-β precursor, ABP, and human sex steroid-binding protein (hSBP). SGP2, a putative apolipoprotein which may be involved in lipid transport, is a major secretory product of Sertoli cells and of the caput epididymis in culture. Immunofluorescence had previously revealed the localization of SGP2 in the cytoplasmic regions of Sertoli cells and on the head and tail of spermatozoa isolated from the testis or distal regions of the male reproductive tract (Sylvestor *et al.*, 1984). This reactivity becomes evident as early as step 11 of spermiogenesis. Since SGP2 mRNA is expressed in Sertoli cells, it is possible that SGP2 associated with the plasma membrane of the head and tail constitutes the secreted form of this protein released by Sertoli cells into the seminiferous tubule (Sylvestor *et al.*, 1991). Receptor-mediated endocytosis in germ cells was demonstrated *in vitro* by O'Brien *et al.* (1989). Glycoproteins bearing the mannose-6-phosphate (M6P) recognition marker, such as TGF-β precursor and testins, were secreted by Sertoli cells and subsequently endocytosed by pachytene spermatocytes and round spermatids. The isolated germ cells were shown to endocytose the Sertoli M6P-glycoproteins, and process them to lower molecular weight forms (O'Brien *et al.*, 1989; O'Brien *et al.*, 1991; O'Brien *et al.*, 1994). It was proposed that these endocytosed glycoproteins may include hydrolytic enzymes which might be targeted to specific organelles such as the acrosome, and/or affect germ cell function. In addition, Gerard *et al.* (1991) have shown

that ABP and hSBP could be endocytosed by germ cells from the monkey (*Macaca fascialasis*), including late spermatids and residual bodies. Germ cells have specialized endocytotic apparatus involving clathrin-like coated pits and vesicles which actively internalize hSBP. It was suggested that steroid-binding proteins play a role locally during spermatogenesis either in concentrating steroids at the germ cell surface, or in transporting them across the plasma membrane. These findings indicate that certain Sertoli cell products may be transferred to meiotic and post-meiotic germ cells by endocytosis *in vivo*. Although, the antiserum used for screening exhibited little or no binding to Sertoli cells, proteins of low abundance produced by these cells might only be detected once they are concentrated in their target organelle, that is, the acrosome.

Finally, the 2 kb 352 cDNA clone was sequenced in order to determine the identity of the gene from which it derives, and thus a possible function of the corresponding protein during spermatogenesis. Due to the lack of 5' sequence, the translational start codon could not be identified, and subsequently an open reading frame could not be established from the cDNA sequence. However, computer translation of the nucleotide sequence revealed a potential open reading frame with a putative stop codon (TGA), and polyadenylation signal (AATGAA). A comparison of this sequence to those of Genbank/EMBL nucleotide database revealed little evidence of significant homology to published sequences, apart from a 53% match with the IgL domain of the *brt* gene (a novel receptor-type protein-tyrosine kinase). The IgL domain is an immunoglobulin-like repeat conserved among extracellular matrix proteins, neural cell adhesion molecules (N-CAM), and cell surface receptors with tyrosine kinase or phosphatase activities. As the acrosomal antiserum used to screen the testis expression library was raised against acrosomal membranes (which might also contain the overlying plasma membrane and some matrix molecules), it is feasible that the 352 clone might contain such domains that play a part in mediating cell adhesion. This is an important feature during sperm development, where differentiating germ cells remain in close contact with Sertoli cells, and with the progression of spermatogenesis, move up through the seminiferous epithelium to be finally released as mature spermatozoa into the lumen of seminiferous tubule. However, translation of 352 nucleotide sequence revealed no significant homology to this IgL domain or to other published protein sequences, and consequently gave little indication of the possible function of 352.

Although conventional polyadenylation of RNA polymerase II transcripts requires an AATAAA hexanucleotide 10 to 29 nucleotides 5' of the poly(A) addition site, this sequence was not present in 352 cDNA. Instead, an AATGAA hexanucleotide was situated between nucleotides 1943 and 1948, 15 bp upstream from the 3' end of the cDNA. This polyadenylation signal sequence has also been found within the 3' untranslated region of various spermatid-specific histone H2b transcripts (Challoner *et al.*, 1989), discussed in chapter 5. This data therefore suggests that 352 represents a novel gene. However, the possibility remains that the presently unsequenced region (from the 5' end of the clone) may be homologous to DNA or protein motifs present in the databases.

In summary, the present chapter shows that the gene encoding 352 is developmentally regulated within the testis of juvenile rats. The 352 mRNA transcripts are present at low levels coinciding with the accummulation of pachytene spermatocytes within the seminiferous epithelium, but becomes more abundant with the appearance of spermatids and throughout spermiogenesis. Therefore, it is likely that 352 protein has a specific function during spermatid differentiation. It was subsequently established that the increase in 352 mRNA expression concomitant with the appearance of spermatids, was not a result of predominant expression in this cell type. From this it was hypothesized that Sertoli cells might be the source of this expression as a result of Sertoli-germ cell interaction, the product of which is important for the development of spermatids. *In situ* hybridization of cross sections through pre- and post-pubertal rat testes would be necessary to identify the cell type(s) expressing 352 mRNA at particular stages of testicular development. Although Northern blot analysis using pre- and post-meiotic germ cells as well as Sertoli and Leydig cells, can be informative, some cell purities are compromised and thus contaminated with other cell types which can affect the interpretation of results (Thomas *et al.*, 1990). Therefore, it is more advantageous to perform *in situ* hybridization to investigate 352 mRNA expression in individual cells. As the 352 cDNA clone might correspond to an acrosomal membrane protein or a plasma membrane protein overlying the acrosome, it is hypothesized that the Sertoli synthesized protein is subsequently transferred to spermatids where it might be associated with the plasma membrane or even targeted to the acrosome. However, this would be confirmed by immunohistochemical staining of equivalent testis sections as those used for *in situ*

hybridization studies, with antibodies raised to recombinant 352 protein. This would establish the site of protein synthesis and subsequent localization of the protein product. Furthermore, the functional role of 352 protein during sperm development could be investigated using antisense transgenics. Antisense RNA or DNA would hybridize to complementary sequences in 352 mRNA, thereby disrupting gene expression by reducing or abolishing the translation of 352 transcript. Alternatively, 352 translation might be prevented by selective amputation of the polyadenylation signal using a technique recently demonstrated by Sheets *et al.* (1995) for c-*mos* mRNA in Xenopus oocytes (described on page 23). If 352 protein appears to be located on the cell surface of developing spermatids following immunohistochemical staining of testis sections, *in vitro* systems may allow us to establish whether 352 protein is important in cell adhesion or cell-cell interaction with the Sertoli cell by adding specific antibodies raised to 352 recombinant protein. The testis cell culture systems that might be used include, Sertoli cell-spermatogenic cell co-cultures (page 40), or the more recent cell culture system which employs the cell line 15P-1 that has been shown to support the meiotic and post-meiotic differentiation of male germ cells in the mouse (Rassoulzadegan *et al.*, 1993, page 43).

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APPENDIX

PREPARATION OF TESTIS CELL SUSPENSION

The testes (3-4 g) from one adult rat were decapsulated by removing the tunica aluginea, and chopped into small pieces. The tissue was placed in a 100 ml conical flask and incubated in a solution (15 ml) of dissociation buffer (136 mM NaCl, 5 mM KCl, 0.95 mM $NaH_2PO_4.2H_2O$, 4.05 mM Na_2HPO_4, pH 7.4, and 0.6% glucose), containing 1 mg/ml collagenase (Type II collagenase, 380 U/mg, Sigma) for 30 minutes at 33^0C in a shaking water bath. The suspension was poured into a 50 ml falcon tube and the dissociated seminiferous tubules were allowed to settle over a 10 minute period. The supernatant was aspirated using a 10 ml pipette and then discarded. The tubules were washed in fresh dissociation buffer (33^0C) and allowed to settle over 10 minutes. This procedure was subsequently repeated twice in order to remove most of the collagenase. The washed tubules were then transferred to a conical flask and incubated in dissociation buffer (15 ml) containing 0.5 mg/ml trypsin (Type III bovine trypsin 10,900 U/mg, Sigma), and 10 µg/ml DNase (Type I, 510 U/mg, Sigma) for 15 minutes at 33^0C in a shaking water bath. The trypsin reaction was inhibited by adding trypsin inhibitor (Type I-S, 10,000 U/mg, Sigma) to a concentration of 0.1%, and foetal calf serum to a concentration of 10%. The cells were dispersed by gentle pipetting with a glass pasteur pipette, and were collected by centrifugation in a 50 ml falcon tube for 10 minutes at 500 g. The supernatant was aspirated and discarded, and the cell pellet was gently resuspended in 50 mls of dissociation buffer (33^0C) using a 10 ml pipette. Finally, the cell suspension was filtered through 80 µm nylon gauze twice to remove cell aggregates.

