13

Changes in DNA Replication during Animal Development

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Throughout animal growth and development, the cell cycle is modified in response to developmental signals. These alterations in the cell cycle influence the control of S phase and the properties of DNA replication (outlined in Table 1). The production of haploid gametes requires a modified cell cycle, meiosis, in which two rounds of chromosome segregation follow a single S phase. Thus, S phase must be prevented between the two meiotic divisions. In the early embryos of many animals, a rapid cell cycle occurs in which S phase oscillates with mitosis without gap phases. This S-M cycle necessitates unique controls for the entry into S phase. Later in embryogenesis in *Drosophila* and *Xenopus*, a G_1 phase is added to the cell cycle, resulting in another developmental alteration of the onset of S phase. There are numerous examples of tissues that become polytene as a consequence of a modified cell cycle with only an S phase and a gap phase.

These developmental changes in the cell cycle require special controls for entry into S phase. The regulation of the onset of S phase has been extensively investigated in the normal cell cycle with G_1-S-G_2-M phases and is reviewed by Nasymth (this volume) and Weisshart and Fanning (this volume). Cyclin-dependent kinases (cyclin-cdk complexes), including cyclins E, D, and A complexed with cdk2, 4, and 6, are all known to play a role in S-phase regulation of higher eukaryotes (Sherr 1993, 1994). G_1 cyclin kinases are thought to phosphorylate the retinoblastoma gene product, pRb, releasing it from the transcription factor E2F, thus allowing the S-phase transcriptional program (Sherr 1994). Other S-phase kinases, perhaps cyclin A kinase, might then inactivate E2F once its transcriptional program is complete (Dynlacht et al. 1994; Krek et al. 1994). In the yeast *Saccharomyces cerevisiae*, three functionally redundant

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DNA Replication in Eukaryotic Cells

Table 1 Alterations in regulation of DNA replication during development

Developmental change	S-phase control	Replication modifications
Meiosis	distinct entry into S phase	slower S phase *S. cerevisiae*: same origins used, asynchronous activation mouse: slower fork rate newt: fewer origins activated
Oocyte activation	*Xenopus*: Mos inhibition of S phase during meiosis	sea urchin: inactive initiation factors in the unfertilized egg
Restart of S phase at fertilization	*Drosophila*: inhibition of S phase prior to fertilization by *plu*, *png*, and *gnu*	
Early embryonic cycles	S/M cycle posttranscriptional control of S-phase genes *Drosophila*: genes that couple S and M phases	*Xenopus* and *Drosophila*: periodic spacing of origins; sequence independent and synchronous activation
Onset of transcription and addition of G_1 phase (MBT)	G_1 phase added; transcriptional control of S-phase genes	longer S phase; asynchronous origin activation and late-replicating heterochromatin
Polyploidy/polyteny	*Drosophila*: endo cell cycle (S/G); spatial and temporal pattern	*Drosophila*: removal of block to rereplication; late replication and underrepresentation of heterochromatin
Amplification		tissue and temporal control; removal of block to rereplication

cyclins, CLN 1, 2, and 3, control the G_1/S transition, and two cyclin-B homologs, CLB5 and 6, also act to control S phase (Richardson et al. 1989; Epstein and Cross 1992; Schwob and Nasmyth 1993). These cyclins all interact with the CDC28 kinase. In addition to these positive regulators, in the normal cell cycle, inhibitors of cyclin kinases exist that are able to inhibit progression of the cell cycle in response to various environmental cues (Sherr 1994; Sherr and Roberts 1995).

In the developmentally modified cell cycles discussed here, entry into S phase is regulated by different mechanisms from those of the normal cell cycle (Table 1). In yeast meiosis, the CDC28 kinase appears to act

later than S phase. During the embryonic S-M cycles, entry into S phase must be controlled posttranscriptionally and must be regulated in the presence of constitutive cyclin E kinase activity. The addition of a G_1 phase during embryogenesis requires that extrinsic developmental cues influence the onset of S phase. In *Drosophila*, this is mediated at least in part through cyclin E. During the S-G cycle that produces polytene cells, entry into S phase can no longer have the completion of mitosis as a prerequisite.

In addition to differential control of the onset of S phase, the actual parameters of S phase are altered during development. By parameters of S phase, we mean the intrinsic properties of DNA replication. The parameters of DNA replication changed in modified cell cycles include replication origin usage and activation, the rate of replication fork movement, and the block to rereplication (Table 1). We discuss how these parameters are changed in each of the variant cell cycles. For a background on the normal intrinsic regulation of DNA replication, we refer the reader to DePamphilis; Heintz; Newlon; Simon and Cedar; and Blow and Chong (all this volume).

S PHASE OF THE MEIOTIC CELL CYCLE

Meiosis is a modified cell cycle in which two rounds of chromosome segregation follow a single S phase. This results in the chromosome number being reduced by half. This is essential so that the chromosome number is restored when the sperm and egg fuse at fertilization. The S phase that precedes chromosome segregation in meiosis has been termed "premeiotic S." The use of the term premeiotic S implies that the term meiosis refers only to the actual segregation of chromosomes. We refer to the S phase of the meiotic cell cycle as premeiotic S, but the reader should realize that premeiotic S phase occurs as part of the same cell cycle in which the two meiotic divisions occur.

