Regulation of the Mitotic and Meiotic Cell Cycles in the Male Germ Line

DEBRA J. WOLGEMUTH,^{*†‡¶||} ERIKA LAURION,[†] AND KAREN M. LELE[†]

**Department of Genetics and Development; †The Institute of Human Nutrition;
‡Department of Obstetrics and Gynecology; ¶The Center for Reproductive Sciences;
and ||The Herbert Irving Comprehensive Cancer Center, Columbia University College of
Physicians and Surgeons, New York, New York 10032*

ABSTRACT

Mammalian gametogenesis provides a unique system in which to study cell-cycle regulation. Furthermore, understanding the genetic program controlling the mitotic and meiotic divisions of the germ line will provide insight into understanding infertility and new directions for contraception. Male and female germ cells have stages of cell-cycle regulation in common, including a mitotic proliferative stage, entry into meiosis, completion of a reductive division, and entry into a quiescent state awaiting signals at fertilization. However, the timing of these events – and, indeed, even the stage of development at which these events occurs – differs in the two sexes. The genes involved in controlling these specialized mitotic and meiotic cycles of mammalian germ cell differentiation are only now being identified. They include a complex array of kinases, phosphatases, regulatory proteins (e.g., cyclins), and an equally complex array of substrates, including components of the nuclear and cytoplasmic structures involved in cell division. This chapter provides an overview of our current understanding of cell-cycle regulation in mammalian mitotic cells and the importance of restriction points. A summary of observations regarding the expression of various cell-cycle regulatory genes in mouse gametes is provided, along with comments on interesting differences between mitotic and meiotic cells. Finally, the role of the novel A-type cyclin, cyclin A1, during male meiosis is discussed in depth.

I. Introduction

Understanding the genetic program controlling the mitotic and meiotic divisions of the germ line represents a unique opportunity for providing insight into cell-cycle control *in vivo* during development and differentiation. Meiosis itself is, of course, restricted to germ line cells and as such represents potential cell-cycle control points that simply do not exist in somatic cells. Furthermore, higher organisms are characterized by sexually dimorphic gametes. Male and female germ cells have stages of cell-cycle regulation in common, including a mitotic proliferative stage, entry into meiosis, completion of a reductive division, and entry into a quiescent state prior to fertilization. However, the timing of these

events – and, indeed, even the stage of development at which these events occurs – differs in the two sexes (reviewed in Wolgemuth *et al.*, 1995) (Figure 1). For example, female germ cells enter meiosis during fetal development, whereas this is a postnatal event in the male. Once the male germ cell has entered meiosis, the process continues without interruption until the haploid spermatozoon is produced. In contrast, the oocyte is arrested in the diplotene stage of meiotic prophase I, where it can remain for months or years. Following a growth period, the oocyte resumes meiosis, only to arrest at a second point, metaphase II. Fertilization then triggers the completion of meiosis and extrusion of the second polar body. Yet another curiosity of mammalian germ cells is the fact that the two haploid pronuclei commence DNA synthesis independently after fertilization, prior to a fusion event. These and other critical control points in the specialized cell cycles of mammalian germ cells are depicted in Figure 1.

The genetic hierarchy controlling mitosis and meiosis in gamete formation is only recently being elucidated, even in less-complicated systems such as the yeasts. A key player in both mitosis and meiosis is maturation promoting factor

FIG. 1. Flowchart of differences in the cell-cycle progression during mammalian spermatogenesis and oogenesis (Wolgemuth *et al.*, 1995). [Modified from Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J 1983 Molecular Biology of the Cell. New York: Garland Publishing, Inc.]

or M-phase promoting factor (MPF), originally described as an activity found in unfertilized frog eggs capable of inducing germinal vesicle breakdown and resumption of meiosis when injected into immature oocytes (Masui and Markert, 1971). MPF is composed of a regulatory subunit, cyclin B, and p34cdc2 (Cdk1), the catalytic serine/threonine protein kinase. These proteins have been detected in all higher eukaryotes examined. Given the high level of conservation of these key molecules across a diverse range of organisms, it is likely that they will also be important for both mitosis and meiosis in mammals, as they are in the yeasts, in which they have been best characterized. However, it is clear that there are control points and “checkpoints” in the cell cycles of higher eukaryotes that do not exist in simpler organisms and that may differ, depending on the developmental and tissue origin of the cells. For example, the oocytes of mammals exhibit unique cell-cycle control points: the signals to enter meiosis are different from those found in yeast, there is an arrest in diplotene of meiosis, and the cell pauses again in metaphase II, awaiting fertilization to complete meiosis (Figure 1). The spermatocyte shares some of these control points but, strikingly, does not normally arrest in either diplotene or at metaphase of meiosis II. It therefore is likely that there will be genes uniquely involved in these regulatory checkpoints that will be distinct from the yeast genes thus far studied.

In this review, we briefly discuss key aspects of cell-cycle control in mammalian cells, most of which have been derived from studies in cultured cells. We then survey what is known about the expression of known mitotic cell-cycle regulators in the testis and ovary, based on previous studies primarily from our lab. We then focus on the A-type cyclins, studies that have occupied much of our attention in the last 5 years and have provided insight into meiosis-specific and sex-specific differences in cell-cycle regulation. Finally, we highlight some of the many unanswered questions regarding the function of the A-type cyclins. These questions are likely to be relevant to other components of the cell-cycle machinery and will provide insight into infertility and possibly identify new targets for contraception.

II. Overview of the Cell Cycle and Components of the Mammalian Cell-cycle Machinery

The mammalian mitotic cell cycle involves a sequence of transitions and can be divided into four discrete phases (Figure 2). These stages include S-phase, in which DNA synthesis occurs, and M-phase, in which the cells complete mitosis. Between these stages are two gap (G) phases, G₂ (S/M) and G₁ (M/S). The progression through these stages is controlled by a series of sequential enzymatic reactions, the activity of which is highly ordered and tightly controlled. Restriction points at the G₁/S and G₂/M boundaries ensure that the requisite steps have taken place properly (discussed below). The primary enzymatic machinery

FIG. 2. Cartoon of key players in regulating the mitotic cell cycle.

orchestrating this progression consists of a regulatory subunit (cyclin) and a catalytic subunit, the cyclin-dependent kinase (Cdk).