CALCULATION OF THE NUMBER OF CELLS PRESENT IN BSA GRADIENT FRACTIONS

An aliquot (200 µl) of solution from each of the relevant fractions was applied to a Neubayer haemocytometer. The haemocytometer consists of a 0.1 mm^3 chamber engraved with a series of lines forming 0.1 x 0.1-mm squares, and a special coverslip. The number of cells were counted in 5 of the 25 squares within a 0.1-mm square, and the average calculated by dividing by 5. This value was multiplied by 25 to obtain the number of cells in 0.1 mm^3, and subsequently multiplied by 10^4 to obtain the number of cells per ml. The cell yield was

calculated by further multiplying by the number of mls of solution in the collected gradient fraction (10 ml).

INDIRECT IMMUNOFLUORESCENT STAINING

The particular cell preparations were fixed on glass slides as described in the appropriate sections. Cells bound to antibody 97.25 on panning dishes were fixed in 4% paraformaldehyde for 10 minutes, washed three times with PBS, and the cells permeabilized in 0.2% Triton X-100 for 2 minutes.

Cells attached to slides or Petri dishes were incubated with one of the following primary antibodies, for 30 minutes at 37^0C in 5% CO_2 in air:

Monoclonal antibody hybridoma supernatant (2-5 µg/ml IgG): 18.6 or 97.25 was used undiluted. Polyclonal antibody R10 (2 mg/ml) purified by adsorption to protein A-Sepharose (Sambrook et al., 1989), was diluted 1:50 in PBS. Antiserum R4 was diluted 1:100 in PBS.

Preimmune serum diluted 1:100 in PBS was used as a negative control for R4 and preimmune serum (purified by adsorption to protein A-Sepharose) diluted 1:50, was used as a negative control for R10. RPMI media was used as a negative control for 18.6 and 97.25 hybridoma supernatants.

The antibody solution was gently washed off the slide with 0.9% saline and subsequently incubated for 15 minutes as before, with FITC-conjugated goat anti-rabbit or anti-mouse antibodies (Dako) diluted 1:100 in 0.9% saline on slides previously incubated with antiserum or hybridoma supernatant, respectively. The slides were washed with saline, and a drop of citifluor was added using a pasteur pipette. A glass coverslip was placed over the slide, and the stained cells were examined under high power epifluorescence using filter block 495-520nm.

PREPARATION OF MONOCLONAL ANTIBODY HYBRIDOMA SUPERNATANT

The method used is described in Harlow and Lane (1988).

Hybridoma cells (producing antibodies 18.6 or 97.25) stored in 8% DMSO (dimethyl sulfoxide) and 92% FCS (4^0C) in liquid nitrogen (-185^0C), were thawed at 37^0C and transferred to a sterile universal tube using standard tissue culture conditions at all times. Tissue culture medium (10 mls) consisted of RPMI 1640 supplemented with FCS and

sodium pyruvate added to a final concentration of 20% and 0.1%, respectively. The medium was filtered through a 0.22 μm millipore filter. For the production of high titre 97.25 antibody used in panning, antibiotics were also added to the medium (Penecillin G and Streptomycin at a final concentration of 100 U/ml and 100 μg/ml, respectively). The cells were recovered by centrifugation for 5 minutes at 500 g, and the supernatant discarded. This procedure was repeated with fresh medium in order to remove all traces of DMSO. The cells were finally resuspended in tissue culture medium (15 mls), transferred to a 50 ml tissue culture flask, and incubated at 37^0C in 5% CO_2 in air. Once the hybridoma cells had reached the approximate concentration of 10^6/ml, they were passaged by transferring 1-2 mls to a 50 ml-tissue culture flask containing 13-15 mls of tissue culture medium. For the production of larger volumes of antibody, 2-3 mls of hybridoma solution was transferred to a 500 ml tissue culture flask containing 30 mls of medium. Cells were passaged every 2-4 days.

Antibody supernatant was collected following centrifugation for 10 minutes at 1000 g. For the collection of higher-titre supernatants (as used for panning), the cultures were allowed to grow until hybridoma cell death, yielding 20-50 μg of antibody/ml of supernatant (Harlow and Lane, 1988). Sodium azide was added to a final concentration of 0.02% to prevent bacterial contamination, and the supernatant was stored at -20^0C.

To store hybridoma cells in liquid nitrogen, the cultures (5×10^6 to 5×10^7) were transferred to universal tubes and centrifuged at 500 g for 5 minutes.The pellet was resuspended in 8% DMSO and 92% FCS. The cell suspension (0.5 ml) was transferred to a freezing vial and frozen in liquid nitrogen.

NORTHERN BLOT ANALYSIS

i) Gel electrophoresis of RNA

Total RNA (5-20 μg) or poly (A+) RNA (1-3 μg) in a volume of 4 μl was denatured in 1x MEA sample buffer (20 mM MOPS, 1mM EDTA, 5mM sodium acetate adjusted to pH 7.2 with NaOH; 50% deionized formamide, 2.5 M formaldehyde) and incubated for 15 minutes at 65^0C. A denaturing 1% agarose gel was prepared in 1x MEA buffer and made 2.2 M with respect to formaldehyde. Prior to loading samples, 0.2 volumes of 6x loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) was added, and

the RNA was electrophoresed until the the bromophenol blue had migrated 8-10 cm from the wells. The gel was run in 1x MEA at 5-6 V/cm .

ii) RNA transfer

The gels were blotted to Hybond-N nylon membranes in 20x SSC by capillary action as described by Sambrook *et al.* (1989). The RNA was fixed by UV irradiation (254 nm) for 3-5 minutes and covered in Saran wrap to prevent the blot drying out.

iii) Hybridization of Northern blot

The membranes were prehybridized in solution (1x Denhardt's solution, 4x SSC, 0.1% (w/v) SDS, 0.05 M phosphate buffer (pH 6.5), 0.001 M EDTA (pH 7), 5% dextran sulphate, 100 µg/ml salmon sperm DNA pre-boiled) for 1-4 hours at 42^0C in Hybaid bottles in a Hybaid oven. The solution was then replaced with hybridization solution (1x Denhardt's solution, 5x SSC, 0.1% (w/v) SDS, 0.05 M phosphate buffer (pH 6.5), 50% de-ionized formamide, 5% dextran sulphate, 500 µg/ml salmon sperm DNA pre-boiled, 100 µg/ml yeast total RNA, 10 µg/ml poly A) containing the radiolabelled cDNA probe (specific activity of 10^6 cpm/ml), and incubated for 15 hours at 42^0C.

^{32}P-labelling of probes was carried out in the same way as described for Southern blot analysis.

iv) Washes

Blots were washed initially using low stringent conditions, in 2x SSC/ 0.1% SDS at 54^0C for 10 minutes, and then repeated. When appropriate, high stringent washes were carried out in 0.1x SSC, 0.1% SDS at the same temperature initially for 10 minutes, or longer as determined by monitoring the activity of the blot with a Geiger Muller counter.

v) Stripping of blots

A solution of 0.1% (w/v) SDS at 95^0C was poured over the blot for 5-10 minutes and subsequently covered with Saran wrap.

METHYLENE BLUE STAINING

In order to assess the integrity of RNA, the membrane was soaked in 5% acetic acid for 15 minutes at room temperature, and subsequently in a solution of 0.5 M sodium acetate (pH 5.2) containing 0.04% (w/v) methylene blue for 5-10 minutes. The membrane was rinsed in deionized H_2O for 5-10 minutes.

The membrane was destained prior to hybridization with radiolabelled probes by incubating in a solution of 0.2x SSC and 1% (w/v) SDS for 15 minutes at room temperature. The blot was placed in fresh solution after 10 minutes.