Regulation of Entry into S Phase

Studies addressing the regulation of premeiotic S phase have been investigated mainly in the yeast, *S. cerevisiae*. Many genes that govern S phase during the mitotic cell cycle have been characterized and are covered by Nasymth (this volume). However, several genes that are required for entry into S phase during the mitotic cell cycle are not required for the control of premeiotic S phase. Interestingly, some of these genes that are involved in S phase during the mitotic cell cycle do have a role in

meiosis, yet are required after replication and before the meiosis I division. One example is the mutation *cdc7*, in which mitotic cells are blocked prior to replication, yet meiotic cells are arrested following premeiotic DNA synthesis (Schild and Byers 1978; Buck et al. 1991). Recently, Cdc7 has been linked to replication origins, in that a protein that binds to and activates the Cdc7 kinase, Dbf4, also binds to *ARS* elements (Jackson et al. 1993; Dowell et al. 1994). If Cdc7 does prove to have a role at the replication origin, this might then suggest that premeiotic replication is differentially regulated. Other similar examples include the *S. cerevisiae* mutations, *cdc28* and *cdc4*, in which DNA replication is blocked in the mitotic cell cycle, yet premeiotic synthesis occurs (Simchen and Hirschberg 1977; Piggott et al. 1982; Shuster and Byers 1989; Reed and Wittenberg 1990). Cdc28 is a component of maturation promoting factor (MPF) and is required both at the G_1/S and G_2/M transitions during the mitotic cell cycle yet does not appear to be required for the G_1/S transition of premeiotic S phase. It should be noted, however, that the *Schizosaccharomyces pombe* homolog of *cdc28*, *cdc2*, is required for premeiotic DNA replication (Iino et al. 1995).

Spatial Control of Meiotic Replication Origins

Comparisons of S phase during the meiotic and mitotic cell cycles using fiber autoradiography indicated that a similar spacing of origins is present and that replication forks travel at a similar rate (Johnston et al. 1982; Newlon 1988). However, the resolution of fiber autoradiography was not precise enough to determine whether the specific origins used were the same origins used during the mitotic cycle. Given the fact that not all *ARS* elements identified by the plasmid assay are active chromosomal origins during the mitotic cell cycle, it is possible that the other *ARS* elements were specifically used during premeiotic S phase. In one study designed to examine *ARS1* function in premeiotic S phase, plasmid loss occurred in both mitosis and meiosis upon induction of transcription through the *ARS1* element (Hollingsworth and Sclafani 1993). Although this study used an indirect assay for *ARS* function, it suggests that the same origin can function in S phase in both the meiotic and mitotic cycles. In another study to identify replication origins active during premeiotic S phase, two-dimensional gel analysis was used to examine origins on chromosome III (Collins and Newlon 1994). The five premeiotic S-phase origins map to the same *ARS* elements as the origins from the mitotic cell cycle. One possible exception was an origin located

at *CEN3* that had weak activity in the mitotic cycle and did not appear to have any activity in the meiotic cycle. No additional premeiotic S-phase origins were found that did not correspond to origins used in the mitotic cycle. Further analysis will be needed to determine if this conclusion is true for all chromosomes, but these studies strongly suggest that meiotic origins are indeed the same as mitotic origins.

In higher eukaryotes, perhaps this is not the case. Using fiber autoradiography in the newt *Triturus*, the spacing between origins used in meiosis is larger than that seen during the mitotic cycle, suggesting that either different origins are used or a subset of mitotic origins are activated (Callan 1974).

Temporal Control of Meiotic Origins

S phase in eukaryotes can vary widely in length, from extremely short S phases during embryonic development (several minutes) to longer S phases in somatic cells (hours) and often very prolonged S phases during premeiotic S phase (at least 24 hours in the *Drosophila* ovary) (Chandley 1966; Grell 1973). S phase seems to lengthen as development places increasingly complex controls on the cell cycle. In *S. cerevisiae*, premeiotic S phase is at least twice as long as S phase in the mitotic cycle (65 min versus 30 min) (Williamson et al. 1983). Despite this lengthening of S phase, origin usage and the rate of fork movement are the same as during mitotic S phase (Johnston et al. 1982).

Two possibilities for the longer premeiotic S phase are either that origins within a chromosome are fired asynchronously or that different chromosomes are replicated at different times. In the study of origins in the meiotic cell cycle on chromosome III mentioned above, the efficiency of usage of specific *ARS* elements and characteristic termination patterns were similar between meiosis and mitosis (Collins and Newlon 1994). This implies that within a single chromosome the kinetics of initiation and termination are roughly the same in meiosis and mitosis. Further studies are needed to determine if this is true for all chromosomes. Collins and Newlon therefore suggest that the longer premeiotic S phase is due to different initiation times for different chromosomes.

In the premeiotic S phase of mouse spermatogonia, the lengthening of S phase was studied by fiber autoradiography (Jagiello et al. 1983). As in yeast, origin spacing during S phase in the meiotic and mitotic cycle is similar. However, the rate of fork movement is slower during meiosis and might account for the longer S phase.

S PHASE DURING EARLY EMBRYOGENESIS

Inhibition of DNA Replication in the Developing Oocyte and Unfertilized Egg

Many animals undergo rapid cell divisions following fertilization, including *Xenopus*, *Drosophila*, sea urchin, starfish, and clam. DNA replication factors are stockpiled in the egg to prepare for the high demand during the rapid embryonic divisions. However, DNA replication must be repressed in the oocyte so as not to occur during meiosis or prior to fertilization. This repression could be at the level of S-phase control such that entry into S phase is blocked during meiosis, or alternatively, at the level of replication parameters, such as initiation factors that might be kept in an inactive state. Two well-characterized mechanisms of replication control in the developing oocyte and unfertilized egg have been found in *Xenopus* and sea urchin.