Cyclins have been identified in a variety of organisms ranging from yeast to man, based on amino acid homology and by functional complementation of yeast cell-cycle mutants. The cyclins have been divided into at least eight classes in higher vertebrates, cyclins A to H, based on their amino acid similarity and the timing of their appearance during the cell cycle (reviewed in Sherr, 1993; Pines, 1995b; Roberts, 1999). The complexity of the mammalian system is underscored by the existence not only of multiple classes of cyclins but also of multiple members of the A-, B-, and D-type cyclin families. Although their role in mammalian germ cell development is only beginning to be investigated, observations from our lab and others have shown that various cyclins exhibit distinct patterns of expression in the male and female germ cell lineages as well as in the somatic compartments of the gonads (Chapman and Wolgemuth, 1992,1993; Chesnel and Eppig, 1995; Ravnik *et al.*, 1995; Ravnik and Wolgemuth, 1996,1999; Sweeney *et al.*, 1996; Taieb *et al.*, 1997; D. Liu *et al.*, 1998). These

studies have revealed patterns of cell-type and cell-cycle-stage specificity of *in vivo* expression of several of the cyclins that had not been detected previously in cultured cell systems (see Section V and Figure 5).

The Cdk's are members of a serine/threonine kinase family that share the common properties of a conserved catalytic domain and phosphorylation sites that can modulate their subsequent activity. At least nine distinct Cdk's have been identified in mammalian cells and been shown to exhibit differences in their preference for a cyclin partner (Pines, 1995a). The choice of cyclin partner may affect the enzymatic activity of the complex and possibly the substrates as well (Roberts, 1999). There are also Cdk-family members for which no cyclin partners have yet been identified. While they share structural homology to the *bona fide* Cdk's, primarily through their shared PSTAIRE motif (or variations thereof), whether they also participate in cell-cycle regulation remains to be determined.

III. Modes of Regulation of Activity of Cyclin/Cdk Complexes

Progression through cell-cycle transitions is due to the sequential appearance of cyclins and activation of their Cdk partner. Several modes of regulating the presence of the cyclins are used, as outlined briefly in the following paragraphs.

A. TRANSCRIPTION

Cyclin abundance is regulated mainly at the level of transcription and proteolysis. The modes of transcriptional regulation vary among and within the different classes of cyclins. For example, transcription of D-type cyclins is growth factor dependent. When mitogens are withdrawn from cultured cells that have not completed a cell cycle, D-type cyclin mRNA and protein are degraded rapidly and the cells arrest in G1 (Sherr, 1993). Expression of genes for other cyclins and the Cdk1, which function later in the cell cycle, is mediated by elements that resemble, in part, E2F binding sites. The promoter of cyclin E contains a bipartite element named the cyclin E repressor module (CERM). The CERM element binds a protein complex that includes E2F-4 and a pocket protein, which then inhibits transcription (Le Cam *et al.*, 1999). *In vivo* footprinting revealed that the CERM repressor is occupied only when the gene is not transcribed (in early G1). The onset of expression of the genes for cyclin A2, Cdk1, and cyclin B2 occurs later in the cell cycle than the gene for cyclin E and is mediated by a different bipartite repressor element termed the cell-cycle-dependent element/cell-cycle genes homology region (CDE/CHR) (Zwicker *et al.*, 1995a; Lange-zu Dohna *et al.*, 2000). The CDE/CHR is occupied when the genes are not transcribed in G1 and a mutation in either the CDE or CHR abolishes repression of corresponding promoter/reporter constructs early in the cell cycle (Zwicker *et al.*, 1995a; Lange-zu Dohna *et al.*, 2000).

B. TRANSLATION

Relatively little is known about translational control of cyclin mRNAs, especially in mammalian cells. In budding yeast, initiation of a new cell cycle requires attainment of a critical cell mass and is responsive to the rate of protein synthesis. *Cln3* translation in early G1 is regulated by the cap-binding protein eIF4E, which controls the rate of G1 protein synthesis and is inhibited by rapamycin (Pyronnet *et al.*, 2001). Given the importance of translational regulation of a wide variety of genes during gametogenesis, especially in postmeiotic cells, it would be surprising if mammalian cyclin mRNAs were not regulated, at least in part, at the translational level. In this light, it was interesting to note that only low levels of mouse cyclin B1 were present in postmeiotic spermatids, as compared to meiotically dividing spermatocytes, even though cyclin B1 mRNA was readily detected (Chapman and Wolgemuth, 1994). Furthermore, recent studies on the translation regulatory factor CPEB revealed that it is involved in synapsis and progression through pachytene of meiosis (Mendez and Richter, 2001).

C. TURNOVER

The timely degradation of cyclins is equally important to exit a transition and appears to involve a ubiquitin-mediated proteolysis (reviewed in Hershko and Ciechanover, 1998). In this process, a ubiquitin-activating enzyme (E1) activates and transfers ubiquitin to a ubiquitin-conjugating enzyme (E2). E2, along with a substrate recognition protein (E3), assembles a ubiquitin chain on its substrate. Multi-ubiquitinated substrates are rapidly degraded by the 26S proteasome. There are two types of E3 complexes: the SCF complex and the anaphase-promoting complex (APC). SCF-mediated proteolysis is best studied in yeast. Its substrates include proteins expressed early in the cell cycle, such as the G1 cyclins. Substrates targeted by SCF must first be phosphorylated, suggesting that their stability is controlled by activity of a protein kinase. In mammals, proteolysis of cyclin D1 and cyclin E is controlled by phosphorylation-dependent ubiquitination (Elledge and Harper, 1998). The mitotic cyclins are proteolyzed after APC recognition, which is essential for exit from mitosis. Cyclin A2 is degraded after breakdown of the nuclear envelope (den Elzen and Pines, 2001) and proteolysis of cyclin B is required for exit from M-phase (Gallant and Nigg, 1992).

