Journal of Cellular Biochemistry Supplements 30/31:1–7 (1998)

The S Phase: Beginning, Middle, and End: A Perspective

Heide L. Ford and Arthur B. Pardee*

Dana-Farber Cancer Institute, Boston, Massachusetts 02115-6084

Abstract Events in the S phase of the cell cycle have been investigated to a relatively limited extent in comparison with those in G1 and M phases. Four aspects of S are briefly discussed in this report: (1) the final biochemical step permitting initiation of DNA synthesis, (2) determination of replication timing of individual genes and its mechanism, (3) S phase processes that lead to the onset of M phase, and (4) resetting the S-phase machinery. J. Cell. Biochem. Suppls. 30/31:1–7, 1998.

Key words: S phase; DNA replication; gene replication

S phase was described in 1951, when Howard and Pelc demonstrated that all nuclear DNA in a growing cell is replicated during only a portion, S, of the interval between cell divisions. This germinal finding created the cell cycle with its well-known four sequential phases: G1, S, G2, and M.

As one cell becomes two during each cycle, all its components ($>10^9$ macromolecules) must be duplicated in amounts and in space. These syntheses take place in a defined sequence throughout the cycle. Numerous biochemical processes are active in S phase, including basal metabolism, RNA and protein synthesis, etc. Nuclear DNA replication is the focus of this perspective. Its beginning is marked by incorporation of the first deoxynucleotides. Then, for a period of several hours, all the DNA is duplicated. Each of the approximately 80,000 different genes replicates during a definite and relatively brief time. Toward the end of S phase, a cell begins preparation for its terminal cycle events of mitosis and division. When DNA synthesis ceases, marking the end of S phase, the cells begin to prepare for the next S phase, while making sure that replication does not occur again until the proper time. Perhaps surprisingly, these four major S phase processes are not well defined molecularly, particularly in mammalian cells.

Because all the above-mentioned areas involve cell growth control, cancer cells could be altered in any one or all of these areas. A better understanding of the general mechanisms that govern replication and determining whether they are modified in cancer cells may prove to be a very fruitful venture. This perspective briefly outlines some existing information on these matters and provides suggestions for further research.

BEGINNING

Preparation During G1 Phase for DNA Synthesis

Cell proliferation is regulated by a series of checkpoints in the cell cycle, each of which depends on the completion of prior events. These checkpoints are responsible for controlling normal cell growth. A key checkpoint is at the restriction point (R) in late G1 [Pardee, 1989], at which effects of positive and negative growth factors commit the cell to another round of DNA replication and subsequent cell division. The R point mechanism is often defective in cancer cells. A great deal has been learned about the molecular biology of events involved in the R point process [Denhardt, 1998].

Checkpoints can be controlled by cyclin dependent kinases (cdk) which are activated by regulatory proteins named cyclins. Cyclin E is essential for G1/S phase progression and binds and activates the cdk2 kinase shortly before entry into S phase. Overexpression of cyclin E accelerates the G1 phase of the cell cycle and dimin-

Contract grant sponsor: National Institutes of Health; Contract grant number: CA-61253; Contract grant sponsor: Dana-Farber Cancer Institute.

*Correspondence to: Arthur B. Pardee, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115-6084.

Received 14 September 1998; Accepted 15 September 1998

ishes the requirement for growth factors. An additional level of control is exerted through the p21 protein, which binds and inhibits cyclin E/cdk2 [for review, see Ohtsubo et al., 1995]. Cyclin E is a strong candidate for controlling the R point because it appears at about the time of R, its half-life is close to that of a protein proposed from cell biological experiments to regulate passage through the R point, and it is overproduced in tumor cells [Keyomarsi et al., 1993]. Furthermore, while cells lacking a functional Rb tumor suppressor molecule no longer require cyclin D/cdk activity [Lukas et al., 1995], cyclin E/cdk2 activity remains essential [Ohtsubo et al., 1995]. Many human cancer biopsy specimens and cell lines are abnormal in amount and size of their cyclin E.

But how cyclin E/cdk2 activates initiation of DNA synthesis remains unclear. The events between the R point and S phase are not at all understood. Cyclin E is known to phosphorylate Rb, whose phosphorylation state regulates members of the E2F family of transcription factors believed to be important for S phase entry. However, overexpression of cyclin E can override a G1 arrest caused by a phosphorylation deficient pRb mutation [Lukas et al., 1997], suggesting other critical downstream targets for cyclin E/cdk2. Transcription of genes just before entry into S phase is necessary but not sufficient, and the cyclin E/cdk2 complex might activate this process by phosphorylation of a transcription factor, which could then activate genes encoding enzymes required for DNA synthesis. In addition, cyclin E/cdk2 could phosphorylate and activate components of the DNA replication machinery, as has been suggested [Voitenleitner et al., 1997].