The inability of *Xenopus* oocytes to replicate DNA is mediated by Mos activity blocking entry into S phase during meiosis (Furuno et al. 1994). Mos is a serine/threonine kinase that is expressed at high levels in germ cells of vertebrates and is maternally loaded into the *Xenopus* oocyte (Sagata et al. 1988). Mos acts at three steps in *Xenopus* oocyte maturation: (1) It activates meiosis; (2) it represses DNA replication between the meiotic divisions; and (3) it helps maintain the metaphase II arrest. The first activity of Mos is that of a meiotic initiator. *Xenopus* immature oocytes (stage VI) are arrested at G_2/M of meiosis I. Following hormone stimulation, germinal vesicle breakdown (GVBD) occurs, and oocytes progress to metaphase II of meiosis. This maturation is dependent on the translation of Mos mRNA following hormone stimulation, after which mature oocytes remain arrested at metaphase II until fertilization.

The second Mos activity, the ability of Mos to block DNA replication during meiosis, is relevant for this review. By examining a precise time course of MPF activity throughout meiosis, it was found that MPF is inactivated early in meiosis I (early metaphase I) and is then reactivated during late metaphase I, well before meiosis II (Furuno et al. 1994; Ohsumi et al. 1994). Mos mediates this reactivation of MPF, which then suppresses DNA replication between the two meiotic divisions. Ablation studies using c-*mos* antisense RNA or Mos antibodies showed that upon inactivation during GVBD, mature oocytes enter S phase inappropriately following meiosis I (Furuno et al. 1994). The role of MPF in suppressing an intervening S phase was also confirmed by studies that block MPF activation at meiosis II, resulting in DNA replication (Furuno et al. 1994; Ohsumi et al. 1994). Once MPF is reactivated, it is stabilized by the ac-

tion of cytostatic factor (CSF) at the metaphase II arrest (Minshull 1993). This is the third meiotic activity of Mos, because CSF is composed of both Mos and an unknown factor. Thus, Mos is needed to maintain the metaphase II arrest in the mature oocyte.

Mos homologs have not been identified in invertebrates, suggesting that different mechanisms might act to control replication during oocyte maturation. In contrast to the control of S-phase entry seen in *Xenopus*, the mechanism that inhibits DNA replication in the unfertilized sea urchin egg is the absence of active initiation factors responsible for DNA synthesis. Following oocyte maturation, sea urchin eggs complete meiosis and arrest at G_1 of the first mitotic cycle. Fertilization then releases this arrest, and replication factors are posttranslationally activated within a short 3 minutes following fertilization (Zhang and Ruderman 1993). The first S phase occurs 20 minutes following fertilization, even in the absence of protein synthesis.

The unfertilized egg of sea urchin is not permissive for DNA replication, as assayed by the inability of egg extracts to replicate added sperm nuclei or double-stranded DNA templates (Zhang and Ruderman 1993). Conversely, embryonic interphase extracts are capable of supporting replication of added templates. Mixing experiments of egg and embryonic extracts showed no evidence of negative factors in the egg capable of repressing replication; the egg extract did not inhibit the ability of the embryonic extract to replicate DNA. Thus, the unfertilized egg does not contain inhibitors of replication, and instead, the inhibition of replication seen is due to inactive initiation factors present at this stage of development.

Restart of S Phase at Fertilization

In many organisms, the unfertilized egg is arrested during or following meiosis, and fertilization then releases the egg from this arrest. This coupling of fertilization with the resumption of the cell cycle ensures that the female and male pronuclei can then enter the first S phase and subsequent cell cycles with the proper timing. In *Drosophila*, starfish, and sea urchin, the completion of meiosis is not coupled to fertilization. In *Drosophila*, the mature oocyte is arrested at metaphase I in the ovary. Upon passage of the egg through the uterus, the egg becomes activated, and meiosis is completed regardless of whether fertilization occurs. Fertilization is necessary to restart the cell cycle in the embryo following the completion of meiosis, and unique regulators may be needed at this developmental step.

In *Drosophila*, three genes that act at this point to couple fertilization with DNA replication are the maternal-effect genes *giant nuclei* (*gnu*), *pan gu* (*png*), and *plutonium* (*plu*). Unfertilized mutant eggs complete meiosis resulting in four meiotic products, yet then undergo improper DNA replication, resulting in large polyploid nuclei (Freeman and Glover 1987; Shamanski and Orr-Weaver 1991). These genes normally act as negative regulators of DNA replication to make the restart of S phase dependent on fertilization. Fertilization must overcome the action of these genes in order to resume the cell cycle.

In many organisms, including starfish, *Xenopus*, sea urchin, and mouse, a transient increase in intracellular calcium (Ca^{++}) occurs at fertilization and is associated with the onset of development including meiotic maturation, pronuclear migration, DNA replication, and nuclear envelope breakdown. Time-lapse confocal imaging was used to study calcium dynamics throughout starfish early development (Stricker 1995). No transient increases in intracellular calcium were observed during oocyte maturation, whereas a single prolonged transient coincided with fertilization, and repetitive calcium oscillations occurred during the early cleavage divisions. The levels of inositol triphosphate also fluctuate during fertilization and might act to trigger the calcium transients seen during sea urchin development (Ciapa et al. 1994). However, the cause and effect relationship between calcium and the second messengers in other systems remains less clear (Whitaker and Swann 1993).

Following the calcium burst in *Xenopus* mature oocytes, the calmodulin-dependent kinase, CaMKII, is responsible for the inactivation of both MPF and CSF, thus releasing the oocyte from a metaphase II arrest (Lorca et al. 1993). How this then regulates the resumption of DNA replication following meiosis is less clear. The role of calcium bursts in controlling DNA replication can be seen in starfish, where the calcium ionophore, A 23187, acts as a parthenogenic agent capable of inducing several rounds of replication in mature oocytes (Picard et al. 1987). The calcium ionophore also triggers the onset of the cell cycle in unfertilized sea urchin eggs (Whitaker and Patel 1990). Whether transient fluxes in calcium levels play a role in *Drosophila* fertilization and the restart of S phase is unclear.