D. PHOSPHORYLATION AND DEPHOSPHORYLATION

The activation state of Cdk complexes is regulated not only by cyclin association but also by Cdk phosphorylation, which can inhibit or enhance its kinase activity and therefore regulate the timing of Cdk activity (Figure 3). For example, MPF is not active until the G2/M transition, although Cdk1 is present

E. INHIBITORS

In mammals and other higher eukaryotes, there are two classes of relatively small (i.e., 16–57 kDa), tightly binding proteins that function as Cdk inhibitors (CKIs) (reviewed in Sherr and Roberts, 1995,1999). They are grouped according to their structure and their Cdk targets and include the CIP/KIP family – consisting of p21CIP1/WAF1, p27KIP1, and p57KIP2 – and the INK family – consisting of p16INK4a, p15INK4b, p18INK4c, and p19INK4d. The CKIs appear to exhibit their inhibitory function by binding in a stoichiometric manner, usually to cyclin-Cdk complexes but sometimes to noncomplexed Cdks. The INK family contains ankyrin repeats and binds only to Cdk4 and Cdk6 but not to other Cdks or to the D-type cyclins. The Cip/Kip families are broader with regard to their inhibiting activity, affecting the activity of the cyclin D-, E-, and

A-type cyclin-dependent kinases. This class contains motifs in their amino-terminal regions that enable them to interact with both cyclin and Cdk proteins. Recent studies suggest that interaction with a Cip/Kip family member may actually positively regulate cyclin D-Cdk complexes but inhibit cyclin A- or E-Cdk complexes (reviewed in Sherr and Roberts, 1999).

F. SUBCELLULAR LOCALIZATION

A final level of control involves the distribution of the cyclin/Cdk proteins within cells, particularly whether they are in the nuclear or cytoplasmic compartments. For example, cyclin A2 apparently is constitutively nuclear in cultured cells (Pines and Hunter, 1991). However, cyclin B1 accumulates almost exclusively in the cytoplasm and undergoes a striking translocation to the nucleus as the cells enter prophase. This nuclear accumulation of Cdk1-cyclin B appears to be required for mitosis to proceed. Several recent studies have implicated the exclusion of Cdk1/cyclin B in the function of checkpoints that prevent entry into mitosis in the presence of DNA damage (Toyoshima *et al.*, 1998). Nuclear transport factors have been identified that appear to be involved in ferrying some of the cyclins and Cdks in and out of the nucleus. Such factors might have been predicted to exist, since no classic nuclear localization sequences have yet been identified in the primary sequences of any of the vertebrate cyclins or Cdks. As noted, they exhibit striking differences in their subcellular localization (reviewed in Yang and Kornbluth, 1999). In fact, deletion studies have identified sequences in the B-type cyclins that may function as both cytoplasmic-retention sequences and/or nuclear-export sequences (reviewed in Yang and Kornbluth, 1999).

The state of phosphorylation also may be important for the nuclear localization of at least some of the cyclin/Cdk complexes. For example, during meiotic maturation in frog oocytes, phosphorylation of four conserved serine residues in cyclin B1 correlates with the nuclear accumulation and activity of MPF (Li *et al.*, 1995). A model for the role of cyclin B1 localization to the nucleus has been proposed in which phosphorylation of cyclin B1 inhibits its interaction with proteins that appear to favor nuclear export, thus promoting the accumulation of cyclin B1 in the nucleus. While the role of changes in the subcellular distribution of cyclin B1 during meiotic maturation in spermatocytes remains to be demonstrated, we note that cyclin B1 is predominantly cytoplasmic in meiotic prophase cells and shifts to the nucleus just before the entry into the first meiotic division (Liu *et al.*, 2000).

IV. Restriction Points During Mitosis and Meiosis

Signaling pathways operate at cell-cycle transitions to monitor genomic integrity and the successful completion of upstream events, which ensures

orderly progress through the cell cycle. In the mitotic cell cycle, response to damaged or improperly replicated or aligned DNA activates pathways that arrest cells at G1-S, G2-M, and at the metaphase-anaphase transition (Figure 2). Recent findings suggest there are two types of cell-cycle checkpoint responses that delay the G1-S transition, both of which target Cdk2 activity. An initial, transient response leads to rapid inhibition of Cdk2 activity due to degradation of Cdc25A (Mailand *et al.*, 2000). A second, sustained response is carried out by p53-dependent production of p21, which binds to and blocks activity of Cdk2/cyclin E complexes (Stewart and Pietsch, 2001). Arrest at the G2-M phase transition targets MPF by preventing the removal of Cdk1/cyclin B inhibitory phosphorylation. Inhibition of Cdc25C phosphatase activity is mediated by damage-induced activation of the protein kinase Chk1, which appears to be a primary mechanism of MPF inhibition, as studied in fission yeast (Lopez-Girona *et al.*, 2001) and in human cells (Blasina *et al.*, 1999). Signaling pathways also activate p53, which activates the gene for p21 (Bunz *et al.*, 1998). Interestingly, DNA damage also may repress transcription of genes for cell-cycle regulators. That is, radiation-induced G2 arrest is accompanied by a p53-dependent downregulation of the gene for Cdk1 via the CDE/CHR element (Badie *et al.*, 2000) and the promoters for cyclin B1, cyclin B2, and Cdc25C, which is mediated by NF-Y-binding CAAT boxes (Manni *et al.*, 2001).

The spindle checkpoint is activated at the metaphase-anaphase transition and prevents entry into anaphase until all chromosomes are properly aligned by monitoring microtubule attachment on kinetochores and their tension (Rieder *et al.*, 1994). Components of the spindle checkpoint inhibit activity of APC, which, in the absence of checkpoint activation, degrades proteins, allowing sister chromatid separation and mitosis exit (Clarke and Gimenez-Abian, 2000).