SOUTHERN BLOT ANALYSIS AND ZOO-BLOT

DNA from plasmid or template preparations electrophoresed on 0.8-1% agarose gels were transferred to Hybond-N membrane as described in Sambrook et al. (1989). The blots were hybridised with ^{32}P-labelled cDNA probes as outlined below for the Zoo-Blot. The Zoo-Blot (CLONTECH laboratories, Inc) is a nylon membrane to which *EcoR* I digested genomic DNAs from 9 eukaryotic species have been transferred by Southern blotting. Each lane contains 8 μg DNA from: Human, Monkey (Rhesus), Rat (Sprague-Dawley), Mouse (BALB/c), Dog, Cow, Rabbit, Chicken, and Yeast (*Saccharomyces cerevisiae*), and an additional lane containing *Hin*d III digested λDNA molecular size markers.

i) Preparation of radiolabelled probe and specific activity determination

This method is taken from Feinberg and Vogelstein (1983). The plasmid preparation of 352 cDNA insert cloned into Bluescript (352/SK+) was digested with *EcoR* I (BRL), and the 2 kb insert was purified using geneclean.

Other cDNA probes such as GAPDH and β-actin were radiolabelled in the same way.

352 cDNA insert (25 ng) was labelled with 15 μCi [a ^{32}P]-dCTP in a 15 μl reaction. Briefly, DNA was made up to a volume of 9 μl with deionized water, in a microfuge screw-cap tube and heated for 5 minutes at 95-100°C to denature the double stranded DNA. After a brief spin to collect droplets formed by condensation, the following components were added:

3.0 μl oligo labelling buffer (described by Feinberg and Vogelstein, 1983).

0.7 μl BSA (10 mg/ml)

1.5 μl [a ^{32}P]-dCTP

0.8 μl Klenow

The mixture was then incubated for 2 hours in a 37^0C water bath, or for at least 5 hours at room temperature, and the reaction stopped by adding 85 μl "Stop" solution (20 mM NaCl, 20mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.25% SDS). The specific activity of the probe was determined prior to use.

Specific Activity Determination

Labelled probe (1 μl) mixture was dotted onto each of two 2.1 cm glass fibre filters (Whatman #1822021), and allowed to air-dry for a few minutes. One filter was then washed 3 times in 5% TCA (trichloroacetic acid) for 5 minutes to remove unincorporated label, blotted onto paper towel to remove excess TCA, and then allowed to air-dry for a few minutes. The washed and unwashed filters were placed in separate scintillation vials containing 2-3 ml scintillation fluid, and measured for incorporated [^{32}P] and total cpm values (incorporated + unincorporated [^{32}P]), respectively, using a beta counter. The cpm value for the washed filter was approximately 50% that of the unwashed filter. The specific activity of the probe (cpm/μg) could then be determined, using the formula below:

Specific activity of probe (cpm/μg) = cpm (washed filter) x 100 μl (final reaction volume) ÷ 0.025 (μg DNA used in labelling reaction).

The specific activity should be >$5x10^8$ cpm/μg DNA.

ii) Hybridization of DNA probes to Zoo Blot

The method used was according to the manufacturer's instructions (Clontech). Briefly, the blot was prehybridized in 10 mls Hybridization/Prehybridization Solution (outlined below), in a small Hybaid roller bottle, for 3-6 hours at 65^0C, with continuous rotation in a Hybaid oven. The Prehybridization/ Hybridization solution was poured off and replaced with fresh solution containing denatured radio-labelled DNA probe at a concentration of

$1\text{-}2\text{x}10^6$ cpm/ml ($2\text{-}4 \times 10^7$ cpm in 20 ml hybridization solution). The hybridization step took place over 18-24 hours with continuous rotation.

A 20 ml volume of solution was prepared and divided into two 10 ml volumes for use in the prehybridization and hybridization steps.

20 ml Solution	Final Concentration
5mls 20x SSPE	5x SSPE
2mls 100x Denhardt's	10x Denhardt's
4mls 10% SDS	2% SDS
9mls H_2O	

100 μg/ml (100 μl) of sheared salmon sperm DNA (10 mg/ml) was added immediately after denaturation by heating to 95-100^{0}C for 10 minutes, to the prehybridization and hybridization solutions.

iii) Washes

The blot was removed from the roller bottle, placed into a sandwich box, and subjected to a series of washes at low and high stringency, with continuous shaking.

Low Stringency wash (for detection of related genes without perfect homology): The blot was rinsed in 2x SSC/0.05% SDS several times at room temperature, followed by a 40 minute wash at 60^{0}C with several changes of solution.

High Stringency wash (for detection of related genes with high homology): The blot was washed as above, followed by a high stringency wash in 0.1x SSC/0.1%SDS. at 60^{0}C for 40 minutes with continuous agitation. The solution was changed once during the high stringency wash.

The blot was exposed to X-ray film (Kodak) at -70^{0}C using intensifying screens.

iv) Removal of probe

The probe was removed from the blot by pouring boiling sterile water directly onto the membrane, and shaking continuously for 20 mins. This was repeated once to ensure complete removal of the probe, after which the blot was removed and allowed to air-dry briefly prior to storage in Saran-wrap at -20^{0}C.

MULTIPLE TISSUE NORTHERN BLOT

Human Multiple Tissue Northern (MTN) blots (Clontech) were probed with ^{32}P-labelled 352 cDNA, following the manufacturer's instructions. The human MTN blots are charge-modified nylon membranes to which poly A+ RNA from multiple tissues has been transferred from a denaturing formaldehyde 1.2% agarose gel. Each lane contains at least 2 µg of pure poly (A+) RNA from human tissues:

Blot I: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

Blot II: spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leucocyte.

RNA size marker bands are indicated in ink in the left margin of the blots, 1.35-9.5 kb.

Quality control data include a representative blot from each lot that has been probed with a radioactively labelled human β-actin cDNA control probe. A single band of 2 kb of equal intensity, is generated in all lanes, except skeletal muscle, which contain two forms of β-actin, 2 kb and 1.8 kb.

Briefly, the blots were prehybridized in 10 mls of hybridization/prehybridization solution (5x SSPE, 10x Denhardt's solution, 50% deionized formamide, 2% SDS, and 100 µg freshly denatured and sheared salmon sperm DNA), at 42^{0}C for 3-6 hours with continual rotation using a roller bottle. The hybridization/prehybridization solution was replaced with fresh solution containing the radiolabelled probe, ^{32}P-labelled 352 cDNA and subsequently GAPDH, at a concentration of 1-2 x 10^6 cpm/ml (specific activity of ≥ 5 x 10^8 cpm/µg), for 18-24 hours.

Following hybridization, the blots were washed at high stringency: 30-40 minutes at room temperature in 2x SSC and 0.05% SDS, followed by a 40 minute wash at 50^{0}C in 0.1x SSC and 0.1% SDS.

The blots were exposed to fast X-ray film at -70^{0}C using intensifying screens, for 1 and 5 days after probing with ^{32}P-labelled GAPDH and 352 cDNA, respectively.

ISOLATION OF POLYSOMES FROM RAT TESTIS

Lysis buffer in all methods of polysome isolation was supplemented with 100 μg/ml of Heparin (Sigma) which acts as an RNase inhibitor.

(I) Discontinuous sucrose gradient

Testis cells were lysed in 1 ml lysis buffer (100 mM KCl, 1.5 mM MgCl$_2$, 20 mM Hepes (pH 7.6), 0.1% (v/v) DEPC, 0.5% (v/v) Triton N-101) in a 1.5 ml Eppendorf tube, and centrifuged at 12000g for 5 minutes to remove nuclei, mitochondria, and cell debris. The post-mitochondrial supernatant was layered onto a discontinuous sucrose gradient consisting of 8 mls lysis buffer containing 40% (w/v) sucrose, and 4 mls 85% (w/v) sucrose, and centrifuged at 40000 rpm in an SW40 rotor, for 105 minutes. The polysomal band was expected to collect at the boundary between the 2 layers of sucrose. Fractions (1 ml) were collected by gentle aspiration using a Gilson pipetman (P1000), and absorbance readings at 260 nm (A$_{260}$) were recorded. Each fraction was blanked with the equivalent fraction aspirated from a gradient overlayed with lysis buffer alone and centrifuged as above. These readings were used to construct a sedimentation profile of the post-mitochondrial supernatant. Polysomes were collected from the boundary between the two sucrose layers and the polysomal recovery estimated by absorbance at 260 nm.