Rapid Early Cycles in *Xenopus* and Flies

Many organisms undergo rapid embryonic cycles following fertilization. The early embryonic cycles of *Xenopus* and *Drosophila* have been well characterized and consist of rapid cycles of alternating S phase and M

phase that are controlled by maternally supplied products present in the egg. DNA replication is differentially regulated during this time in development. Unique regulation occurs at both the entry into S phase and in the replication parameters used. S-phase entry is regulated posttranscriptionally due to the absence of zygotic transcription. DNA replication parameters are also developmentally controlled such that many synchronous origins are activated to ensure complete replication within the very rapid 3- to 10-minute S phases.

Posttranscriptional Control of S Phase

The rapid early cycles of *Xenopus* and *Drosophila* are controlled by posttranscriptional modifications of regulators during S phase and M phase. In *Drosophila*, the first 13 divisions in the fertilized embryo are rapid, synchronous nuclear divisions within a common shared cytoplasm, where S phase occurs in an extremely short 3- to 4-minute period. The early cycles in *Xenopus* are similar in that the first 12 divisions consist of rapid, synchronous cycles with S phase occurring in a brief 10 minutes. During these early embryonic cycles of both *Xenopus* and *Drosophila*, transcription of the zygotic nucleus does not occur. In *Drosophila*, maximal zygotic transcription occurs following cellularization at cycle 14, whereas in *Xenopus*, zygotic transcription occurs at the midblastula transition (Newport and Kirschner 1982b; Edgar and Schubiger 1986).

Known regulators of S phase are present at high levels in *Drosophila* and *Xenopus* embryos. In *Drosophila*, the S-phase cyclin E-cdk2 kinase is present throughout early embryonic development (Richardson et al. 1993; Knoblich et al. 1994). Similarly, a large pool of maternal cyclin E is present during the early cleavage divisions of *Xenopus* (Jackson et al. 1995). The constitutive high levels of these proteins indicate that control of entry into S phase during the early division cycles must be regulated differently than during the later cycles when S phase is preceded by a G_1 phase.

In addition to the posttranscriptional control of known S-phase regulators, unique regulators might also be used early in development for the rapid cell cycle. In *Drosophila*, three maternal-effect genes, *giant nuclei* (*gnu*), *pan gu* (*png*), and *plutonium* (*plu*), appear to be novel cell cycle regulators. As discussed above, these genes are needed to couple S phase with fertilization such that mutant unfertilized eggs undergo inappropriate DNA replication. When fertilized, these embryos also give rise to giant polyploid nuclei, suggesting that these genes control S phase during the early cycles. Although fertilized, these embryos fail to proper-

ly couple S phase and M phase, and some aspects of mitosis such as centrosome duplication continue to cycle independently from nuclear division (Freeman et al. 1986; Freeman and Glover 1987; Shamanski and Orr-Weaver 1991). *png* is unique in that several presumably leaky alleles transiently couple S phase and M phase, resulting in embryos containing many more giant nuclei. However, defects in mitotic figures and DNA condensation can be seen as early as the first division, and the uncoupling of replication and mitosis progresses as development proceeds (J. Carminati, unpubl.). The unfertilized and fertilized phenotypes can be explained by the proposal that these genes inhibit DNA replication. In unfertilized eggs they function to make S phase dependent on fertilization, whereas in fertilized embryos these gene products make S phase dependent on the proper completion of mitosis. However, at what level these genes act is unclear. They might control either the entry into S phase, the block to rereplication, or other aspects of the cell cycle, such as chromosome condensation, that link mitosis to replication.

Molecular data confirm that *plu* is a unique regulator that acts solely during the early *Drosophila* divisions and not in later canonical cell cycles. RNA null alleles are maternal-effect alleles, and expression of the *plu* transcript is not present during later stages of development (Axton et al. 1994). Plu encodes a 19-kD protein consisting of three ankyrin repeats. Interestingly, another small ankyrin repeat protein is the cdk4 inhibitor, $p16^{INK4}$, which acts to inhibit cdk4, thus disrupting its association with the S-phase cyclin D protein (Serrano et al. 1993). Plu might act analogously in its role in repressing DNA replication, possibly by inhibiting S-phase cyclin kinases until their proper time of action.

Replication Origins

Studies in both *Xenopus* and *Drosophila* suggest that in the extremely rapid cycles of the early embryo, origins are controlled by a unique mechanism perhaps involving chromosome folding or attachment to the nuclear envelope. This could ensure the complete replication of the genome during the brief S phase. This mechanism acts temporally and spatially, such that periodically spaced origins are activated synchronously at the beginning of each S phase.

Experiments using *Xenopus* eggs showed that plasmid replication upon injection into the egg is under cell cycle control. Replication is not dependent on specific sequences, but instead depends on the size of the plasmid injected (Harland and Laskey 1980; Méchali and Kearsey 1984). Two-dimensional gel analysis of replication intermediates revealed that

in both *Xenopus* eggs and extracts, plasmids containing either rDNA repeats or single-copy sequences initiated and terminated replication at random sites throughout the plasmid (Hyrien and Méchali 1992; Mahbubani et al. 1992). It was also determined that although initiation could occur at random sites on the plasmid, a single initiation event gave rise to complete replication of each plasmid molecule.

