

# Replication origin plasticity, Taylor-made: inhibition vs recruitment of origins under conditions of replication stress

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**Abstract** Among his many contributions to the field of chromosome structure and dynamics, J. Herbert Taylor showed that eukaryotic cells have many more potential replication origins than they use, which they can recruit when replication forks are slowed to complete S-phase in a timely fashion. Thirty years later, his findings raise an important but largely overlooked paradox. Although new data have confirmed his results, a larger body of data has revealed that slowing replication forks activates an S-phase checkpoint cascade that inhibits initiation from unfired origins until the stress is relieved. In this paper, in celebration of Taylor's work published in *Chromosoma* 30 years ago, I draw attention to this paradox and offer some plausible models to explain how replication stress can both inhibit and recruit new origins. I hope that this essay will stimulate further experimentation into the basis of Taylor's original findings.

## Introduction

Eukaryotic cells must not only limit replication to only one round per cell cycle but also must ensure the complete synthesis of all DNA within the time frame of a single S-phase. Cell division with unreplicated segments of DNA leads to chromosome breaks (Lengronne and Schwob 2002; Syljuasen et al. 2005). In fact, whenever sufficient numbers of replication forks stall, either by encountering DNA

damage or under conditions that reduce fork processivity, cell cycle checkpoint pathways are activated that prevent cells from proceeding through S-phase. Depending upon the nature of the replication block, these checkpoints can prevent any further initiation of replication and/or maintain the integrity of the stalled replication forks so that they may resume elongation rapidly after the problem is corrected (Dimitrova and Gilbert 2000; Lopes et al. 2001). Cells are routinely confronted with challenges to the replication fork, yet the overall length of the mammalian S-phase is remarkably resilient, with most of the variability in cell cycle length accounted for by the length of G1-phase. Hence, an important question for those interested in cell growth and proliferation is: How do cells balance the need to maintain genome integrity with the need to complete replication within a timely fashion under environmental conditions that routinely cause fluctuations in replication fork elongation rates (often referred to as replicational stress)?

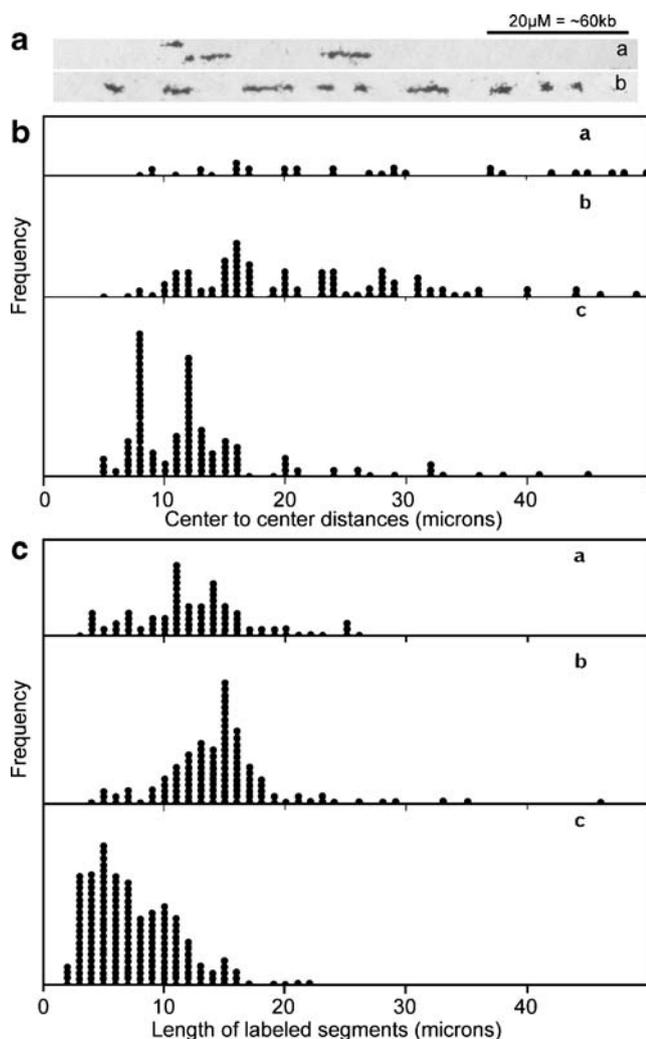
## Old wine: how to maintain a constant S-phase when starved for nucleotides?