Some evidence exists for the presence of checkpoints in meiotic cell cycles, primarily from studies in yeast. Cells that divide meiotically can arrest in prophase of metaphase I. In yeast, arrest occurs at the pachytene stage of meiotic prophase in response to incomplete chromosome synapsis or recombination (reviewed in Roeder and Bailis, 2000). There may be an analogous checkpoint in mammalian meiotic cells, since failure to enter into meiosis I has been observed when defects occur in prophase (Figure 4; Table I). Male mice lacking genes that function in recombination, DNA repair, or cell-cycle regulation exhibit defects in chromosomal synapsis or recombination during prophase that, unlike observations in yeast, result in apoptosis (Table I). However, it should be noted that the cells arrest at different stages of prophase, suggesting multiple rather than a single checkpoint. In response to low levels of radiation, a G2 delay is observed in spermatocytes that is not observed in spermatocytes that are p53 deficient, suggesting a role for p53 in the process (Schwartz *et al.*, 1999). A spindle checkpoint may be active during meiosis in *Drosophila* spermatocytes. Entry into

FIG. 4. Possible restriction points during mitosis and meiosis.

anaphase is delayed in spermatocytes that are either colchicine treated or contain misaligned chromosomes (Rebollo and Gonzalez, 2000).

V. Which of the Cell-cycle Components Are Expressed in the Testis?

A. OVERVIEW OF THE EXPERIMENTS

We initiated our studies on identifying the cell-cycle genes involved in mammalian gametogenesis with a very simple hypothesis having two major points: 1) meiosis is a highly conserved process across organisms, thus the regulatory components may also be conserved; and 2) control points exist in mammalian meiosis that simply do not exist in lower forms, hence, novel cell-cycle regulators may have evolved to fulfill these specialized functions. As will be discussed in the following sections, both parts of this hypothesis have been shown to be true.

B. SUMMARY OF CELL-CYCLE GENES EXPRESSED IN THE GERM LINE

A variety of experimental approaches have been used to identify cell-cycle genes expressed during gametogenesis, from mRNA expression and

TABLE I
Genetic Models in Male Mice Exhibiting Meiotic Prophase Arrest and Apoptosis

Mutation	Arrest point	Synapsis phenotype	Reference
A-myb	Early pachytene	Not reported	Toscani <i>et al.</i> , 1997
Atm	Zygotene/early pachytene	Frequent asynapsis	Xu <i>et al.</i> , 1996
Dmcl	Zygotene/early pachytene	Asynapsis	Yoshida <i>et al.</i> , 1998
Msh4	Zygotene	Frequent asynapsis; Nonhomologous pairing	Kneitz <i>et al.</i> , 2000
Msh5	Zygotene/early pachytene	Frequent asynapsis; Nonhomologous pairing	Edelmann <i>et al.</i> , 1999
Scp3	Zygotene	Asynapsis	Yuan <i>et al.</i> , 2000
Spo11	Zygotene	Asynapsis	Baudat <i>et al.</i> , 2000; Romanienko and Camerini-Otero, 2000
XSxr(a)0	Late prophase/meiosis I	Asynapsis of the sex chromosome	Odorisio <i>et al.</i> , 1998
Hsp70.2	Late pachytene	Failure to desynapse	Dix <i>et al.</i> , 1997
Mlh1	Late pachytene	Failure to desynapse	Edelmann <i>et al.</i> , 1996
Cyclin A1	Late pachytene/diplotene	Incomplete desynapsis	D. Liu <i>et al.</i> , 1998

protein analysis using several methods and known probes and reagents to screens to identify new genes. A summary of the overall expression patterns of various categories of cell-cycle regulators that have been investigated in our lab is presented in Figure 5. It should be recognized that the data were obtained from studies in the rodent model system, where precise identification of cell types is easier than in other mammals (e.g., humans). However, in several studies, we have been able to extend the expression analysis to humans. For example, cyclin A1 is expressed in human late pachytene to diplotene spermatocytes, very much as it is in the mouse (C. Liao, S.Q. Li, S. Mulrad, X.Y. Wang, and D.J. Wolgemuth, submitted).

The genes examined generally fall into four classes. First, genes that are expressed in both germline and somatic cells of gonads and are rather ubiquitously expressed in other cells. Second, genes that are much more abundant in the germ line and third, those that are apparently specific to the germ line. Finally,

FIG. 5. Summary of the expression of selected cell-cycle-regulating genes in the mouse testis and ovary. This figure represents a composite of the results of studies from our laboratory on the levels of expression of the genes denoted on the left. The references containing the data supporting this compilation may be found in a review by Wolgemuth *et al.* (1995) and in references cited in this review. The relative levels of expression are depicted by shading of the bars: solid black, most robust expression of a particular gene; white bar, no detection of expression.

genes that are specific to one or the other germ line (i.e., exhibiting sexual dimorphism in their patterns of expression).

C. HIGHLIGHTS AND INTERESTING FEATURES OF THESE EXPRESSION PATTERNS THAT MAY BE IMPORTANT FOR SPERMATOGENESIS

One of the earliest observations of unique differences between meiotic and somatic cells came from the observations of expression of the B-type cyclins (Chapman and Wolgemuth, 1992). The two mammalian B-type cyclins had been shown to be coexpressed in virtually all cultured cells analyzed. Surprisingly, their expression at the mRNA level was shown to be uncoupled in prophase spermatocytes. Cyclin B2 transcripts were detected earlier in prophase, at about the zygotene to early pachytene stage, and were followed by the appearance of cyclin B1 later in prophase. Further, cyclin B1 transcripts were observed in early round spermatids, which are postmeiotic. Whether these transcripts have any functional significance is not known, since subsequent studies using highly specific antibodies against the two B-type cyclins did not reveal significant accumulation of B1 protein in spermatids (Brandeis *et al.*, 1998; D. Liu *et al.*, 1998). The cyclin B1 null mutation results in embryonic lethality (Brandeis *et al.*, 1998), thus obviating a direct test of its function in early spermatids in the adult. In contrast, cyclin B2 does not appear to be essential in that cyclin B2 knockout animals are viable and fertile, although a bit smaller and yielding slightly reduced litter sizes (Brandeis *et al.*, 1998).