(II) Continuous gradient

A post-mitochondrial supernatant was prepared as previously described for the discontinuous sucrose gradient. The 1 ml of supernatant was layered onto an 11.5 ml 10-40% (w/v) linear sucrose gradient, underlayed with a 0.5 ml 85% (w/v) sucrose cushion. The gradient was then centrifuged at 40000 rpm for 90 minutes in an SW40 rotor. Fractions (1 ml) were collected from the top of the gradient, and absorbance readings at 260 nm were recorded. Each fraction was blanked with the equivalent fraction aspirated from a gradient overlayed with lysis buffer only, and centrifuged as above. These readings were used to construct a sedimentation profile of the post-mitochondrial supernatant, and subsequently to pool the fractions (9 to 12) that were expected to contain polysomal RNA. Non-polysomal RNA should be present in the upper part of the gradient, with the heavier polysomes sedimenting further down the gradient.

(III) Ultracentrifugation

Testis cells (1×10^8) were lysed by incubating in 8 mls HKM buffer (75 mM KCl, 5 mM MgCl$_2$, 50 mM Hepes (pH 7.4), 0.1% (v/v) DEPC, 0.5% (v/v) Triton N-101) at 4^0C for 5 minutes. The solution was then centrifuged at 14000 g for 10 minutes in a Beckman JA20.1 rotor, to remove debris, mitochondria and nuclei. The 8 ml post-mitochondrial supernatant was layered onto a 5 ml solution of HKM buffer containing 65% (w/v) sucrose, and centrifuged at 40000 rpm for 2 hours in an SW40 rotor, in order to pellet the polysomes. The polysomal pellet was finally resupended in 1 ml HKM buffer and absorbance readings at 260 nm were recorded.

(IV) Precipitation with MgCl$_2$

Testis cells (1×10^6 to 1×10^8) were pelleted by centrifugation for 10 minutes at 500 g. After removal of the supernatant, the cells were lysed in 1 ml of HKM buffer. The suspension was dispersed by aspiration using a Gilson pipetman (P1000), following a 5 minute incubation at 0^0C. The lysed cell suspension was then transferred to a 1.5 ml Eppendorf tube and centrifuged at 12000 g for 10 minutes at 4^0C, to pellet cell debris, nuclei, and mitochondria. The post-mitochondrial supernatant (1 ml) was transferred to a 10 ml plastic Falcon tube, and 1 ml HKM buffer containing 200 mM MgCl$_2$ was added to give a final concentration of 100 mM (in 2 mls). The supernatant was incubated for 1 hour at 0^0C in order to allow the polysomes to precipitate. The polysomes were subsequently collected by centrifugation at 27000 g for 10 minutes at 4^0C through a 2 ml layer of 0.2 M sucrose in HKM buffer.

EDTA control:

A post-mitochondrial supernatant was prepared as before. However, 1 ml of HKE buffer (75 mM KCl, 50 mM EDTA, 50 mM Hepes (pH 7.4), 0.1% (v/v) DEPC, 0.5% (v/v) Triton N-101) was then added, in which the 200 mM MgCl$_2$ was replaced with 50 mM EDTA.

POLYSOMAL RNA EXTRACTION

The protocol used was a modification of the method outlined by Palmiter (1974).

Polysomes isolated using the precipitation method described above, were resuspended in Hepes (1 ml) and aliquoted (250 µl) into 1.5 ml Eppendorf tubes. An equal volume (250 µl) of 0.1 M NaAc (pH 5), and SDS at a final concentration of 0.5% (w/v), were added to each tube and vortexed for 5 seconds. An equal volume of phenol (500 µl) pre-equilibrated as (described in later in the Appendix) was then added, and the mixture was shaken vigorously for 10 seconds. Chloroform (500 µl) was added, the mixture was shaken as before, and then centrifuged for 1 minute at 12000 g in a microfuge, to separate the organic and aqueous phases. The lower organic phase was carefully aspirated, discarded, and then replaced with 500 µl choroform. The process was repeated, followed with the removal of the lower phase. This chloroform extraction process was usually repeated to remove any remaining interphase material. Finally, the upper aqueous phase which contained RNA extracted from polysomes was removed and the nucleic acid precipitated with 2 volumes of 100% ethanol at -20^0C overnight. The RNA was recovered by a 10 minute centrifugation at 12000 g in a microfuge. The pellet was washed in 70% ethanol, air-dried, and resuspended in a small volume of DEPC-treated water (5-10 µl). The RNA was quantified by measuring absorbance at 260 and 280 nm, and the purity was assessed by calculating 260/280 ratios.

PREPARATION OF PROTEIN A-SEPHAROSE COLUMN

The protocol used was according to the manufacturer's instructions (Protein A-Sepharose, Pharmacia).

Protein A-Sepharose (0.35 g) was equilibrated in 20 mM Hepes buffer, pH 7.4 (10 ml) in a sterile universal tube. The beads were allowed to swell at 4^0C, and the remaining solution was poured off. The swollen beads were transferred to a 2 ml disposable syringe plugged with non-absorbant cotton wool, and allowed to set. The protein A-Sepharose was washed with 10 column volumes of Hepes buffer to remove the dextran and lactose stabilizers. Column chromatography was carried out at 4^0C. After use, the column was washed with 1 M acetic acid to dissociate the protein A-Sepharose-antigen complex, and finally re-equilibrated with polysome buffer (20 mM Hepes, pH 7.4).

REMOVAL OF RNases FROM SOLUTIONS USED IN RNA PREPARATION

All solutions were treated with 0.1% DEPC (diethylpyrocarbonate) for 1 hour and then autoclaved for 30 minutes at 15 lb/sq in liquid cycle.

Quartz cuvettes (50 μl and 500 μl) were treated with a 1:1 solution of methanol and concentrated HCl for 1 hour and washed with DEPC-treated H_2O.

NUCLEIC ACID PRECIPITATION

Nucleic acid was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at -20^0C overnight. The DNA or RNA was recovered by a 10 minute centrifugation at 12000 g in a microfuge. The pellet was washed in 70% ethanol, air-dried, and resuspended in a small volume of DEPC-treated water (5-10 μl).

PREPARATION OF R4 ANTISERUM

Hamster spermatozoa recovered from the cauda epididymis were cooled to 4^0C in hypotonic MEM medium (200 mOsm) and vortexed to remove acrosomal caps. Centrifugation at 300 g for 5 minutes allowed the separation of the acrosomal caps from spermatozoa, and their subsequent purification on a 10-40% discontinuous Percoll density gradient formed in isotonic medium and centifuged at 1500 g for 10 minutes. The acrosome membrane preparation was then treated with 0.1% (v/v) Nonidet-P40 and 8 M urea, and subjected to heat denaturation in order to elicit the production of antibodies to a broad spectrum of epitopes, in particular primary structural determinants. The material was then emulsified with an equal volume of Freund's complete adjuvant and was administered intramuscularly to rabbits monthly for 3 months. Serum, designated R4 antiserum, was collected and heated to 56^0C for 1 hour before use.

ANALYSIS OF IMMUNOREACTIVE PROTEINS WITH R4 ANTISERUM BY WESTERN BLOT ANALYSIS

i) Deglycosylation of acrosomal membranes

Acrosomal caps were isolated as described above, and recovered by centrifugation for 10 minutes at 11,600 g. Aliquots (100 μg) were resuspended in 100 mM $NaH_2PO_4.2H_2O$ (pH 6), containing 1 mM phenylmethylsulphonylfluoride (PMSF), and endoglycosidase F (200 mU) or endoglycosidase H (50 mU) in a final volume of 25 μl, in order to remove

high-mannose, N-linked carbohydrate chains. Acrosomes were also treated with trifluoromethanesulphonic acid (TFMS) which removes O-linked carbohydrate. Controls in which enzyme was omitted were processed in parallel. All samples were incubated at 37^0C for 16 hours, and subsequently used for Western blot analysis, as described next.

ii) Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Acrosomal samples were subjected to SDS-PAGE electrophoresis using 10% discontinuous gels (Sambrook *et al.*, 1989), subsequently transferred to nitrocellulose (Hybond-C). Blots were blocked in buffer composed of 10 mM Tris.Cl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20 (TBST) containing 5% (w/v) skimmed milk powder for 1 hour, and subsequently incubated with either anti-β-galactosidase antibody (diluted 1:500 in TBST) or R4 antiserum (diluted 1: 100 in TBST) overnight at 4^0C. Blots were washed in TBST and incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (diluted 1:800 in TBST) for 2 hours at room temperature. The blots were then washed in TBST and TBS and bands were visualized using 4-chloro-1-napthol and hydrogen peroxide.