In the 1970s, J. H. Taylor was interested in a question that we are still debating to this day—whether or not specific DNA sequences dictate the sites where replication initiates in mammalian cells (Gilbert 2004). Toward this goal, Taylor had developed a protocol in which Chinese hamster ovary (CHO) cells could be highly synchronized at the very beginning of S-phase by mitotic selection followed by blocking DNA synthesis with the drug fluorodeoxyuridine (FdU; Fig. 1). FdU inhibits the conversion of dUMP to dTMP and hence starves cells for thymidine. The high degree of synchrony allowed him to use very short pulse-labeling

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**Fig. 1** Increase in origin density in response to nucleotide starvation. **a** CHO cells were synchronized in mitosis and released into medium lacking thymidine for 3.5 h and then FUdR was added for and additional 3 (*a*) or 8 h (*b*). Cells were then released from the FUdR block and pulse-labeled with  $^3\text{H}$ -TdR for 12 (*a*) or 8 min (*b*), and DNA fiber autoradiography was performed. **b** Histograms showing the center-to-center distances between labeled segments on the same DNA fiber (one dot per event) in cells blocked with FUdR as in **a** for 3 (*a*), 8 (*b*), or 16 h (*c*). **c** Histogram of replication fork elongation rates (one dot per event) in cells blocked with FUdR as in **b**. Interpretation: The longer cells are starved for TdR, the slower are their fork elongation rates and the more origins they utilize to complete S-phase in a similar amount of time (figure adapted from Taylor 1977)

times, ensuring that the observed DNA synthesis was very close to replication origins. To detect origins, Taylor used DNA fiber autoradiography, a method made possible by Taylor's prescient development of tritiated thymidine as a tool for metabolic labeling (Taylor 1953). His 1977 *Chromosoma* paper (Taylor 1977) was an attempt to reconcile the enigma that when his FdU-synchronized cells were transferred to medium without FdU but containing low concentrations of high specific activity tritiated thymidine (TdR), the total amount of labeling was

increased, although the rates of fork movement were significantly reduced (nearly half) from their normal  $\sim 1,500$  nucleotides/min (Kurek and Taylor 1977; Taylor et al. 1973). The most logical way for this to have occurred is through an increase in the number of initiation sites, thereby assembling a greater number of replication forks. In fact, this is exactly what Taylor found (Taylor 1977). The longer he held cells at the beginning of S-phase with FdU, the closer together were the sites of labeling on DNA fibers, indicating a higher density of replication origins. At the longest blocking times, origins seemed to be present in intervals of approximately 12 kb (4 microns), leading him to conclude that cells normally use only 1 in 15 to 20 of the potential sites that they can recruit for initiation of replication (average spacing without FdU  $\sim 220$  kb). Because the length of the labeled tracks from different origins was very similar, he concluded that many sites are fired simultaneously when cells are released. Hence, cells have many more potential origins than they use and can adjust the number of active origins in accordance with fork elongation rates to complete S-phase in a timely fashion.

#### New bottles: improved methodologies allow identification of specific origin sites

Recently, Anglana et al. (2003) used modernized methods to confirm Taylor's findings and extend them by establishing "the number and nature of the sites," as Taylor wished he could have done (Taylor and Hozier 1976). Novel fluorescence microscopy methods made it possible to simultaneously visualize sites of DNA synthesis and specific DNA sequences on individual fibers to determine the approximate locations of replication origins. Moreover, because fluorescence can be visualized directly, results could be recorded immediately rather than after 6 months of autoradiography. Finally, through sequential pulse labels with two different halogenated nucleotides, iododeoxyuridine (IdU), and chlorodeoxyuridine (CldU), which can be distinguished with specific antibodies, the direction of replication fork movement along the length of the DNA fiber could be unambiguously determined. Naturally, during the course of three decades, many other methods were developed to map replication origins. However, DNA fiber methods remain the only approach that can reveal the distribution of origins that simultaneously fire on a given DNA molecule.