Similar conclusions were reached upon examination of the replication of chromosomal rDNA repeats in early embryos prior to the midblastula transition (Hyrien and Méchali 1993). Initiation occurred at random positions, and the estimated replicon size was 9–12 kb. To complete replication in the rapid S phase, all origins were presumed to be activated synchronously at the beginning of S phase. Whereas replication initiation is random with respect to sequence, the periodic spacing of replicons suggests that initiation is not random with respect to higher-order chromatin folding. If initiation were entirely random, there could be instances where some replicons were too far apart to finish replication in the short S phase. Thus, the authors suggest that chromosomal folding might specify a periodic spacing of origins, guaranteeing complete replication within the 10-minute S phase.

A similar periodic spacing of replicons was seen in the *Drosophila* early cleavage nuclei in which replication occurs in a 3- to 4-minute S phase. By electron microscopy (EM) studies, the average replicon size was 7.9 kb with a preferred periodicity of 3.4 kb and a maximum size of 19 kb (Blumenthal et al. 1974). An estimated 20,000 bidirectional origins must be activated nearly synchronously to finish S phase in the extremely short time. The maximum replicon size correlates with the amount of DNA that can be replicated in 3–4 minutes, given the rate of fork movement observed. However, these studies did not resolve whether initiation occurs at defined or random sequences. Using two-dimensional gel analysis of replication intermediates from early embryos, it was found that random initiation occurs both within the histone repeats and within a 40-kb single-copy sequence (Shinomiya and Ina 1991). Therefore, the periodicity of replicon spacing and the sequence-independent nature of replication are similar to that found in early *Xenopus* embryos. Thus, there appears to be a specific control of origin usage in the early rapid cycles.

S-phase Slowdown

Following the rapid embryonic cell cycles, the cell cycle lengthens to allow certain developmental processes to occur, such as the onset of zygotic gene transcription and gastrulation. In both *Xenopus* and *Drosoph-*

ila, the increase of the nuclear-to-cytoplasmic ratio results in the slowing of the cell cycle (Newport and Kirschner 1982a,b; Edgar and Schubiger 1986). During this time in development, both the control of replication origins and the entry into S phase are altered. In *Xenopus*, this is a one-step process that occurs at the midblastula transition (MBT), whereas in *Drosophila* these events occur in two distinct steps. First, following cellularization at cycle 13, alterations in the temporal control of origins occur. At this time, a G_2 phase is added to the cell cycle, and zygotic transcription commences. Following three more divisions, a G_1 phase is added, permitting transcriptional control of S phase. This transcriptional control in *Drosophila* and the MBT in *Xenopus* are discussed below.

In *Drosophila*, replication proceeds from the fast synchronous S phase of the early cycles to a prolonged S phase in which origin activation becomes asynchronous. A change in condensation of both euchromatin and heterochromatin occurs during this lengthening of S phase (Foe et al. 1993). During the first 13 cycles of the *Drosophila* embryo, the euchromatin remains decondensed throughout interphase. The heterochromatic regions decondense for a short time in S phase, decondensing progressively later in interphase as the cycle slows during cycles 11 to 13. By cycle 13, S phase has lengthened from 3–4 minutes to 13 minutes. During the three post-blastoderm cycles 14 to 16, S phase is eight times as long as the early S phases, taking 35–45 minutes.

In contrast to the early cycles, at least 200 particles of highly condensed euchromatin can be seen during interphase of cycles 14, 15, and 16 (Foe et al. 1993). EM studies show that new forks appear throughout the first 20 minutes of S phase during cycle 14, indicating that origin activation becomes asynchronous during interphase of these later cycles (McKnight and Miller 1977). Heterochromatin remains condensed throughout most of interphase and is not replicated until euchromatic replication has finished (Edgar and O'Farrell 1990; Foe et al. 1993). This altered regulation of origins and concurrent lengthening of S phase correlates with the onset of zygotic transcription and tissue-specific expression of certain genes. Replication must be coordinated with other processes now occurring during the cell cycle. In the post-blastoderm cycles, replication is followed by a G_2 phase of varying length, whereas mitosis occurs in distinct temporal and spatial domains.

Mouse

Similar to *Xenopus*, mouse embryos also are able to support the replication of injected double-stranded DNA templates, whereas injected plasmids are not replicated in oocytes (Wirak et al. 1985). Replication in em-

bryos is dependent on specific *cis*-acting origin sequences of polyomavirus (PyV) or simian virus 40 (SV40) present on the plasmids. This is in contrast to results in *Xenopus* eggs, in which replication is independent of *cis*-acting sites and initiates at random sequences.

Using mouse embryos, an added layer of control is seen when comparing arrested one-cell embryos containing the unfused male and female pronuclei with two-cell embryos containing zygotic nuclei (Martinez-Salas et al. 1988, 1989). One-cell embryos are able to replicate DNA containing a minimal origin core sequence from PyV, whereas the two-cell embryo requires enhancer sequences present in *cis* to the PyV origin. Enhancers are also required for gene expression in the zygote as opposed to the one-cell embryo (Martinez-Salas et al. 1989; Majumder et al. 1993; Melin et al. 1993). The cytoplasmic factors that act on promoters and enhancers are not present in the one-cell embryo, but rather appear during the formation of the two-cell embryo (Henery et al. 1995). Enhancers are postulated to prevent the repression of origins and promoters by altered chromatin structure that is thought to occur upon zygote formation.

Replication in the mouse embryo is distinct from that occurring in the rapid early embryonic cycles of *Xenopus* and *Drosophila*, possibly due to the difference seen in zygotic transcription in these organisms. *Xenopus* and *Drosophila* have an early period lacking zygotic transcription, so are able to support fast cycles of alternating S and M phase. In contrast, transcription occurs in the early mouse embryo following zygotic formation, and thus replication must be coordinated with transcription. Enhancers might be associated with origins to overcome chromatin effects due to a transcriptionally active genome.