ISOLATION OF λGT11 cDNA CLONES

i) Screening a human testis λgt11 cDNA expression library (Clontech) with antiserum R4, using standard protocols (Maniatis et al., 1982)

The library was plated out at a density of 4×10^5 plaques per 20 cm plate on *E.coli* Y1090, and overlayed with Hybond-C membrane impregnated with with 10 mM IPTG to induce protein expression from the clones. The filters were then removed and processed as for a Western blot (described in previous section) using antiserum R4 as primary antibody and peroxidase-conjugated anti-rabbit IgG as secondary antibody.

Positive primary plaques (21) were selected and the phage titre for each was determined by plating out a dilution series on 90 mm LB agar plates. Subsequently, secondary screening was carried out as before on plates containing 1-2 x 10^2 pfu's (plaque forming units) per 90 mm plate. Positive plaques were selected, titred and replated for tertiary screening, and the final 30 positive plaques were selected and each considered to derive from a single phage. Another 6 clones were selected as negative controls.

ii) Fusion protein production from recombinant phage

The production of a fusion protein larger than β-galactosidase alone assessed by Western blot analysis, would establish the presence of a cDNA insert at the *Eco*R I site of the Lac Z gene in the correct orientation and reading frame. High titre phage lysates were prepared for positive and negative clones and used to infect *E.coli* Y1090. Fusion protein was generated as a result of inducing expression of β-galactosidase with IPTG (Sambrook *et al.*, 1989). Bacterial lysates containing fusion proteins were subjected to SDS-PAGE using 8% discontinuous gels, and transferred to nitrocellulose Hybond-C. The blot was probed with antiserum R4 or anti-β-galactosidase as described previously.

RESTRICTION DIGESTS

Restriction endonucleases were obtained from NBL, and were each supplied with a 10-fold concentration reaction buffer. The reaction mixture was incubated for 1-2 hours at 37^{0}C.

i) Single digests

Typically, reactions were set up in a final volume of 10 μl, containing 500-1000 ng of DNA, 1 μl each of *Eco* R1 and enzyme buffer (x10).

ii) Double digests

Typically, reactions were set up in a final volume of 20 μl containing up to 1000 ng of DNA, 1 μl of each enzyme, and 2 μl of enzyme buffer (x10) compatable with both enzymes. If the buffer was not compatable, universal buffer was used.

GEL ELECTROPHORESIS OF DNA

DNA fragments were separated on 0.8% to 1.2% agarose gels (prepared with electrophoresis grade agarose in 1x TBE) containing ethidium bromide (1 μg/ml), depending on the size of the fragment. Prior to loading, 0.1 volume of 6x loading dye (0.25% bromophenol blue, 15% ficoll 400) was added to each DNA sample. The gels were run in 1x TBE buffer at 10-20 V/cm for approximately 1 hour or until the bromophenol blue had run two thirds of the length of the gel. Where smaller fragments were to be subcloned, gels were prepared using low melting point agarose prepared in 1x

TAE buffer, and electrophoresed in the same buffer at 5-10 V/cm for approximately 1 hour.

The DNA was visualized on an ultraviolet transilluminator (wavelength 254 nm) and photographed using polaroid 667 film through an orange filter.

POLYMERASE CHAIN REACTION USED TO AMPLIFY λGT11 352 INSERT

Oligonucleotide primers: 5' GAT TCG GTG GCG ACG ACT CCT GG 3' and

5' GAT TCC AGA CCA ACT GGT AAT C   3'

complementary to the vector sequences flanking the *EcoR* I site of the λgt11 genome, were combined with 10^6 to 10^7 phage under the recommended conditions (Perkin-Elmer Cetus Corporation). Denaturation was carried out at 95^0C for 1 minute, annealing at 55^0C for 1 minute, and extension at 72^0C for 2 minutes for 29 cycles. In cycle number 30, the 72^0C step was extended to 5 minutes.

PURIFICATION OF DNA

i) Geneclean

The protocol used was according to the manufacturer's instructions.

The band of interest was cut from the gel and placed in a pre-weighed Eppendorf tube, and the weight of the gel slice was calculated. Sodium iodide (3 volumes) was added and the tube was incubated at $45\text{-}50^0C$ for 5-10 minutes to melt the agarose. Glassmilk (5 µl for up to 1 µg of DNA) was added, and incubated for 10 minutes at room temperature to allow binding of DNA to the glassmilk, with periodic agitation by flicking the tube. The glassmilk was then pelleted by centrifuging at 12,000 g for 5 seconds in a microfuge. The supernatant was removed and the pellet washed 3 times with 10-50 volumes of "New buffer" by resuspending the pellet and centrifuging for 5 seconds each time. After removing the last traces of supernatant, the pellet was resuspended in 5 µl of Tris-EDTA (TE) using a Gilson pipetman (P20), and the solution heated to 45-50 ^0C for 2-3 minutes, to elute the DNA from the glassmilk. The glassmilk was finally pelleted by centrifuging at 12,000 g for 30 seconds, and the supernatant containing purified DNA was carefully removed and transferred to a sterile Eppendorf tube. The final step was repeated and the eluates combined to give a final volume of 10 µl.

ii) Mermaid

The protocol used was according to the manufacturer's instructions for the purification of low MW DNA (10-200 bp).

The DNA was run on gels made with highly purified agarose (BIOGEL) and MERmaid electrophoresis buffer supplied with the kit.

The band of interest was cut from the gel and placed in a pre-weighed Eppendorf tube. High Salt Binding Solution (3 volumes) was added, followed by Glassfog (8 µl for up to 1 µg of DNA). The tube was vortexed continuously for 10 minutes, to allow the gel to disperse and the DNA to bind to the Glassfog. The Glassfog was then pelleted by centrifuging at 12,000 g for a few seconds, and the supernatant was removed. The pellet was washed once in High Salt Binding Solution (200 µl) by resuspending the pellet and centrifuging for 5 seconds. Following the removal of the supernatant, the pellet was resuspended in Ethanol wash (300 µl) by vortexing for a few seconds to remove the salt. After a brief centrifugation as before, the supernatant was removed and discarded. The Ethanol wash step was repeated two more times, and the last traces of ethanol were removed. Finally, the DNA was eluted from the Glassfog, by resuspending the pellet in 5 µl of water and incubating at 45-50^{0}C for 5 minutes. The Glassfog was pelleted by centrifuging for 1 minute, and the supernatant containing the DNA was transferred to a fresh tube. The elution step was repeated making the final volume 10 µl.

PHOSPHATASE TREATMENT OF VECTORS

Following restriction endonuclease digestion of vector DNA, 1 µl of calf intestinal alkaline phosphatase (1000 U/ml) was added and the mixture incubated at 37^{0}C for 30 minutes. The solution was made up to a final volume of 200 µl with TE, and phenol/chloroform extracted to remove the alkaline phosphatase and restriction endonuclease, as follows. An equal volume (100 µl) of phenol and chloroform/isoamylalcohol (24:1) was added to the mixture and vortexed for 5 seconds. The aqueous and organic phases were separated by centrifugation at 12,000 g for 2-5 minutes in a microfuge. The upper aqueous phase was transferred to a fresh Eppendorf tube, and 200 µl of chloroform/isoamylalcohol (24:1) then added, and the mixture vortexed and centrifuged as before. The DNA in the upper phase was precipitated at

-20°C for 2 hours, following the addition of 20 μl of 3 M sodium acetate (pH 5.2), and 500 μl of ice cold ethanol (final concentration of 0.3 M sodium acetate, and 2.5 volumes of ethanol). The DNA was recovered by centrifuging for 15 minutes at 12,000 g in a microfuge, and the pellet washed in 70% ethanol, and then dried in air. The DNA was finally resuspended in 10 μl of TE, and the recovery estimated by running 1 μl on a 1% agarose gel.