The Debatisse group had mapped a specific origin near the adenosine deaminase 2 (AMPD2) gene in a Chinese hamster lung (CHL) fibroblast cell line in which this gene and  $\sim 200$  kb surrounding it had been amplified through stepwise selection in coformycin (an inhibitor of AMPD2) to approximately 100 copies per cell. They were interested

to know whether the same origin would be used in other, independently derived cell lines in which the amplicons (segments of amplified chromosomal DNA) had different structures. Surprisingly, one of these cell lines (474) initiated replication at several origins in addition to the previously mapped origin that was preferentially used in two other lines (471 and 422), and frequently initiated at more than one of these origins simultaneously (Fig. 2). This same cell line also displayed a replication fork speed approximately half that of the other cell lines, although its S-phase length was similar. In agreement with the Taylor results then, slow fork progression in 474 appears to be compensated for by the activation of otherwise silent replication origins.

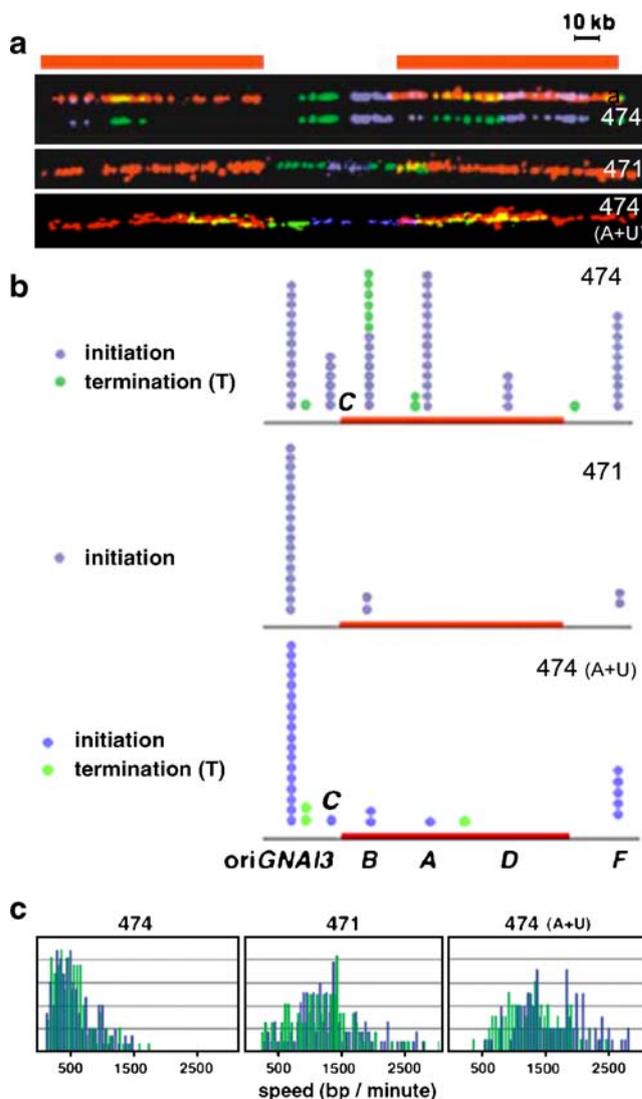
To further probe this “compensation” hypothesis, Anglana et al. searched for growth conditions that could increase fork progression rates in cell line 474 and found that they could more than double the fork speed in the presence of adenine and uridine without affecting the overall length of S-phase. Consistent with the “compensation” hypothesis, the density of replication origins was reduced by approximately half, and the specificity of origin usage near the AMPD2 gene was altered to remarkably resemble the other two cell lines, 471 and 422 (Fig. 2). The authors then treated 471 and 422 with hydroxyurea (HU), which starves cells for nucleotides through inhibition of ribonucleotide reductase. These cell lines responded to the reduced replication fork rates by initiating replication at the same additional origins used by 474. Together, these results confirm Taylor’s conclusions and extend them by suggesting that the adjustment of origin choice is not stochastic. When forks progress slowly, a specific group of origins is activated; under conditions of rapid fork rates, initiation is focused to a specific subset of potential sites.