A Compartmentalization Hypothesis

The nuclear envelope serves as a selective barrier to macromolecular exchanges with the cytoplasm and thereby provides compartmental regulation to eukaryotic cells [Ohno et al., 1998]. During the 2 h between R and S phase, newly produced enzymes involved in DNA synthesis are transported from cytoplasm to nucleus, the site of their activity [Reddy et al., 1982]. Thus, the final G1 event before initiation of DNA synthesis could be activation of essential proteins' transport across the nuclear membrane, which permits their access to sites of DNA synthesis. Cyclin E/cdk2 might activate entry of these enzymes, by catalyzing either their phosphorylation or that of the elements of the nuclear membrane transport system. The basis for this idea is that (1) cyclin E/cdk2 activity is the final known event of R point control, (2) kinases can activate transport of proteins into the nucleus, and (3) enzymes necessary for DNA synthesis are transported into the nucleus at the time between the R point and the start of DNA synthesis. Interestingly, a membrane-based regulation mechanism has been described at the end of prophase, when phosphorylation of nuclear lamins by the cyclin B/cdc2 complex results in opening the nuclear barrier by membrane disintegration [Peter et al., 1991].

The mechanism of nuclear import may be either selective phosphorylation of substrate molecules, or global effects on components of the transport system such as cytosolic factors and nuclear pore proteins. Cdks can phosphorylate substrates of nuclear import and thereby modify their nuclear translocation, as reported for the SV40 large T antigen NLS. Such phosphorylations can modulate the cell cycle-dependent translocation of proteins such as Rb, cyclin B1, v-jun, and the yeast transcription factor SW15.

Molecular exchanges between the cytoplasm and nucleus are through highly complex and uniform nuclear pores, via both passive diffusion and active transport. The numerous components of the giant pore complex are incompletely described. The aqueous channel in the center of the nuclear pore complex permits diffusion of molecules less than 90 Å in diameter. Those molecules that are larger than ~60 kD can only be actively transported across the pores, based on their appropriate nuclear localization sequences (NLS).

Nuclear pores can control entry of proteins through selective phosphorylation of components of the transport system, such as cytosolic factors and nuclear pore proteins. Evidence exists linking cell cycle proteins to such phosphorylations. The NBP60 NLS receptor protein, present in the nuclear envelope, is highly phosphorylated by cdc2 mitotic kinases. Cdc2 also phosphorylates the 200- and 97-kD nuclear pore glycoproteins in a *Xenopus* egg cell free system [Macaulay et al., 1995]. However, the functional consequences of these modifications are not known.

A group of cytosolic factors is responsible for docking a substrate protein to the cytoplasmic

surface of the nuclear pore. Then translocation across the nuclear pore requires the small protein Ran/TC4, which belongs to the Ras protein family. Ran has intrinsic GTPase activity, which is critical for cell cycle progression in G1 → S and is also important for DNA synthesis. These data raise the possibility that Ran regulates G1/S phase transition via its nuclear transport activity. In addition, the GTP-bound form of Ran is required for cyclinB/cdc2 activation, thus demonstrating its role in G2 → M [Clarke et al., 1995].

As import of proteins may be important in controlling DNA synthesis, so may export. Export of the cdc47 protein that coincides with the onset of DNA replication has been observed in budding yeast [Dalton and Whitbread, 1995]. It is possible that many other such exported proteins exist.

MIDDLE: TRANSIT THROUGH S PHASE

Gene Replication Timing

Although molecular events before the G1/S boundary have been examined in considerable detail, less is known about mechanisms permitting initiation of individual gene replication during S phase. Studies in yeast have provided us with a much greater understanding of the proteins associated with origins of replication, as well as the cell cycle control exerted on them [for review, see Toone et al., 1997]. However, much remains unexplored, particularly in mammalian systems where replication may be more complex. What happens to permit replication of a specific subset of genes just as cells enter S phase? One interesting and relatively unexplored question of S phase progression revolves around the issue of replication timing.

S phase is characterized by the temporally ordered replication of the eukaryotic cell genome. Gene sequences studied to date have definite replication times within S phase. Active alleles, regardless of their chromosomal position, replicate earlier than do inactive alleles [Boggs and Chinault, 1994]. Almost all housekeeping genes replicate within the first half of S phase. Carbamyl phosphate synthetase, dihydrofolate reductase, and glucose 6-phosphate dehydrogenase (G6PDH) replicate early in S phase in HeLa cells [Goldman et al., 1984]. Genetically inert genes, such as satellite DNA and genes on the inactive X-chromosome, often replicate very late in S phase [Goldman et al., 1984]. These data demonstrate the tight cell cycle control of replication and suggest a relationship between replication and transcription.