LIGATIONS

Ligation reactions were performed to ligate 352 cDNA insert to cut Bluescript, or M13 vector. Typically, reactions were set up using 3:1 or 5:1 molar excess of insert to vector. T4 ligase and x5 ligase buffer were obtained from Gibco BRL.

Ligation reactions were generally carried out in a final volume of 10 μl. Insert and vector DNA, were heated to 65°C for 10 minutes, cooled rapidly on ice for 2-3 minutes to denature the DNA, and microfuged briefly (to recover droplets formed from condensation). The DNA mixture was subsequently incubated at 6-10°C overnight following the addition of 1 μl of T4 ligase (1 U/μl) and 1 μl of ligase buffer (x5).

Control ligations were also set up to assess the efficiency of phosphatase treatment of the vector. These included:

1) phosphatase treated vector without insert DNA (20 ng in 2 μl), containing T4 ligase, and ligase buffer, as above

2) Phosphatase treated vector without insert DNA (20 ng in 2 μl), omitting T4 ligase, but including the ligase buffer.

To assess the efficiency of the ligase reaction, the following control was set up:

Cut vector, without phosphatase treatment (20 ng), containing T4 ligase, and ligase buffer, as above.

Following transformation of ligation mixes in JM101 bacteria (see Transformation section below), the results would confirm whether or not the phosphatase treatment, and ligation reactions were efficient. If phosphatase treatment was not effective, but the ligation reaction itself was, the out come would be the presence of blue plaques or colonies, resulting from the religation of the vector. However, if religation did not occur, very few plaques or colonies, if any at all, would be present, as transformation efficiency

for cut DNA is very low. Plates containing successful ligations: vector + insert, would give rise to clear plaques or colonies, due to the interruption of the Lac Z gene. The ligation control, however, would give rise to blue plaques or colonies, as the religated vector has restored the function of the Lac Z gene.

PREPARATION OF COMPETENT CELLS

A single colony of JM101 was grown to stationary phase overnight by incubating in 2 ml of 2TY media in a 37^0C rotary shaker. The culture was then diluted 1:100 in 2TY (500 µl of overnight culture added to 50 ml of 2TY in a 50 ml sterile tube), and grown to mid log phase (A_{550} 0.4-0.6) at 37^0C in a rotary shaker. The cells were collected by centrifugation at 500 g for 10 minutes at 4^0C. The supernatant was discarded and the cell pellet was gently resuspended in 1/2 volume (25 mls) of 50 mM ice-cold calcium chloride ($CaCl_2$). The cells were centrifuged as above, resuspended in 1/10 volume of 50 mM $CaCl_2$. (5 ml) and stored on ice until required, or at 4^0C for use the next day.

TRANSFORMATION

Once in the bacterial cell, the phage or plasmid DNA will replicate using the host's machinery, and each template DNA will be packaged to become either phage or plasmid, which are now capable of reinfecting neighbouring bacterial cells. The phage will then continue replicating using the lytic life cycle, that is, until it causes lysis of the host cells forming as clear plaques. However, plasmids will replicate in the host cell to the appropriate copy number, and then infect the neighbouring cells containing the F plasmid, without killing the host cell. Instead, colonies of bacteria containing plasmid are formed by positive selection, that is, only bacteria containing plasmid will survive on agar plates containing ampicillin, as Bluescript has the ampicillin resistance gene and JM101 does not.

i) Transformation of recombinant M13 phage into JM101

Control DNA (1-10 ng of uncut M13) or half the ligated samples, were added to 100 µl of "competent" JM101 cells in a 1.5 ml Eppendorf tube and incubated on ice for 30 minutes. The mixture was heated at 42^0C for 3 minutes and incubated on ice for 30

minutes. The transformed cells were transferred to a sterile plastic 7 ml tube, and the following solutions were added sequentially:

200 µl of plating cells (1:100 dilution of an overnight culture of JM101, grown for at least 2 hours),

40 µl of 100 mM IPTG (Isopropyl-β-D-thio-galactopyranoside) in H_2O,

3 ml H-top agar, and

40 µl of 2% (w/v) X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) in DMF (dimethylformamide).

The mixture was immediately poured onto an H-agar plate and allowed to set. The plate was then inverted and incubated at 37^0C overnight. If the cells were competent, the positive control plate would give rise to blue plaques, whereas a ligated sample would give rise to clear plaques, as a result of the interruption of the lac Z gene.

ii) Transformation of recombinant Bluescript into JM101

Control DNA (10 ng of uncut Bluescript), or half of the ligated samples (Bluescript + insert), were added to 200 µl of "competent" JM101 bacteria. The solution was gently mixed by tapping the tube, incubated on ice for 40 minutes, and heated at 42^0C for 2 minutes. After cooling on ice for 10 minutes, L-broth (300 µl) was added and the transformed cells were incubated at 37^0C for 30 minutes. Prior to plating out, 70 µl each of 100 mM IPTG, and 2% X-Gal, was added. The mixtures were immediately spread onto L-broth plates containing Ampicillin (50 µg/ml), allowed to adsorb, and the plates then inverted and left at 37^0C overnight

TEMPLATE PREPARATION

E.coli JM101 cells were grown overnight in 2TY, and a dilution of 1:100 in 2TY was prepared. Aliquots (1.5 ml) were transferred to 30 ml plastic tubes. Phage from individual plaques were picked using separate yellow Gilson pipet tips, and transferred to separate plastic tubes. The cultures were grown for 5 hours at 37^0C with vigorous shaking (200 rpm), to allow the phage to infect the bacterial cells, and generate single strand phage. The cultures were then transferred to Eppendorf tubes, and centrifuged for 5 minutes in a microfuge at 12,000 g, to pellet bacteria containing phage DNA in it's

double stranded replicating form, and cell debris. The supernatant contains phage that have been packaged into phage coat particles while in the host cell, and then released following lysis of the cell. Supernatant (1 ml) was transferred to fresh Eppendorf tubes, and 200 μl of PEG-NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to disrupt the phage coat particles. After incubating for 15 minutes at room temperature, the precipitated phage DNA was collected following centrifugation at 12,000 g for 5 minutes. The supernatant was carefully removed and discarded, and the pellets spun again for 2 minutes to remove all traces of supernatant. The pellet was dissolved in 100 μl of TE (10 mM Tris.Cl, pH 7.5 and 1 mM EDTA, pH 8), and protein was removed by a phenol/ chloroform step: 50 μl of phenol, and 50 μl of chloroform/ isoamylalcohol (24:1) were added to the tube and vortexed for 15 seconds. Following a 15 minute incubation at room temperature, the tubes were vortexed again as before. The aqueous and organic phases were separated by centrifugation at 12,000 g for 5 minutes. The upper aqueous layer (90 μl) containing phage DNA was transferred to a fresh Eppendorf tube and precipitated overnight at -20⁰C, following the addition of 10 μl of 3 M Sodium acetate (pH 5.2), and 250 μl ice cold ethanol (final concentration of 0.3 M sodium acetate, and 2.5 volumes of ethanol). Template DNA was recovered by centrifugation at 12,000 g for 10 minutes. The DNA pellet was washed in 1 ml of 70% ethanol, and re-centrifuged for 5 minutes to pellet DNA again. After removing the ethanol, the pellet was allowed to dry in air prior to resuspending in 25 μl of TE. For sequencing reactions, 5 μl of template DNA was used.

In all cases, 5 μl of template DNA was electrophoresed on a 1% agarose gel, with uncut M13 in one lane, to assess recovery and distance travelled in relation to the uncut DNA. In many cases, if the cloned insert in M13 was large enough, there would be an obvious difference in distance travelled, with the latter travelling a shorter distance in the gel. If size difference was not obvious, as is the case with smaller inserts, a Southern blot would routinely be performed, and positive templates would then be used for sequencing.