### A paradox: S-phase checkpoints inhibit unfired origins

At face value, these two papers seem remarkably consistent and are supported by apparently sound cellular logic. However, recent work from many laboratories has demonstrated that the stalling of replication forks activates an intra-S-phase checkpoint cascade that inhibits unused origins to prevent further initiation of replication until the source of the fork stalling (e.g., collisions with damaged DNA, low nucleotide pools, inhibitors) can be corrected. Numerous studies have probed the molecular mechanisms by which this checkpoint is transduced from the stalled fork to the repressed origins, revealing highly conserved signaling pathways (Nedelcheva-Velleva et al. 2006; Pasero et al. 2003). This presents a contradiction to the findings that fork stalling activates additional replication origins. To illustrate this paradox most clearly and identify models of

reconciliation, I will focus on recent reports that used DNA fiber studies to examine origin usage under conditions of replicational stress.

The first paper, from Merrick et al. (2004), looked for the firing of new replication origins in human HeLa and IMR90 cells after reducing nucleotide pools with HU. In



**Fig. 2** Nucleotide pools modulate origin choice. **a** CHO cell lines 471 and 474 or cell line 474 grown in medium supplemented with adenine and uridine (A+U) were sequentially labeled with IdU and CldU. Isolated DNA fibers were stained with fluorescent antibodies to IdU (blue) and CldU (green) and were hybridized with probes specific to DNA near the AMPD2 gene (red). Fibers were aligned by their red signals. Blue tracks flanked by green tracks demarcate origins of bidirectional replication; green tracks flanked by blue represent termini. **b** Histogram of the number of initiation (blue) or termination (green) events (one dot per event) observed at each indicated position. **c** Histograms showing the percentage of fibers presenting a given replication rate for either blue or green tracks. Interpretation: Replication forks move more slowly, and initiation takes place at more sites in 474 than 471; the addition of A+U to 474 produces an initiation pattern and elongation rate that is similar to 471 (figure adapted from Anglana et al. 2003)

these experiments, cells were first enriched in early S-phase (albeit not necessarily at the very beginning of S) and then briefly pulse-labeled with IdU, followed by a pulse label with a combination of CldU and HU. In this way, replication forks that were active before replication stress (HU) are tagged with IdU, the degree of extension of those forks in the presence of HU is revealed by consecutive IdU/CldU tracks, and any newly fired origins generate tracks labeled exclusively with CldU. This method determines the ratio of newly fired origins after stress to replication forks before stress. The study clearly demonstrated that the addition of HU, over a range of concentrations that slowed replication fork growth from approximately fivefold to nearly complete stalling, severely inhibited the firing of new origins. These authors concluded that a partial depletion of nucleotide pools *inhibits* new origins from firing, rather than increasing the number of origins.