Gene replication times are not irrevocably fixed. The replication timing of the β-globin genes is not fixed during development, where it is replicated early in expressing cells and late in nonexpressing cells [Dhar et al., 1989]. Chromosomal rearrangements can activate cryptic origins of replication [Leu and Hamlin, 1992], suggesting that altered replication patterns may be observed in cancer, in which chromosomal rearrangements are frequent [Goldberg et al., 1991]. Indeed, exons of the c-*myc* proto-oncogene display altered temporal replication after chromosomal rearrangements [Stanton et al., 1983].

Study of Mechanisms

What mechanisms govern each gene's replication timing in transit through S phase? Does this process depend on chromosomal territories, in which DNA domains are localized in the nucleus, perhaps to membrane regions [Cremer, 1996]? Perhaps replication timing depends on a spectrum of proteins that become sequentially associated with DNA domains during S phase [Chang et al., 1995]. Numerous DNA replication inhibitors can be used to synchronize cells to allow their passage through the cycle to be investigated. Replication timing mechanisms could be studied with cells containing chromosomal translocations, which should allow one to determine whether DNA sequences or structures governing replication timing are 5′ or 3′ of a gene.

Replication of Selected Genes

When does replication of a specific gene occur during S phase? Do alterations in replication timing of some genes in cancer cells correlate with alterations in their transcriptional activities? A useful tool would be a simple and general method for determining the replication timing of any given gene. One approach uses the fluorescence in situ hybridization (FISH) method [Cremer, 1996], in which a given gene on its chromosome is labeled by hybridization to a fluorescent probe. The number of labeled dots per cell doubles when the gene is replicated. Another approach might depend on a twofold increase in transcription rate when a gene replicates, although such a change could be difficult to detect.

One could determine replication timing of cell cycle related tumor suppressors and oncogenes in cancer versus normal cells, since these genes often exhibit altered expression levels in cancer. For example, cyclin A, which is involved in two major checkpoints of the cell cycle—the G1-S transition and the G2-M transition—has increased mRNA levels in breast tumor cells, as compared with normal cells [Keyomarsi and Pardee, 1993]. It may therefore be of interest to determine whether its increased transcriptional activity correlates with an earlier replication timing in cancer, although the mRNA increase is at least in part due to greater stability. Any gene with altered transcriptional activity (whether in cancer vs normal or in early vs late stage of development) may serve to examine its replication timing in relation to transcriptional activity. While considerable data exist correlating the two, it remains to be determined whether this relationship is direct or indirect.

Coupling of Gene Duplication With Transcription

Is the correlation between transcriptional activity and replication timing due to an actual direct coupling of the two processes to each other, or rather because the two processes are each coupled to underlying cell cycle processes? Possible mechanisms for the relationship between replication and transcription have been suggested. However, this relationship is complex and warrants further study. No conclusive data exist showing that replication timing per se directly influences transcriptional activity or vice versa, although the relationship has been examined in some detail.

Several studies suggest that transcription promotes replication [DePamphilis et al., 1988]. Binding of a transcription factor to a promoter site activates transcription, and similarly this binding near an origin of replication could activate replication. Eukaryotic chromosomal origins of DNA replication are organized into modular arrays; some of the modules serve as binding sites for transcriptional activators [Marahrens and Stillman, 1992]. When these sites are changed to sites binding unrelated transcriptional activators, origin function remains intact as long as the transcription factor binding to that site is active. Furthermore, enhancers that stimulate transcription are known to function as components of origins of DNA replication [DePamphilis et al., 1988].

To address whether transcriptional activity directly affects replication timing, simple transfection studies could be used in which a reporter gene is stably transfected into mammalian cells. The reporter would be inactive until transfection with a second gene (the activating transcription factor). Replication timing of the reporter gene could be examined before and after the second transfection to see whether transcriptional activation alters the replication timing. This assay could be extended to utilize a mutated activator that could bind the DNA but not activate transcription. This would address whether binding at the promoter (and thus altering DNA structure) is sufficient to affect replication timing, or whether transcriptional activation per se is necessary. A caveat to such an experiment would involve the effect of the insertion site of the reporter plasmid, since chromosomal context is increasingly acknowledged as an important regulator of gene function. This suggests that the relationship may not be so simple.

It would be particularly interesting to examine the role of DNA methylation in coupling replication timing with transcriptional activity. Differential methylation is believed to play a role in the formation of active and inactive chromatin and has been linked to both transcription and replication [for review, see Haaf, 1995]. Indeed, recent evidence suggests that CpG islands (G + C-rich regions that are free of methylation) are initiation sites for both transcription and replication [Delgado et al., 1998].

END: PREPARING FOR M PHASE

Events initiating mitosis, after the G2 phase, have been intensively studied. They principally involve production of cyclin B and changes of phosphorylation and activation of cdc2 kinase. But preparatory mitotic events may occur in the preceding S phase and are therefore of relevance in this perspective.