PLASMID PREPARATION

E.coli JM101 colonies containing recombinant Bluescript plasmid were grown to saturation overnight in an orbital shaker, in 400 ml L-broth containing ampicillin (50 μg/ml in H_2O, sterilized by passage through a 0.22 μm nalgene filter). The bacteria were sedimented at 7000 rpm for 10 minutes at 4^0C in a Beckman JA10 rotor, and the pellet resuspended in 4 ml ice cold 50 mM Tris.Cl (pH 8) containing 25% (w/v) sucrose. Freshly prepared lysozyme was added to a final concentration of 1 mg/ml, and the cells allowed to lyse during incubation on ice for 15 minutes. The solution was made 10 mM with respect to EDTA, and following a further 30 minutes on ice, a 0.5 volume of Triton lysis buffer (150 mM Tris.Cl, pH 8 containing 375 mM EDTA and 3% (v/v) Triton X-100) was added. After incubating on ice for a further 30 minutes, the cell debris was removed by centrifugation at 18,000 rpm for 60 minutes in a Beckman JA20 rotor. The supernatant was removed, and NaCl added to a final concentration of 0.5 M. Phenol/chloroform and chloroform extractions as described for Template preparation, were performed to extract DNA. Nucleic acid was precipitated overnight at 4^0C with 10% (w/v) PEG 6000, and DNA was collected following centrifugation for 20 minutes at 4^0C in a Beckman JA20 rotor. DNA was resuspended in 500 μl of 0.1 M Tris.Cl (pH 8), and RNA was removed by treatment with 0.2 mg/ml RNase A (previously heated to 100^0C for 10 minutes to inactivate DNases) for 30 minutes at 37^0C. DNA was precipitated with an equal volume of PEG buffer (10 mM Tris.Cl, pH 8 containing 1 mM EDTA, 1 M NaCl and 20% (w/v) PEG 6000) for 1 hour on ice, and collected by centrifugation in a microfuge for 10 minutes. The DNA was then redissolved in 400 μl of 10 mM Tris.Cl (pH 8) containing 0.5 M NaCl. DNA was then sequentially extracted with phenol, phenol/chloroform, and chloroform prior to ethanol precipitation.

An aliquot (2 μl) of plasmid DNA was electrophoresed on a 0.8% agarose gel, with a known quantity of uncut Bluescript plasmid in one lane, to assess recovery. To ensure insert was present, the DNA was digested with the appropriate restriction endonuclease(s), and analysed by agarose gel electrophoresis

EXTRACTION OF RNA WITH GUANIDINIUM THIOCYANATE AND CESIUM CHLORIDE SOLUTIONS

The protocol used was essentially that described in Sambrook *et al.* (1985) with some modification.

Up to 1 g of fresh or frozen tissue from the appropriate testis, was added to a sterile plastic tube containing 10 ml of Guanidinium thiocyanate homogenization buffer (4 M guanidinium thiocyanate, 0.1 M Tris.Cl (pH 7.5), and 1% β-mercaptoethanol), and homogenized with a Polytron at high speed for 2 minutes to shear nuclear DNA. The detergent Sodium lauryl sarcosinate was added to a final concentration of 0.5% to solubilize membranes, and the homogenate then transferred to a fresh plastic tube and centrifuged at 5000 g (3000 rpm in a Beckman JA20 rotor), for 10 minutes at room temperature to pellet cell debris. The supernatant was transferred to a fresh 30 ml plastic tube, passed 2 or 3 times through a 23-gauge needle to ensure complete shearing of DNA, and overlayered on a 3 ml cushion of 5.7 M CsCl, and 0.01 M EDTA (pH 7.5) in an SW40 polyallomer tube. All tubes were weighed accurately, and brought to the same weight by addition of guanidinium thiocyanate homogenization buffer, prior to centrifugation in an SW40 rotor at 33,000 rpm for 18 hours at room temperature. The supernatant above the level of the CsCl cushion was withdrawn using a glass pasteur pipette (previously baked to remove RNases). 1ml of 70% ethanol was added to the pellet to remove residual CsCl, the tube inverted to drain off the ethanol, and the pellet allowed to dry at room temperature for a approximately 10 minutes. The pellet was resuspended in 300 μl TE (p H7.6) containing 0.1% SDS (TE/SDS), by drawing the solution repeatedly into a Gilson pipetman (P1000). As this often proved difficult, the RNA suspension was transferred to a 1.5 ml Eppendorf, frozen and thawed twice, and then heated to 45°C for 5 minutes to disperse the pellet, and thus allow the RNA to be easily resuspended. The RNA was made up to a volume of 600 μl with 300 μl TE (pH 7.6), and precipitated at 0°C on melting ice for 1 hour, following the addition 60 μl of 3 M sodium acetate (pH 5.2) and 900 μl of ice-cold ethanol. The RNA was recovered by centrifugation at 12,000 g for 10 minutes at 4°C. The resulting pellet was washed in 70% ethanol and recentrifuged for 3 minutes at 7,500 g. The ethanol was removed and the pellet (allowed to dry in the air for a few minutes) resuspended in 200 μl RNase-free

water. The recovery and purity of RNA was assessed by measuring OD readings at 260 nm, and 260/280 ratios, respectively. RNA (5-10 µg) extracted from each sample, was electrophoresed on a 1% agarose gel containing formaldehyde and stained with ethidium bromide, to assess the integrity of the RNA prior to isolation of mRNA.

EXTRACTION OF mRNA WITH OLIGO (dT) CELLULOSE COLUMNS

Prepared oligo (dT) cellulose (100 mg) columns were obtained (Gibco BRL) and used according to the manufacturer's instructions.

Total RNA obtained from 1 g of tissue (0.5-2 mgs total RNA) was resuspended in 3 ml of Binding buffer (10 mM Tris.Cl (pH 7.5), containing 1 mM EDTA, 0.3 M NaCl, and 0.1% (w/v) SDS), and denatured by heating to 70^{0}C for 5 minutes and cooling on ice for 5 minutes. The RNA was immediately applied to the oligo (dT) cellulose column, previously equilibrated with Binding buffer, to allow hybridization of the poly A tails to the oligo (dT). The column was then washed with Binding buffer to remove unbound RNA (ribosomal and transfer RNA). The mRNA was eluted with Elution buffer (10 mM Tris.Cl (pH 7.5), containing 1 mM EDTA, and 0.1% (w/v) SDS) preheated to 70^{0}C, the combination of reduced salt concentration and elevated temperature which destabilizes the hybrids of oligo (dT) and poly (A). The mRNA was precipitated with 0.3 M NaCl and 2 volumes of ice-cold ethanol at-20^{0}C overnight, and recovered by centrifugation at 7000 g for 20 minutes at 4^{0}C. The mRNA pellet was briefly washed in 70% ethanol, allowed to dry in air and finally resuspended in 50 µl Rnase-free water. Absorbance readings at 260 and 280 nm were taken to assess the recovery and purity of mRNA. Routinely, 1 µg of mRNA was used for Northern blot analysis, and the blot was stained with methylene blue prior to probing.

SMALL SCALE EXTRACTION OF RNA

The method used was according to Chomczynski and Sacchi (1987) to extract RNA from smaller amounts of tissue or cells (100 mg of tissue or 10^{8} cells). Cells recovered from BSA gradients were collected by centrifugation at 500 g for 10 minutes immediately prior to RNA extraction. Tissue samples were used immediately following dissection, or frozen in liquid nitrogen and stored at -70^{0}C until required.

The tissue or cells were homogenized in 400 µl of denaturing solution (Solution D) in small sterile plastic pestles (Anachem.). Sequentially, 40 µl of 2 M sodium acetate (pH 4), 400 µl of Aqua phenol (water saturated), and 80 µl of chloroform:isoamyl alcohol mixture (24:1) were added to the homogenate with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds, incubated on ice for 15 minutes, and centrifuged at 12,000 g for 20 minutes at 4^0C in a microfuge. The upper aqueous phase, containing the RNA, was transferred to a fresh Eppendorf tube, and the RNA was precipitated by adding 400 µl of isopropanol and incubating at -20^0C for at least 1 hour. The RNA was then recovered by centrifugation at 12,000 g for 20 minutes at 4^0C, and the resulting pellet resuspended in 200 µl of Solution D. The RNA was reprecipitated with 200 µl of isopropanol at -20^0C for 1 hour, and centrifuged as before. The final pellet of RNA was washed in 70% ethanol, and allowed to dry in air prior to resuspension in 20 µl RNase-free water. An aliquot (1 µl) of the RNA solution was used to quantify yield and purity by diluting 1:100, and measuring OD readings at 260 and 280 nm. Routinely, 5 µg of total RNA extracted was electrophoresed on a 1% agarose gel containing formaldehyde, and stained with ethidium bromide to assess the integrity of the RNA.

DNA SEQUENCING

Sequencing was performed according to the manufacturer's instructions using the Sequenase Version 2 kit supplied by United States Biochemical Corporation (USB).