A second series of studies took advantage of the versatile *Xenopus* egg extract cell-free DNA replication system (Marheineke and Hyrien 2004; Woodward et al. 2006 and references therein). In these experiments, *Xenopus* sperm chromatin was introduced as a substrate into extracts supplemented with aphidicolin, a drug that directly impedes the action of DNA polymerase, and the density of origins on DNA fibers was determined by pulse-labeling the earliest sites of DNA synthesis with biotinylated dUTP. Under these conditions, aphidicolin had no effect on the local density of earliest firing origins, but instead inhibited the initiation of replication at later firing origins. These investigators went on to inhibit the checkpoint-signaling pathway with caffeine, which is known to inhibit at least two critical mediators of the S-phase checkpoint cascade. This caused a dramatic increase in the density of newly fired replication origins, whether or not aphidicolin was present. Apparently, in this system, a checkpoint control mechanism inhibiting origin firing is activated even in the absence of external inhibition of replication. The authors concluded, as did Taylor and Debatisse, that there exist many more potential origins than are typically used in a normal cell cycle and these origins can be recruited to complete DNA synthesis more rapidly. However, activation of these origins required conditions in which checkpoints were inhibited, emphasizing in the words of Woodward et al., “the counterintuitive nature of the intra-S-phase checkpoint that globally blocks further origin firing (when) it might seem better for cells to respond by using more origins.”

### Testable working models

How can slow replication fork movement both inhibit and recruit unfired origins? Are there differences in these

experimental systems that provide possible explanations for this paradox? Three possible models seem worthy of further experimentation (Fig. 3).

First, the two responses may result from different *degrees* of replicational stress imposed by each experimental system. It may be advantageous for cells to ignore minor fluctuations in nucleotide pools or low levels of DNA damage that occur frequently and can be quickly repaired, whereas severe blockages may require more extreme measures including cell cycle arrest or even cell death. Biological signaling pathways that produce qualitatively different outputs at different signal strengths are commonplace in developmental biology (Ashe and Briscoe 2006; Schreiber and Bernstein 2002) and in cell cycle responses to DNA damage (Sancar et al. 2004). In support of this “threshold” model, the cell lines studied by the Debatisse group differed by only about twofold in their fork elongation rates (Fig. 2c), much less than the fivefold or more seen in the Diffley study. Similarly, the FUdR treatment used by Taylor studies reduced fork elongation rates by approximately half, albeit only after releasing cells from conditions of nearly complete fork arrest. In extracts from *Xenopus* eggs, the S-phase checkpoint is activated even in the absence of inhibitors of replication. However, in this system, both the rates of replication and the response to the checkpoint inhibitor caffeine are dependent upon the batch of extract and the concentration of nuclei (Marheineke and Hyrien 2004; Woodward et al. 2006). Hence, some factor(s) may be limiting in egg extract preparations, and/or there may be variability in the activities of proteases or other enzymes during extract preparation, providing a trivial explanation for why the checkpoint becomes activated in the absence of inhibitors. In fact, robust preparations of extracts that replicate sperm nuclei with kinetics similar to the *in vivo* situation show a minimal change in replication rate in response to caffeine (see Fig. 3b of Luciani et al. 2004).