Another curiosity is the expression of cell-cycle genes, which are intimately tied to proliferation in mitotic cells, in testicular cells that are not dividing. In addition to the detection of cyclin B1 in spermatids, several of the Cdks have been observed to be expressed in nondividing cells in the testis. For example, both Cdk4 (Rhee and Wolgemuth, 1997) and Cdk5 (Q. Zhang, K. Rhee, and D.J. Wolgemuth, unpublished observations; Session *et al.*, 2001) have been shown to be expressed in Sertoli cells in the adult mouse and human testis — cells that are no longer dividing and are highly differentiated.

Although the cyclins and Cdks are the major focus of this review, other pathways, possibly acting in parallel with cyclin/Cdk complexes, could play roles in mitosis and possibly in meiosis. For example, we and others have investigated the role of the putative homologues of the *Aspergillus never in mitosis* (NimA) gene (Osmani *et al.*, 1991; Rhee and Wolgemuth, 1997; reviewed in Fry and Nigg, 1995). Although there appears to be as many as seven mammalian homologues of NimA (designated Nek genes), based on structural similarities, none has been shown to complement NimA *in vivo* (Schultz and Nigg, 1993; Kandli *et al.*, 2000;). The pattern of expression of Nek1 and Nek2, in particular, during mammalian gametogenesis strongly

suggests roles in meiosis (Letwin *et al.*, 1992; Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998).

VI. Focus on the A-type Cyclins

A. IDENTIFICATION OF TWO A-TYPE CYCLINS IN HIGHER ORGANISMS

Of the mammalian cyclins identified to date, cyclin A has remained somewhat enigmatic, in part due to the fact that no homologues exist in the yeasts. We have shown that there are two distinct cyclin A genes in mammals, one of which, cyclin A1, is most highly expressed in the testis where it is restricted to the germ line (Ravnik and Wolgemuth, 1996; Sweeney *et al.*, 1996). In contrast, cyclin A2 was expressed in all tissues analyzed. Two A-type cyclin genes have been documented in human and frog (Howe *et al.*, 1995; Yang *et al.*, 1997). Mouse cyclin A1 and A2 share 44% identity at the amino acid level; however, they share a much higher level of identity (84%) within the two highly conserved cyclin boxes (Ravnik and Wolgemuth, 1996; Sweeney *et al.*, 1996).

B. EXPRESSION OF THE A-TYPE CYCLINS IN MAMMALIAN GAMETOGENESIS

In the adult mouse, Northern blot hybridization analysis revealed that expression of cyclin A1 was restricted to the testis, specifically, to meiotic germ cells. *In situ* hybridization analysis and immunohistochemistry were used to localize their expression to specific testicular cells. The levels of cyclin A1 mRNA (Sweeney *et al.*, 1996; D. Liu *et al.*, 1998) and protein (D. Liu *et al.*, 1998; Ravnik and Wolgemuth, 1999) rise dramatically in late pachytene spermatocytes and become undetectable soon after completion of the first meiotic division. Thus, its expression is cell-cycle regulated. At both the RNA and protein levels, the predominant sites of cyclin A2 expression in the adult testis were the mitotic germ line stem cells, the spermatogonia, and in preleptotene spermatocytes, cells in which premeiotic DNA synthesis occurs (Ravnik and Wolgemuth, 1996, 1999). Cyclin A2 expression also is detected in Leydig cells (Ge and Hardy, 1997). The concurrent localization of mRNA and protein for both of the A-type cyclins further suggested that both are regulated primarily at the level of transcription in the testis. The observed cellular specificity of cyclin A2 expression is consistent with its function during mitosis in the stem-cell stage of this lineage, while the restricted preleptotene stage localization suggests function in G1/S or S but not in the meiotic divisions *per se*. No cyclin A1 mRNA was detected in ovaries or oocytes by Northern or *in situ* hybridization analysis (C. Liao, S.E. Ravnik, Q. Zhang, S. Mulrad, and D.J. Wolgemuth, in preparation).

However, both somatic and germ cells of the adult ovary express cyclin A2 mRNA and protein. The different expression patterns suggest distinct functions for cyclin A1 and cyclin A2 in the somatic and germinal lineages, which also differ between the male and female.

Cyclin A2 is expressed ubiquitously in cultured cells and in a broad variety of tissues in the adult mouse and during embryogenesis (Ravnik and Wolgemuth, 1996; Sweeney *et al.*, 1996). Perhaps not surprisingly then, targeted mutagenesis of encoding murine cyclin A2 resulted in early embryonic lethality, apparently around the peri-implantation stage (Murphy *et al.*, 1997). This embryonic lethality has obviated understanding the role of cyclin A2 in other aspects of mammalian development, including the germ line. In contrast, the strikingly restricted expression of cyclin A1 led us to hypothesize that its primary site of function is in the male germ line, specifically, at the first meiotic division.