DEVELOPMENTAL SHIFT TO TRANSCRIPTIONAL REGULATION OF S PHASE

Following the rapid cycles of *Xenopus* and *Drosophila* that are controlled by posttranscriptional modifications of cell cycle regulators, a developmental shift occurs and transcription of the zygotic genome becomes active. At this time in development, S-phase regulators can be controlled at a transcriptional level, a mode of regulation characteristic of the somatic cell cycle.

MBT in *Xenopus*

The cell cycle of *Xenopus* is modified during the MBT, which occurs after the first 6 hours of development. The early rapid cycles of S and M

phase slow, giving rise to a longer cell cycle in which both G_1 and G_2 gap phases are added to the cell cycle. This process then allows for the resumption of zygotic transcription (Kimelman et al. 1987). This slowing of the cell cycle is thought to occur due to a mitotic initiation factor that becomes rate-limiting at the MBT (Kirschner et al. 1985; Newport et al. 1985). Blastula cleavage becomes less synchronous, cells become motile, and zygotic transcription turns on. Recent results determined that the excess of histones present in the early embryo is responsible for the repression of transcription prior to the MBT (Prioleau et al. 1994).

As mentioned previously, the developmental regulation of S phase is altered at two different levels during the MBT in *Xenopus*. First, similar to *Drosophila*, origin activation presumably becomes asynchronous, giving rise to a lengthened S phase. DNA replication becomes asynchronous between cells, as evidenced by a variation of proliferating cell nuclear antigen (PCNA) staining, with some nuclei showing a peripheral staining and others showing a homogeneous staining (Leibovici et al. 1992). This is in contrast to the early cycles in which PCNA staining is homogeneous throughout S phase. Second, the entry into S phase is altered such that a G_1 phase is added to the cell cycle. Zygotic transcription resumes, permitting S-phase regulators to become transcriptionally controlled.

Addition of G_1 in *Drosophila*

The developmental shift to asynchronous origin activation has already occurred by cycle 14 of the *Drosophila* embryo. The second developmental control placed on S phase occurs following cycle 16 when a G_1 phase is added to the cell cycle. Following cycle 16, mitotic embryonic cells either arrest in G_1 and divide later in development (imaginal cells) or continue to divide (neuronal cells). The cells giving rise to most of the larval tissues become polytene, and as described below, these cells go through additional cycles that have only S and gap phases, but no mitosis. Thus, cycle 17 is the first time during embryogenesis there is a G_1 phase and, with the exception of a small group of cells on the ventral epidermis, it occurs in cells that are becoming polytene. At this point in embryogenesis, the cyclin E gene changes from being constitutively transcribed to a dynamic pattern of transcription in which the appearance of cyclin E transcripts precedes S phase. The down-regulation of cyclin E is needed for the arrest of cells in G_1, and cyclin E is then necessary for the G_1/S transition (Richardson et al. 1993; Knoblich et al. 1994). Both the cyclin E transcript and protein are down-regulated following the last

mitotic division of epidermal cells during cycle 16 and remain off in these G_1-arrested cells (Knoblich et al. 1994).

During *Drosophila* development, the transcriptional control of cyclin E during the added G_1 phase is a new mode of S-phase regulation that occurs specifically following embryonic cell cycles 1–16. Mutations in the E2F transcription factor block DNA replication beginning at cycle 17, correlating with the first appearance of G_1 (Duronio et al. 1995). In embryonic cells that have a G_1 cell cycle phase, cyclin E is the downstream target of E2F, as evidenced by the observation that ectopic expression of cyclin E can overcome the requirement for E2F for S phase (Duronio and O'Farrell 1995). Thus, E2F appears to control the developmental pattern of cyclin E transcription that is responsible for the G_1 to S transition. Cyclin E in turn activates the transcription of a set of genes encoding essential replication factors, including polymerase-α, *PCNA*, and ribonuclease reductase 1 and 2 (*RNR1, 2*) (Duronio and O'Farrell 1994). E2F and cyclin E exert a different role in the cells becoming polytene than in the neuronal cells that lack a G_1 phase (Duronio and O'Farrell 1995). In the nervous system, cyclin E continues to be constitutively expressed, and this expression is independent of E2F. Rather, in the nervous system, cyclin E appears to regulate E2F.

POLYPLOIDY/POLYTENY

Changes in S-phase Regulation

Polyploid cells exist in a number of organisms including plants, ciliates, and dipteran insects, as well as in some mammalian cell types such as the trophoblasts that give rise to the mammalian placenta. Polyploidy is often associated with cells or tissues in which a requirement for increased protein production is needed; multiple chromosome copies is one way in which evolution has met that demand. An area of current research concerns the identification of regulators that govern the developmental transition leading to polyploidy. Similar to the developmental alterations of DNA replication already discussed, polyploid replication is controlled at two basic levels. These include changes in regulation of the cell cycle as well as alterations at the level of replication origins and other parameters used during DNA synthesis. In *Drosophila*, the transition to polyteny results in an altered cell cycle, termed the endo cell cycle, which consists of an alternating S phase and gap phase (Smith and Orr-Weaver 1991). Parameters of replication also become altered, and the block to rereplication is overcome during the endo cell cycle.

Polyploid and polytene cells are defined as those in which DNA replication has become uncoupled from mitosis, giving rise to cells with greater than diploid content of DNA. The degree to which replication is uncoupled from mitotic aspects of the cell cycle can vary. Polytene cells uncouple replication from all aspects of mitosis; 1000 or more chromosome copies remain synapsed, forming the large polytene chromosomes characteristic of the *Drosophila* salivary gland. Some polyploid cells do not uncouple replication from all aspects of mitosis, and chromosome segregation or cycles of chromosome condensation still occur. This is referred to as endopolyploidy or endomitosis. Finally, multinucleate cells have been referred to as polyploid cells. The *Drosophila* larval polytene cells are among the best characterized in terms of alterations in S phase that occur during this developmental transition.