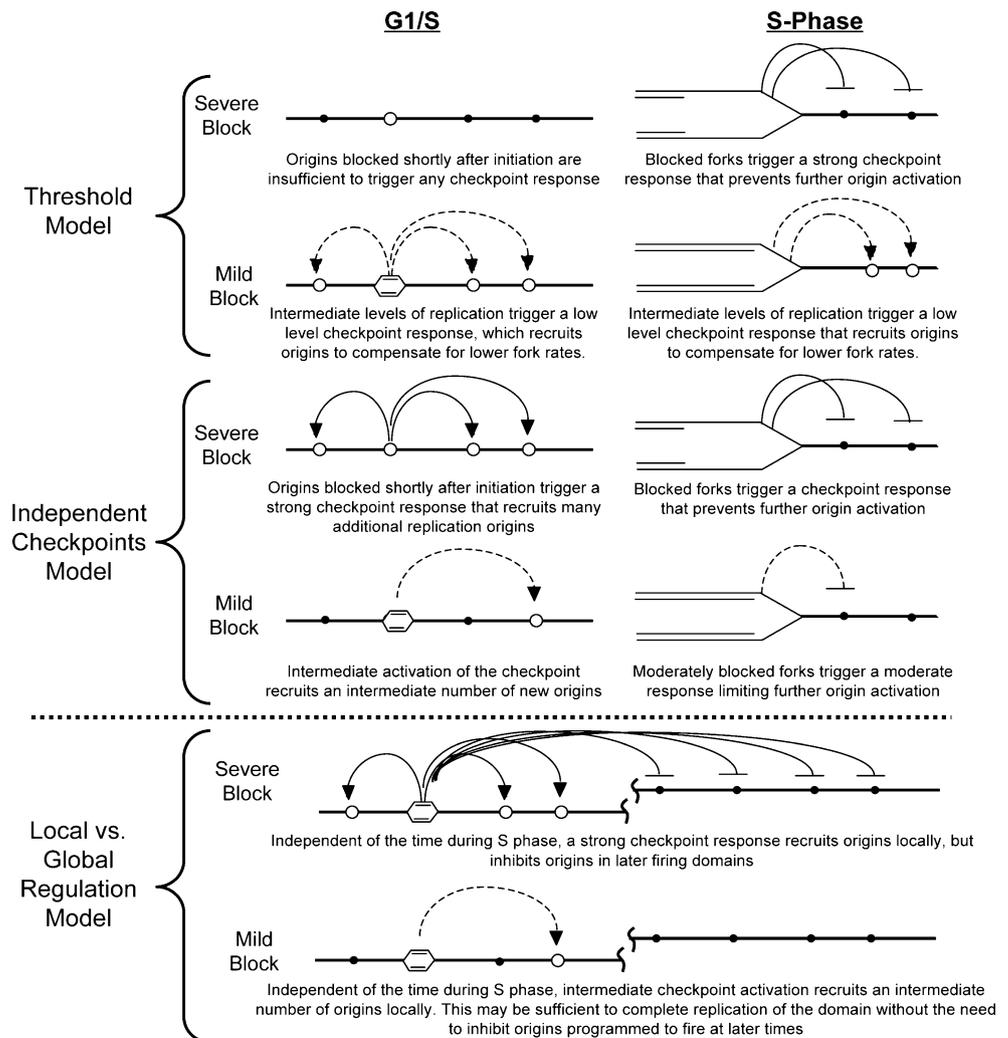
A second possibility is that the different experiments are revealing qualitatively different checkpoints, one that blocks further origin firing when pre-assembled replication forks are arrested, and a second that increases the number of replication origins to compensate for too few replication forks. The Taylor study, in particular, used conditions in which G1-phase cells were held at the beginning of S-phase with barely detectable DNA synthesis before release. Under these conditions, it is possible that there are insufficient numbers of replication forks to surpass the threshold necessary for S-phase checkpoint activation (Cobb et al. 2004; Shimada et al. 2002), or that primers are not of sufficient length for checkpoint activation (Byun et al. 2005). Cells may then generate an alternative response to the insufficient number of replication forks. Consistent with this “independent checkpoints” model (Fig. 3), Taylor

found that the longer he held cells at the beginning of S-phase, the higher was the density of origins observed upon release. In the *Xenopus* cell-free system, the threshold for S-phase checkpoint activation may be exceeded too rapidly to witness origin recruitment without disabling the checkpoint. If this model is correct, then the S-phase checkpoint, once activated, must override the signal to activate new origins. This would make it very difficult to observe origin recruitment unless cells were highly synchronized at the G1/S boundary.

A third model is that origin recruitment is a localized effect to complete the synthesis of individual replicons, while origin inhibition affects more distal origins. In other words, under conditions of replicational stress, cells may recruit flanking origins to complete synthesis of local replicons while inhibiting the initiation of replication at DNA segments that are programmed to replicate at later times during S-phase (Fig. 3). When forks are moving slowly, it may be important to stimulate the completion of

replicon clusters that have already initiated, while holding off on initiating at other sites to insure the proper temporal sequence of events during S-phase. This would also allow the cell to adjust for frequent minor fluctuations in fork rates locally, without the need to activate a global checkpoint response. Localized cross talk between origins makes sense when one considers that chromosomes replicate via the synchronous firing of clusters of replication origins that together form coordinately replicating units of chromosome structure that may be regulated from a common replication complex (or “factory”) that engages multiple replicons simultaneously (Gilbert and Gasser 2006). Local stimulation could occur through a positive recruitment mechanism that stimulates nearby origins to fire. Alternatively, local origin recruitment could be a passive process; fork stalling may simply increase the length of time (and hence the probability) that additional origins have to fire before being replicated by a passing fork. This model accounts for the differences between the