C. REGULATION OF EXPRESSION OF THE A-TYPE CYCLINS

Regulation of cyclin A2 has been extensively studied in cultured cells and shown to be first expressed during the mitotic cell cycle at the G1/S transition (reviewed in Pines and Hunter, 1991). The regulatory elements required for efficient cell-cycle-regulated transcription are contained within a small fragment of the proximal promoter (89 base pairs upstream and 11 base pairs downstream from its transcriptional start site) (Schulze *et al.*, 1995). Periodic occupation of the CDE/CHR element, located in this fragment, by a repressor controls the timing of its expression (Zwicker *et al.*, 1995b). Activities have been isolated from nuclear extracts that bind to the CDE/CHR (Liu *et al.*, 1997) or to the CHR (Philips *et al.*, 1999) in gel-shift experiments. E2F can bind the CDE of CDE/CHR (Zerfass-Thome *et al.*, 1997). The CDE/CHR binding activity, termed CDE/CHR binding factor-1 (CDF-1), could not be supershifted by antibodies directed against E2F or DP-1 proteins. Mutating the first nucleotide of the CDE can abrogate E2F binding, while having little effect on the binding of CDF-1 in gel-shift experiments (N. Liu *et al.*, 1998). The corresponding mutant reporter construct showed unchanged repression in G0, suggesting that E2F does not mediate repression of the gene for cyclin A2. This led to the hypothesis that E2F may compete with and displace CDF-1 in late G1/S phase, leading to transcriptional activation of the gene (N. Liu *et al.*, 1998). Transcriptional activation upon E2F binding also can explain the observed upregulation of the cyclin A2 promoter following ectopic expression of E2F-1 (Schulze *et al.*, 1995). The CHR-binding activity, CHR-binding factor (CHF), was isolated from nuclear extract from quiescent cells (Philips *et al.*, 1999). CHF, when microinjected into cells, was able to downregulate the cyclin A2 promoter/reporter construct (Philips *et al.*, 1999).

It is speculated that the repressor occupying the CDE/CHR may interfere with the activity of factors bound upstream. *In vivo* footprinting revealed upstream elements that were protected constitutively during the cell cycle (Desdouets *et al.*, 1995; Zwicker *et al.*, 1995a). These elements bind Sp1, NF-Y, and CBP/cycA (CCAAT binding protein for cyclin A gene) and members of the ATF/CREB/CREM family (Desdouets *et al.*, 1995; Zwicker *et al.*, 1995a; Kramer *et al.*, 1997). CBP/cycA is implicated in adhesion-dependent cyclin A2 transcription (Kramer *et al.*, 1997), while members of ATF/CREB/CREM mediate regulation of the cyclin A2 promoter by cAMP (Desdouets *et al.*, 1995). There may be cell specificity of binding of ATF/CREB/CREM family factors to the cAMP response element in the cyclin A2 promoter, as shown in gel-shift assays using extracts from various types of cells (Blanchard, 2000).

Almost nothing is known about the regulation of the cyclin A1 gene, especially *in vivo*. A 1.3-kb fragment of the human cyclin A1 promoter appears to reproduce the highly restricted tissue expression pattern in three of four transgenic mouse lines carrying human cyclin A1 reporter gene constructs (Muller *et al.*, 2000a). Potential regulatory elements and factors that control expression have been studied in the context of a smaller promoter fragment that is comprised of 190 base pairs of upstream promoter sequence in cultured cells. This fragment can induce expression of a reporter gene mediated by GC boxes that can bind Sp1 in HeLa cells (Muller *et al.*, 1999) and by coexpression with B-myb and cyclin A1 in CV-1 cells (Muller *et al.*, 2000b). Although it was initially suggested that the tissue-specific expression of mouse cyclin A1 correlates with methylation of its CpG-rich proximal promoter (Muller *et al.*, 2000a), subsequent studies revealed that this is not likely (Muller-Tidow *et al.*, 2001). Cyclin A1 frequently is overexpressed in human acute promyelocytic leukemia, a subtype of acute myeloid leukemia that is characterized by the presence of the PML-RAR α fusion protein (Yang *et al.*, 1999). A recent study has suggested that PML-RAR α ectopically expressed in human myeloid leukemia cell lines led to induction of co-transfected human cyclin A1 promoter/reporter constructs with either 190 base pairs of promoter or with 1.3 kb (Muller *et al.*, 2000b). Whether such constructs will be activated in myeloid cells *in vivo* remains to be determined, as does the question of whether these flanking regions are active in spermatocytes.

D. PREFERENCE OF CDK PARTNER FOR THE A-TYPE CYCLINS IN TESTICULAR CELLS

To extend our studies on the function of the A-type cyclins during meiosis in the male at the biochemical level, we examined the developmental and cellular distribution of their candidate cyclin-dependent kinase partners, Cdk1 and Cdk2, in the spermatogenic lineage (Ravnik and Wolgemuth, 1999). As noted previ-

ously, immunohistochemical localization revealed that cyclin A1 is present only in male germ cells just prior to or during the first, but not the second, meiotic division. By contrast, cyclin A2 was expressed in spermatogonia and was most abundant in preleptotene spermatocytes, cells about to enter the meiotic pathway. Immunohistochemical detection of Cdk1 was most apparent in early pachytene spermatocytes, while staining intensity diminished in diplotene and meiotically dividing spermatocytes, the cells in which cyclin A1 expression was strongest. Cdk2 was highly expressed in all spermatocytes. Notably, in cells undergoing the meiotic reduction divisions, Cdk2 appeared to localize specifically to the chromatin. This was not the case for spermatogonia undergoing mitotic divisions. We also showed that, in the testis, cyclin A1 binds both Cdk1 and Cdk2 (Sweeney *et al.*, 1996; D. Liu *et al.*, 1998,2000). In contrast, cyclin A2 strongly preferred to bind to Cdk2 in testicular lysates. These results suggest that the A-type cyclins have differences in their partner preference and may exhibit unique target substrate specificity.

E. TARGETED MUTAGENESIS OF CYCLIN A1

To begin to address possible redundancy of the two A-type cyclin genes, we generated cyclin A1-deficient mice by targeted mutagenesis of the cyclin A1 gene (D. Liu *et al.*, 1998). The remarkable restriction of cyclin A1 suggested a very specific function only at the G2/M transition of the first meiotic division. Cyclin A1-deficient males were sterile due to a block of spermatogenesis before the first meiotic division, whereas females were normal. Meiosis arrest in males lacking cyclin A1 was associated with increased germ-cell apoptosis and desynapsis abnormalities (D. Liu *et al.*, 1998,2000). There was a striking reduction in the activation of MPF kinase at the end of meiotic prophase, although both Cdk1 and cyclin B proteins were present. Cyclin A1 is therefore essential for spermatocyte passage into the first meiotic division in male mice, a function that cannot be complemented by the concurrently expressed B-type cyclins.