Regulators of the Endo Cell Cycle

One of the best-understood examples of polyteny is in the *Drosophila* larval tissues (Smith and Orr-Weaver 1991). Following embryogenesis, *Drosophila* larval growth is due to an increase in cell size upon polytenization, because only cells in the nervous system and imaginal tissues undergo mitosis during larval development. In *Drosophila*, most tissues enter the endo cell cycle during late embryogenesis, and this transition is temporally and spatially regulated (Smith and Orr-Weaver 1991). The first transitions to the endo cell cycle occur in tissue-specific domains that replicate at characteristic times after cycle 16, with the salivary gland being the first tissue to enter the endo cell cycle, followed by the midgut, hindgut, and Malpighian tubules. It was determined for the hindgut that cells enter the endo cell cycle from the G_1 phase of the cell cycle, whereas salivary gland cells may enter the endo cell cycle following G_2. Gap phases of the endo cell cycle can vary widely in length, from 3 hours for the midgut to 18 hours for the salivary gland (Smith and Orr-Weaver 1991).

The spatially and temporally regulated pattern seen in *Drosophila* polytene tissues argues that a novel factor controls these cycles. However, regulators of the endo cell cycle could in theory be known mitotic cell cycle regulators that also act to control the endo cell cycle. In this case, the dependency between S phase and M phase must be disrupted in this altered cell cycle. In *Drosophila*, the mitotic cell cycle regulators, cyclin A, cdc2, and the cdc25 phosphatase homolog, *string*, are not needed for the endo cell cycle. Endoreplication proceeds in embryos lacking any of these regulators (Smith and Orr-Weaver 1991; Smith et al. 1993; Stern et al. 1993).

The cyclin E gene is essential for polytenization. The endo cell cycle does not occur in embryos lacking cyclin E or E2F, and cyclin E expression parallels S phase in endoreplicating tissues (Knoblich et al. 1994; Duronio et al. 1995). Thus, developmental regulation of the transcription of the cyclin E gene can account for the pattern of polytene S phases. Cyclin E transcription in endoreplicating cells is subject to a negative feedback, because ectopic expression of cyclin E down-regulates endogenous cyclin E transcription in these cells (Sauer et al. 1995). This negative feedback loop may be responsible for the transient appearance of cyclin E transcripts in the endo cell cycle and could be necessary for cyclic, rather than continuous, DNA replication in these cells. It may simply be that periodic transcription of cyclin E in the absence of cyclins A and B results in a cycle of S phase in the absence of mitosis (Sauer et al. 1995). However, it is possible that novel regulators couple developmental signals to the regulation of the endo cell cycle.

An interesting gene, *escargot*, is a transcription factor that appears to maintain the diploid state of arrested imaginal cells in the *Drosophila* larva (Hayashi et al. 1993; Fuse et al. 1994). In certain allelic combinations, a group of imaginal cells known as the histoblast nests overreplicate. Inappropriate expression of *escargot* in the polytene salivary gland represses endoreplication. A model has been proposed by which *escargot* maintains diploidy via transcriptional repression of regulators of the endo cell cycle. However, an alternate model might be that *escargot* plays a more direct role in controlling the cell fate of imaginal cells.

In the fission yeast, *S. pombe*, the absence of cyclin B due to a *cdc13* deletion causes cells to undergo multiple rounds of S phase, resulting in high levels of polyploidy (Hayles et al. 1994). These results have led to a model whereby high levels of cyclin B-cdc2 kinase promote entry into M phase and, conversely, low levels cause the entry into S phase. Thus, by simply disrupting the cyclin B kinase activity, cells are able to reset to a G_1 phase and enter S phase. In this example, *S. pombe* has created a cell cycle leading to polyploidy solely by altering the control of mitotic cell cycle regulators. The cell cycle of *S. pombe*, however, represents a very simplified cell cycle. In higher eukaryotes a more complex set of regulators controls the cell cycle and ensures the proper coupling of replication and mitosis. Therefore, in most organisms, the transition to the endo cell cycle most likely requires more than simply inactivating G_2/M regulators, although this might be a necessary step for the transition to polyteny. Other regulatory changes must also occur, such as the alteration of checkpoints that act to couple S phase and mitosis, as well as the block to rereplication which must be removed.

Removal of the Block to Rereplication

A major alteration in the parameters of replication that must occur during the endo cell cycle is the removal of the block to rereplication. One postulated mechanism that normally limits DNA replication to once per cell cycle is the existence of licensing factor. This factor is proposed to permit one round of DNA replication and to then become inactivated following replication (Blow and Laskey 1988). Active licensing factor is then excluded from the nucleus, and reentry into the nucleus is only permitted following nuclear envelope breakdown in mitosis (see Blow and Chong, this volume). Perhaps the actions of licensing factor or other factors involved in replication initiation are altered in the endo cell cycle, but the nature of this alteration is unclear. A mutation has been identified in the *Drosophila MCM2* gene, a proposed component of licensing factor (Treisman et al. 1995). It is interesting that this mutation inhibits proliferation of the diploid imaginal and neuronal cells but has almost no effect on the polytene cells. The polytene cells grow and undergo DNA replication, but the chromosomes may be more fragile in the mutant. Thus, licensing factor may become dispensable in the endo cell cycle. In the case of the multiple rounds of replication that occur in *S. pombe* as described above, the block to rereplication was removed solely by the disruption of cyclin B without a direct effect on licensing factor.