**Fig. 3** Models and predictions. The models developed in the text are depicted, with their predicted effects on checkpoint responses and origin activation in cells synchronized at the very beginning of S-phase (G1/S) or examined at arbitrary times during S-phase. In the case of the “Local vs Global Regulation Model,” the outcome should not vary at different times during S-phase so long as the experimental design focuses on newly fired origins



three mammalian cell studies discussed above (Taylor, Debatisse, and Diffley), since the methods used in the Diffley study were not designed to examine initiation at new origins fired within the same fibers that contained arrested forks.

### DNA fibers pave the way

Whether one of these models—or aspects of all three models—can resolve the paradox raised in this essay will require more experimentation using DNA fiber methods, which are the only methods that can directly measure changes in origin density. An important question to address is whether the S-phase checkpoint is activated in experiments where origin recruitment vs inhibition is observed. A simple experiment would be to vary the degree of inhibition of replication forks in cells before and after they have assembled replication forks (i.e., G1 vs S-phase). All three models predict that severe blockages already present upon entry into S (G1/S) would not activate an S-phase checkpoint because cells could not form sufficient numbers of arrested fork structures (due to insufficient synthesis of primers) necessary to trigger the checkpoint (Byun et al. 2005; Cobb et al. 2004; Shimada et al. 2002), whereas the same severe blockages during S-phase would trigger checkpoint activation mediated by pre-existing forks. When cells are grown under conditions of intermediate replication fork blockage, where forks can extend but at reduced rates, the distinction between the models would become clear. If the threshold model is correct, below a certain level of replication fork arrest, the S-phase checkpoint will not be activated, and an increase in the number of active origins will be observed regardless of whether the stress is experienced during entry into S or after S-phase has already begun. Above that threshold, origin firing will be inhibited as soon as sufficient forks are assembled to trigger the checkpoint. However, if the independent checkpoints model is correct, the localized density of origins fired at the onset of S-phase will increase proportionately to the severity of the stress, whereas the density of newly fired origins in cells already in S-phase when the stress is applied will decrease in proportion to the severity of the stress. To address the local vs global regulation model, experiments must be designed to detect both localized densities of replication origins and global effects on normally later replicating origins. Perhaps most important of all, there is a need for experiments that compare different conditions within the same experimental system, as cells may respond differently to different fork-arresting agents (Liu et al. 2003) and different cell lines may respond differently to various forms of replication stress.

### Origin plasticity past, present, and future

The implications of Taylor's observations extend beyond cell cycle regulation of DNA replication. Early DNA fiber studies of replication in *Xenopus* and *Drosophila* embryos detected numerous closely spaced replication sites (Blumenthal et al. 1974; Wolstenholme 1973), and it was generally assumed that these additional sites were somehow inactivated during development. As Taylor pointed out, his study suggests that these additional origins are not inactivated during development but rather remain as potential origins to be recruited under certain cellular conditions. Today, it is clear that cells choose a subset of potential origins, possibly during G1-phase of each cell cycle (Sasaki et al. 2006), and that these choices vary during development (Gilbert 2005; Norio 2006; Norio et al. 2005). Whether replication origin plasticity is important to differentiation or to the maintenance of genome integrity under conditions of replicational stress are questions of great interest for the next generation of replication researchers. But as we progress with our understanding of origin plasticity, we must pause now and again to recall contributions such as those made by Herb Taylor's experiment, published in *Chromosoma* 30 years ago—contributions so far ahead of their time that they risk going unnoticed when their significance finally comes to light.

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