For each sequencing reaction, 2-3 µg of double-stranded DNA insert subcloned into Bluescript plasmid vector, or single-stranded DNA subcloned into M13mp18 or mp19 phage vector were sequenced according to manufacturer's instructions. The products of the sequencing reactions were denatured and separated on 6% polyacrylamide gels prepared from pre-made sequa-gel acrylamide solutions (33 ml sequagel diluent, 12 ml sequagel concentrate and 5ml 10x TBE, polymerized with 220 µl of 25% ammonium persulphate (APS) and 12 µl N,N,N',N'-tetramethyl-ethylene diamine (TEMED). The samples were electrophoresed and subsequently dried according to manufacturer's instructions. The activity of the blot was monitered with a Geiger Muller counter and exposed to X-ray film at room temperature usually overnight.

PREPARATION OF PARTICULAR SOLUTIONS

De-ionized formamide

High reagent grade formamide (200 mls) was added to resin beads and stirred continuously for 1 hour in a fume hood. The mixture was filtered twice through Whatman paper 1, and stored at -20^0C.

Equilibration of phenol

Phenol was equilibrated before use by heating to 65^0C and mixing with an equal volume of 0.5 M Tris.Cl (pH 8), and hydroxyquinoline (Sigma) to a final concentration of 0.1% (w/v). The upper (aqueous) phase was aspirated, and the process was repeated two or three times until the pH of the phenol was greater than 7.8. After the final aqueous phase had been removed, 0.1 volume of 0.1 M Tris.Cl (pH 8) containing 0.2% β-mercaptoethanol was added to the equilibrated phenol, and stored at 4^0C for up to 4 weeks.

MATERIALS

Animals

A breeding nucleus of Sprague-Dawley rats were obtained from Bantin and Kingman Ltd. (Grimston, Aldbrough, Hull, HU11 4QE) and were subsequently maintained at the Institute of Zoology.

Animals were maintained on a 12:12 hours light:dark cycle.

Bacteria

Y1090: *E.coli*, lacU169, proA+, lon, araD139, strA, supF (trpC22: :Tn10) (pMC9). Y1090 stock bacteria were maintained on LB agar plates containing ampicillin (50 μg/ml) at 4^0C.

JM101: E.coli, lacpro, thi, supE, F'traD36, proAB, lacI^{q}z M105. JM101 stock bacteria were maintained on minimal media plates at 4^0C.

DNA

Adult human testis λgt11 cDNA library- Clontech, U.S.A.

Mouse β-actin cDNA- provided with the Human Multiple Tissue Northern Blot (Clontech, U.S.A.).

Rat GAPDH cDNA- obtained from Andy Calver, University College London.

Zoo Southern blot- Clontech, U.S.A.

Vectors:

Bluescript SK+ - obtained from Dr Alison Moore, Institute of Zoology, Regent's Park, London.

M13 mp18 and M13 mp19- Boehringer Mannheim Corporation Ltd., U.K.

Deoxyribonucleotides- Pharmacia LKB.

RNA

Human Multiple Tissue Northern blot- Clontech, U.S.A.

Enzymes

Restriction endonucleases, T4 DNA ligase, DNA polymerase Klenow fragment- obtained from NBL.

Proteinase K- obtained from Gibco BRL.

Collagenase Type II (380 u/mg), Trypsin Type III Bovine (10,900 u/mg), Trypsin inhibitor Type I-S (10,000 u/mg), DNase Type I (510 u/mg)- obtained from Sigma.

Radiochemicals

Radiochemicals were purchased from Amersham International, Amersham, UK.

For preparation of radiolabelled DNA probes: [α-^{32}P] dCTP (3000 Ci/mmol).

For DNA sequencing: [α-^{35}S] dATP (800-1500 Ci/mmol).

Tissue

Human testis was obtained from the Department of Urology, University of Sheffield, for RNA extraction and Northern blot analysis.

Kits

Sequenase kit- United States Biochemicals, obtained through Cambridge Bioscience, U.K.

GeneAmp PCR reagent kit- Perkin Elmer Cetus.

Expresep™ Total RNA/mRNA Isolation kit- AMS Biotechnology U.K. LTD.

B.C.A. Protein Assay kit- Pierce Co. U.S.A.

Geneclean and MERmaid™ kits- Bio 101 Inc., California.

Micro-Fast Track™ mRNA Isolation kit- Invitrogen Corporation, California.

Other materials and reagents

Nylon and nitrocellulose filters (Hybond-N and Hybond-C, respectively)- Amersham International, U.K.

X-ray film- X-omat AR (Kodak, U.K) or Fuji RX film (Fuji Photofilm Co., U.K.).

Polaroid 667 film- Polaroid, U.K.

Ilford K5 emulsion, Ilfospeed paper developer, fixer and microphen film developer- Ilford Scientific Products, U.K.

Sequagel concentrate and Sequagel diluent- National Diagnostics, Bucks., U.K.

Nalgene filters (0.22 μm and 0.45 μm)- Millipore, U.K.

Glass fibre filters (DE81)-Whatman

Oligo (dT) cellulose columns- Gibco BRL

RNA ladders (high and low molecular weight)- Gibco BRL

Petri dishes (90 mm x 15 mm and 140 mm x 15 mm)- Sterilin

Tissue culture flasks- Falcon

Foetal calf serum- Gibco BRL

Other chemicals, solvents and materials were obtained from one of the following: British

Drug House (BDH), Sigma chemical company or Fisons Laboratories.

Antibodies and lectins

Rabbit anti-mouse immunoglobulins- Dako Ltd., Bucks., U.K.

Macrophage mouse monoclonal antibody ED2- Serotec, U.K.,

Goat anti-rabbit, and Rabbit anti-mouse immunoglobulins (FITC-conjugated)- Sigma chemical company,

Datura stramonium lectin (DSL), and *Dilochus bifluoris* lectin (DBL)- Sigma chemical company.

COMPONENTS OF PARTICULAR BUFFERS AND SOLUTIONS

Tissue culture media was obtained from Gibco BRL. All other chemicals were obtained from Sigma chemical company, British Drug House (BDH) or Fisons.

Growth media:

L-broth (LB)- 1% (w/v) bactotryptone, 0.5% (w/v) bacto yeast extract, 1% NaCl in H_2O, adjusted to pH 7.2 with NaOH.

LB-agar and top agarose were prepared with LB and 1.5% (w/v) bactoagar or 0.7% (w/v) ultrapure agarose, respectively.

Minimal media- M9 salts (0.6% (w/v) $NaHPO_4$, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl and 0.05% (w/v) NaCl) and 1.5% (w/v) bacto-agar.

2TY media- 1.6% (w/v) bacto-tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) NaCl.

H-broth- 1% (w/v) bactotryptone, and 0.8% (w/v) NaCl.

H-agar and H-top agar were prepared with H-broth and 1.2% or 0.8% (w/v) bacto-agar, respectively.

Buffers: (all solutions were made in H_2O and autoclaved at 15 lb/sq.in unless otherwise stated).

Phosphate buffer (pH 6.4)- 1 M Na_2HPO_4 (2.5 mls) and 1M NaH_2PO_4 (7.45 mls) diluted to 100 mls with H_2O (Sambrook *et al.*, 1989).

Phosphate buffer (pH 7.4)- 0.5 M $Na_2HPO_4.7H_2O$, adjusted to pH 7.4 with phosphoric acid (H_3PO_4).

Phosphate buffered saline (PBS)- 135 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 15 mM KH_2PO_4 (pH 7.2).

20x SSC- 3 M NaCl and 300 mM trisodium citrate, adjusted to pH 7 with HCl.

TE- 10 mM Tris.Cl (pH 7.5) and 1 mM EDTA.

Tris-borate electrophoresis buffer (TBE)- 100 mM Tris.Cl (pH 8.3), 100 mM boric acid, and 2 mM EDTA.

Tris-acetate electrophoresis buffer (TAE)- 40 mM Tris.Cl, 0.114% (v/v) glacial acetic acid, and 1 mM EDTA, adjusted to pH 8 with glacial acetic acid.

100x Denhardt's solution: 2% (w/v) polyvinyl pyrolidine 360,000, 2% (w/v) BSA and 2% (w/v) Ficoll 400,000. The solution was sterilized through 0.22 nalgene filter and stored at -20^0C.