Late-replicating Heterochromatin/Underrepresentation

Another aspect of altered replication during the endo cell cycle is the temporal control of polytene replication. Fiber autoradiography of polytene chromosomes shows a pattern of late-replicating heterochromatic regions (Spradling and Orr-Weaver 1987). In *Drosophila virilus* the replicon size in polytene chromosomes is similar to diploid brain cells, yet the rate of fork movement is three times slower (Steinemann 1981a,b). Similar studies of *Drosophila nasuta* show that shorter and slower replicons are seen in late-replicating regions (Lakhotia and Sinha 1983). Perhaps the chromatin organization of polytene heterochromatin inhibits replication fork movement. This slower replication might account for the late replication of the heterochromatin.

A second characteristic alteration of DNA replication in the endo cell cycle is that 20–30% of the genome is underrepresented, including the centric heterochromatin and rDNA and histone repeats. Less underrepresentation of rDNA is seen, however, in the polyploid nurse cells, most likely due to the function of the nurse cells in producing the rRNA for the developing oocyte. Regions of euchromatin are generally repli-

cated to the same extent during polyploidization. In *Drosophila* polytene chromosomes, both bands and interbands also replicate to the same extent, as confirmed by quantitative Southern blots (Spierer and Spierer 1984).

Heterochromatic underrepresentation might be caused by incomplete replication of specific sequences during polytenization, or alternatively, by elimination of sequences from the chromosome (Karpen and Spradling 1990; Glaser et al. 1992). One hypothesis is that elimination of specific regions is caused by the excision of transposable elements. Interestingly, many transposable elements are found solely in heterochromatic regions. A testable prediction of the elimination model is that novel DNA junctions should be formed upon elimination. The role of underrepresentation during the endo cell cycle is unclear; perhaps these sequences are not needed for the high level of protein expression characteristic of most polytene tissues.

Amplification

Amplification of specific genomic sequences is a developmentally regulated mechanism that allows the production of large amounts of protein products in a short developmental time frame. Amplification control occurs at the level of the block to rereplication of specific sequences, whereby reinitiation of replication leads to multiple copies of genomic sequences. Amplification provides a model system to study the developmental regulation of a eukaryotic replicon. Two well-characterized examples of developmental amplification are the *Drosophila* chorion genes and the *Sciara* DNA puffs. This is reviewed more extensively by Gerbi and Urnov (this volume).

Drosophila Chorion Genes

During oogenesis, the somatic follicle cells surrounding the egg chamber are responsible for the secretion of the chorionic eggshell layers encompassing the developing oocyte. Following polyploidization of these cells, a further tissue-specific mechanism ensures an increased copy number of the chorion genes, so that proteins can be made in a rapid developmental time window. The major chorion genes are organized into two chromosomal clusters present on the X and third chromosomes, which amplify to levels of 15-fold and 60-fold, respectively (Orr-Weaver 1991). Amplification within these clusters occurs by repeated reinitiation

of an origin as shown by multiple eye forms (bubbles within bubbles) in EM spreads (Osheim et al. 1988).

Studies of both clusters have identified *cis*-acting regions responsible for amplification, termed the amplification control element (ACE). ACE3 of the third chromosome cluster has been delineated to a 320-bp region that acts in a distance- and orientation-independent manner (Orr-Weaver et al. 1989). Replication intermediates in this region have been analyzed, and a predominant replication origin has been mapped 1.5 kb downstream from ACE3. This lies in a region important for high levels of amplification known as amplification enhancing region-d, (AER-d) (Delidakis and Kafatos 1989; Heck and Spradling 1990). However, initiation events also occur throughout a 12-kb region surrounding both ACE3 and AER-d.

ACE3 is able to direct the autonomous amplification of sequences when inserted throughout the genome, albeit at lower amplification levels (Carminati et al. 1992). These studies suggest a model by which ACE3 controls the reinitiation of nearby origins, perhaps by capturing limiting replication factors and overcoming a block to rereplication.

Sciara DNA Puffs

Amplification also occurs in the fungus fly *Sciara coprophila* within puff regions of the larval salivary gland polytene chromosomes. Similar to the *Drosophila* chorion genes, amplification is temporally and tissue-regulated. Amplification as well as transcription of the *Sciara* DNA puffs is developmentally regulated by the steroid hormone, ecdysone (Bienz-Tadmor et al. 1991; Gerbi et al. 1993). Amplification presumably allows the rapid production of proteins needed during late larval development such as those for the formation of the pupal case. Similarly, in *Rhynchosciara*, puffs encode polypeptides necessary for the production of the pupal cocoon and undergo amplification (Glover et al. 1982).

Amplification occurs in several major puff regions in *Sciara*, and puff expansion is due to a burst of transcription following amplification of these sequences to approximately 20-fold levels (DiBartolomeis and Gerbi 1989; Wu et al. 1993). DNA amplification within one of the major puffs, II/9A, has been well characterized and is consistent with an onion-skin mechanism, similar to the *Drosophila* chorion genes. Puff II/9A encodes two genes that share 85% sequence similarity, and a major amplification origin has been mapped to a 1-kb region lying upstream of the two genes (Liang et al. 1993; Liang and Gerbi 1994). Replication from this origin occurs bidirectionally.

SUMMARY

DNA replication is regulated by a wide variety of mechanisms that act throughout development to coordinate S phase and replication with developmental transitions. Replication can be controlled both at the level of key S-phase regulators and at the level of parameters of DNA synthesis, such as origin usage. By studying the unique ways that different developmental events control and alter S phase, we will broaden our understanding of the regulators and mechanisms involved in DNA replication.

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