We then explored the possibility that a cyclin A1-dependent process dictates the activation of MPF (D. Liu *et al.*, 1998). There are several pieces of evidence in the literature that supported a role for A-type cyclins in the activation of MPF. *Xenopus* cyclin A1 is synthesized before cyclin B and accumulates as an active kinase complex (Minshull *et al.*, 1990). Cyclin A1 bound to Cdk1 in *Xenopus* egg extracts is immediately active, independent of Cdc25 activity (Clarke *et al.*, 1992). The addition of cyclin A1 into such cell-free extracts potentiated MPF activity (Devault *et al.*, 1992). Further, blocking Cdk2 activity, either by p21 or by immunodepletion, prevented activation of MPF and mitosis in cultured cells (Guadagno and Newport, 1996).

We asked whether the expression of the B-type cyclins that comprise MPF was retained in cyclin A1-deficient spermatocytes (Liu *et al.*, 2000). Both cyclin

B1 and B2 proteins were readily detected, although their associated kinases were kept at inactive states. We then turned to an *in vitro*, short-term culture system developed by Handel and colleagues (Wiltshire *et al.*, 1995) in which treatment of pachytene spermatocytes with the protein phosphatase type 1 and type 2A inhibitor okadaic acid can induce premature chromosome condensation and entry into the first meiotic division. We asked if treatment of the arrested diplotene spermatocytes in cyclin A1-deficient testes could drive the cells into meiosis I and activate MPF activity. Indeed, okadaic acid treatment restored MPF activity and induced entry into M phase and the formation of overtly normally condensed chromosome bivalents (Figure 4), concomitant with hyperphosphorylation of Cdc25 proteins (Liu *et al.*, 2000).

Conversely, inhibition of tyrosine phosphatases, including Cdc25s, by vanadate suppressed the okadaic acid-induced metaphase induction. The highest levels of Cdc25A and Cdc25C expression and their subcellular localization during meiotic prophase coincide with that of cyclin A1. When overexpressed in HeLa cells, cyclin A1 coimmunoprecipitates with Cdc25A. Furthermore, the protein kinase complexes consisting of cyclin A1 and either Cdk1 or Cdk2 phosphorylate both Cdc25A and Cdc25C *in vitro* (Liu *et al.*, 2000). These results suggest that, in normal meiotic male germ cells, cyclin A1 participates in the regulation of other protein kinases or phosphatases critical for the G2-M transition. In particular, it may be directly involved in the initial amplification of MPF through the activating phosphorylation on Cdc25 phosphatases (Figure 3).

VII. Unanswered Questions

Our investigations on the role of the A-type cyclins in the development, differentiation, and function of the germ line have provided some clues as to their *in vivo* function; however, much remains to be elucidated. Outlined below are several of the questions we would like to address.

A. IS CYCLIN A2 REQUIRED IN MITOSIS IN SPERMATOGONIA AND FOR PRELEPTOTENE DNA SYNTHESIS?

The striking differences in the patterns of expression between cyclin A1 and cyclin A2 in spermatogenic cells suggested that they would have distinct functions (Ravnik and Wolgemuth, 1999). Our targeted mutagenesis of the cyclin A1 gene revealed the clear requirement for this gene, specifically in meiosis in the male but not in the female germ line. The role of cyclin A2 is more difficult to assess because, as shown very elegantly by Murphy *et al.* (1997), loss of cyclin A2 results in early embryonic lethality. Embryos lacking cyclin A2 can develop from the two-cell to blastocyst stage but then die (Winston *et al.*, 2000). Therefore, to study its function during spermatogenesis in the adult animal, a

cell-specific targeted loss of function strategy will be needed. One such approach would involve the generation of transgenic mice expressing the Cre recombinase uniquely in spermatogonia and/or preleptotene spermatocytes, under the direction of a promoter specific to these cells. These animals would then be crossed to mice carrying the cyclin A2 gene into which loxP sites have been introduced (floxed cyclin A2). Excision of portions of the cyclin A2 gene between the loxP sites would result in a mutant cyclin A2 gene but only in spermatogonia or preleptotene spermatocytes. The critical missing link in this approach is the lack of well-characterized, spermatogonia-specific promoters.

B. IS CYCLIN A2 ESSENTIAL FOR OOGENESIS?

Ongoing studies in our lab and the work of others have shown that cyclin A2 is expressed in the mouse oocyte (Winston *et al.*, 2000; C. Liao, S.E. Ravnik, Q. Zhang, S. Mulrad, and D.J. Wolgemuth, in preparation). As noted previously, the early embryonic lethality of the cyclin A2 null mutation (Murphy *et al.*, 1997; Winston *et al.*, 2000) obviates a direct test of its function in the germ line in either male or female. The Cre-lox strategies discussed earlier – using, in this case, oocyte-specific promoters to drive Cre recombinase – might be fruitful to address the role of cyclin A2 in oocyte mitosis and especially meiosis. Alternatively, the recent, very exciting application of double-stranded RNAs (RNAi) to interfere with gene expression in oocytes might permit a direct test of the cyclin A2 protein in the resumption of meiosis (Svoboda *et al.*, 2000). In this approach, double-stranded RNAs for the c-mos proto-oncogene were shown to inhibit the translation of c-mos mRNA in a time- and concentration-dependent manner. Importantly, this inhibition resulted in parthenogenetic activation of the oocytes, similar to the phenotype observed in c-mos knockout mouse oocytes (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994).