We have recently embarked on a study to identify genes whose expression increases toward the end of S phase, with the idea that some of these transcriptional changes may be important for events at the end of the cell cycle. For this purpose, a mammary carcinoma cell line was synchronized in late G1/S phase with mimosine; differential display analysis was performed as the cells progressed through S phase. One cDNA band that was isolated was initially very low and increased in late S phase. It was

sequenced and identified as HSIX1, a homeobox gene recently cloned from human adult skeletal muscle [Boucher et al., 1996].

Expression of the HSIX1-related *sine oculis* (*so*) gene during *Drosophila* eye development precedes a burst of mitosis [Cheyette et al., 1994]. This result, together with the late S phase cell cycle-regulated expression of HSIX1 in human cancer cells, supports the suggestion that HSIX1 plays a role in preparing the cell for division. To test this hypothesis, the MCF7 mammary carcinoma cell line was transfected with HSIX1. When these cells were irradiated, the DNA damage-induced G2 cell cycle checkpoint was changed as compared with (transfected) controls. The data demonstrated that overexpression of HSIX1 leads to an abrogation of the DNA damage-induced G2 cell cycle checkpoint [Ford et al., 1998]. HSIX1 provides an example of a gene expressed at the end of S phase that has an effect later in the cycle. We expect that others will soon be found.

CODA: RESETTING THE S PHASE MACHINERY

Why can only one S phase occur per cycle? And what preparations must be made for the subsequent S phase? The original licensing model proposed that a replication factor present only during G1 is required for initiation of DNA synthesis, is destroyed during S phase, and then is resynthesized after the nuclear membrane breaks down at mitosis [Blow and Laskey, 1988; Chong et al., 1995]. This would prepare the cell for another round of DNA synthesis.

Recent data suggest that loss of cyclin B/cdc2 activity is necessary to reset the system and make chromatin permissive for initiating duplication. In yeast, this step occurs at the end of mitosis, where the decrease in cyclin B/cdc2 activity allows for the formation of a pre-replicative protein complex over the origin of replication. The subsequent rise in G1 cdk activity as the cells pass START leads to the initiation of DNA replication from the preformed initiation complexes. Finally, as cyclin B/cdc2 levels rise again, not only is origin firing triggered, but formation of new pre-replicative complexes is inhibited, ensuring that the DNA is only replicated once per cell cycle [for an excellent review, see Toone et al., 1997].

Recent evidence in mammalian cells suggests that cell cycle proteins such as p53, Rb, and p21 may be involved in the coupling of S and M phases, ensuring that DNA replication does not occur in the absence of cell division [Khan et al., 1998, Niculescu et al., 1998, Waldman et al., 1996].

FUTURE PROSPECTS

Mechanisms

The molecular bases of most of the processes outlined here are not well understood. For example, does cyclin E influence nuclear transport? If so, how does it do this? Does the time of duplication of a gene depend on specific binding of proteins to adjacent DNA motifs? Are such proteins transcribed and translated when preceding genes are duplicated? Or is the timing of duplication more dependent on DNA methylation or chromosomal architecture, or both? What other transcriptional changes at the end of S phase prepare the cell for upcoming cell division? These are fertile fields for future investigation.

Cancer

Several of the above-mentioned areas have already been identified as altered in cancers. Alterations of nuclear architecture in cancer cells are well recognized [Nickerson, 1998]. Furthermore, a few studies suggest that changes in DNA replication timing may be observed in cancer [Adolph et al., 1992; Calza et al., 1984; Stanton et al., 1983]. For example, murine T-cell lymphomas replicate the lymphocyte antigen-6 and the neighboring thyroglobulin gene early in S phase. When these cells are fused with normal fibroblasts, however, resulting in nontumorigenic cells, these two genes are replicated later in S phase. The entire region between these two genes might switch to early replication in the tumor cells [Adolph et al., 1992]. In addition, while examining four pairs of homologous loci in normal versus chronic myeloid leukemia and lymphoma patients, Amiel et al. [1998] demonstrated that all samples derived from normal patients showed high levels of synchrony in replication timing of alleles, whereas those from cancer patients showed large temporal differences, with both early and late replicating alleles.

We also have observed changes in cancer with respect to genes transcribed at the end of S phase. The HSIX1 gene is overexpressed in 44% of primary breast cancers and 90% of metastatic lesions examined to date [Ford et al.,

1998]. Expression of HSIX1 was not found in human blood, so this gene may be a useful marker for both detection and prognosis.

This review highlights the need for more emphasis on studies of S phase. This will help us to gain a more complete understanding of the growth regulatory mechanisms of normal cells and of cancerous cells.

ACKNOWLEDGMENTS

This work is dedicated to Sir Michael Stoker on his 80th birthday, with respect and affection. H.L.F. was supported by DFCI Tumor Biology grant T32-CA 09361.

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