C. ARE THE A-TYPE CYCLINS FUNCTIONALLY REDUNDANT?

One hypothesis regarding the possible functional redundancy of the two mammalian A-type cyclin proteins is that they have essentially identical functions as part of cyclinA/Cdk complexes and that differences in their function are reflected only by where they are expressed. A prediction of this hypothesis is that the two A-type cyclins would have identical biochemical properties as part of a Cdk1 or Cdk2 complex and be functionally interchangeable *in vivo*. An alternative hypothesis is that the two mammalian A-type cyclins have distinct functions as well as distinct regulation of expression. Yet a third possibility is that they may have both common and distinct functions, including unique functions of cyclin A1 in meiotic progression.

One approach for assessing these possibilities would ask whether functional differences between cyclin A1- and A2-containing kinase complexes can be

detected with regard to their activity on specific substrates. One might also examine the interaction of cyclin A1 with proteins known to interact with cyclin A2 *in vivo*, including upstream inhibitors and activators and/or downstream targets. A systematic screen of known targets of cyclinA2/Cdk complexes for differences in the specificity of kinase activity of cyclin A1-containing versus cyclin A2-containing complexes might be quite informative. In such a scenario, particular attention would be paid to substrates whose expression and function, if known, might suggest a role during spermatogenesis.

A second strategy might utilize a molecular genetic approach to address the question of functional redundancy between the cyclin A1 and A2 proteins. As noted before, we have generated a targeted mutation in the cyclin A1 gene that resulted in an arrest in spermatogenesis specifically at the late pachytene-diplotene stage of meiosis. The first meiotic division is prevented and the cells undergo a wave of apoptosis. One might now ask if cyclin A2 can rescue the meiotic arrest caused by the lack of cyclin A1 expression in the knockout mice. The use of either a transgenic approach using regulatory sequences that drive the expression of cyclin A1 to the appropriate cell types *in vivo* (K.M. Lele and D.J. Wolgemuth, unpublished observations) to drive coding sequences for cyclin A2 or alternatively, a knock-in approach (Hanks *et al.*, 1995) could be envisioned. The technically more-complicated “knock-in” strategy would involve placing cyclin A2 coding sequences within the context of the cyclin A1 gene and then substituting into the genome by targeted mutagenesis.

D. WHAT ARE THE SUBSTRATES FOR CYCLIN A1/CDK COMPLEXES IN GERM CELLS?

As mentioned before, cyclin A2 functions during both G1-S and G2-M phases in cultured cells (Girard *et al.*, 1991; Pagano *et al.*, 1992; Pines and Hunter, 1994). The activity of the cyclin A2/Cdk2 kinase complex has been shown to be regulated by either inhibitory or activating proteins. Cyclin A2/Cdk2 shares some common properties with other G1/S or G2/M-phase cyclin/kinase complexes as well as possessing several features that are unique. Unlike cyclin B1, cyclin A2 can bind and activate both Cdk1 and Cdk2 proteins (Elledge *et al.*, 1992; Kobayashi *et al.*, 1992; Rosenblatt *et al.*, 1992). While both cyclin A2/Cdk and cyclin B/Cdk1 phosphorylate histone H1, only cyclin A2/Cdk complexes were shown to associate with and phosphorylate the Rb-related protein p107 *in vitro* (Peeper *et al.*, 1993). Differences in the consensus phosphorylation site for cyclin A2 versus cyclin E/Cdk2 complexes have been determined (Higashi *et al.*, 1995). Cyclin A2/Cdk2 was more efficient than cyclin E/Cdk2 in phosphorylating the transcription factor E2f-1 (Kitagawa *et al.*, 1995). Cyclin A or E/Cdk2 and cyclin D/Cdk4 can all phosphorylate pRB; however, the phosphorylation sites are different (Kitagawa *et al.*, 1996). Much less is known about cyclin A1,

in particular as to how it may differ from cyclin A2 with respect to its interacting proteins and substrates. Our studies on the role of okadaic acid in overcoming the block in the first meiotic division in the absence of cyclin A1 led us to explore the role of the Cdc25 phosphatases as potential downstream targets of cyclin A1/Cdk. We previously had shown that Cdc25C is expressed in late meiotic prophase cells in the mouse testis (Wu and Wolgemuth, 1995) and, recently, that Cdc25A is also expressed in spermatocytes at this stage (as is cyclin A1) (Liu *et al.*, 2000). We have gone on to demonstrate that cyclin A1 can co-precipitate Cdc25A in co-transfection experiments and that testicular cyclin A1/Cdk2 kinase complexes can phosphorylate recombinant Cdc25 A and Cdc25C *in vitro* (Liu *et al.*, 2000). Whether the Cdc25s are critical substrates *in vivo* in pachytene spermatocytes remains to be determined.

E. WHAT ARE THE MECHANISMS BY WHICH THE LACK OF CYCLIN A1 TRIGGERS APOPTOSIS?

A striking aspect of the loss of cyclin A1 function during male meiosis is the abrupt entry of the late-pachytene/diplotene spermatocytes into cell death or apoptosis, as assessed by TUNEL (terminal-deoxy UTP nick end labeling) assay (D. Liu *et al.*, 1998). In fact, we have noted that apoptosis is characteristic of loss of function of several genes that have been shown by targeted mutagenesis to be required for the progression of meiotic prophase. Table I lists several of these genes and the stage at which the cells arrest. There are several pathways by which cells can undergo cell death, including pathways mediated by p53, members of the Bcl2/Bax family, Fas/Fas ligand, and the TNF-R superfamily (reviewed in Raff, 1998, and Strasser *et al.*, 2000). A role for p53-mediated pathway in the apoptosis observed in Atm-deficient spermatocytes is suggested by the elevated levels of p53, Bax, and p21 observed in Atm-deficient testes and the decrease in apoptosis seen in mice lacking both Atm and p53 function (Barlow *et al.*, 1997). There was not a complete loss of apoptosis, however, which raises the possibility of multiple pathways. The pathways of apoptosis for the other mutant strains listed in Table I remain to be elucidated.

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