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Establishment of a Spatial and Temporal Program for Mammalian Chromosome Replication

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Key Words: DNA replication; cell cycle; replication origin; replication timing

ABSTRACT

It has been 55 years since the elucidation of the structure of DNA, suggesting an elegantly simple means for its self-replication. Who would have dreamed in 1953 that it would take longer for us to understand DNA replication than it would for us to uncover the basic rules of animal development? Without question, the mechanisms regulating where and when DNA replication initiates in the cells of our own body is the greatest remaining fundamental mystery in molecular biology. Cis-acting sequences that function as replication origins in mammalian cells have not been identified and the mechanisms that regulate where and when origins will fire during S-phase remain elusive. Indeed, the problem has been so difficult that most researchers move on to more lucrative fields. In this essay, I will summarize my laboratory's humble attempts to make some progress in this area. In doing so, I hope that I can inspire a few young scientists to breath fresh energy into this challenging field.

The challenge

Studies of DNA replication in mammalian cells have been plagued by two overall problems: 1) a lack of *in vitro* assays with which to dissect mechanism (in any eukaryote) and 2) the lack of specific DNA sequences serving as assembly sites for the essential players (in nearly all eukaryotes).

Success of any biochemical or molecular approach is directly proportional to the robustness (speed and ease of interpretation) of the devised assay. Much of what we know about bacterial and viral DNA replication was derived from clever biochemical assays. A major challenge with eukaryotes is that chromosomal DNA replication is regulated by two separate and mutually exclusive steps: first, a pre-replication complex (pre-RC) must be assembled on DNA under conditions that prevent initiation of replication and, second, replication must initiate under conditions that prevent pre-RC formation (2, 3, 10). This two-cycle engine is critical to prevent DNA re-duplication. Hence, it is not possible to make a simple soluble cellular extract that is conducive for both steps (6). In the near future, we anticipate the development of transfer systems in which pre-RC assembly is followed by a transfer of the substrate to conditions that elicit initiation.

It is even more difficult to envision how the precise events taking place at the initiation site can be elucidated without DNA sequence specificity to identify specific protein:DNA contacts and the early events leading to nascent strand synthesis. Imagine studies of transcription without specific start sites and when virtually every piece of DNA can function as a promoter. Scores of graduate student and post-doc years have been squandered trying to devise assays that can convincingly identify specific DNA sequences required for initiation of replication (9). Frustratingly, in some cases replication initiates at highly specific sequences and those sequences are required for initiation in their native chromosomal context (1, 22). In other cases replication initiates rather haphazardly throughout large regions where no sequences seem to be required

(19). Moreover, studying mammalian origins in their native context is an extremely laborious task; most initiation sites are used in only a fraction of cell cycles so the abundance of nascent strands is extremely low.

Another challenge with eukaryotic origins is their diverse properties. In addition to the wide variability in their efficiency of usage, many origins are active only in certain tissues, adding a layer of developmental regulation (12, 20). The most diverse property of replication origins however is that each is activated at a different and characteristic time during S-phase. Indeed, we have no working knowledge of what regulates replication timing, even in budding yeast where sequence-specificity has greatly facilitated our understanding of the players involved in the initiation process. It is clear in most cases that the determinants of timing are not encoded within the origin sequences, but that origins fire at times dictated by their chromosomal location (13, 25).

Our Approach

Our approach is premised with the following logic: since the initiation of replication is context-dependent, retaining the context is important in studies of regulation. We have exploited a cell-free replication system in which nuclei from mammalian cells (Chinese Hamster Ovary or mouse C127 fibroblasts) staged at various times during G1-phase are introduced into extracts from *Xenopus* eggs. *Xenopus* egg extracts provide a rich source of S-phase promoting factors that rapidly and efficiently initiate replication within nuclei from cells synchronized at any time during G1-phase. The two-step problem

discussed above is overcome because pre-RC assembly takes place in the mammalian cells, and extracts are prepared in such a way that they can only carry out the initiation step of replication. Hence, sites of initiation in the extract are entirely dependent upon the locations of pre-RCs assembled in the mammalian cell. Extracts can be immunodepleted or supplemented with inhibitors of specific components of the replication machinery to determine when mammalian nuclei have completed steps that require these components. We can also manipulate the mammalian cells in culture prior to nuclear isolation (e.g. by expressing proteins or treating cells with inhibitors) and we can manipulate the nuclei during isolation (e.g. inactivate, remove or change the activities of chromatin proteins) to characterize the properties of complexes assembled at each stage of G1-phase.

Using this approach we've shown that the establishment of a spatial and temporal program for replication takes place in several discrete steps during early G1-phase (Figure 1). First is the assembly of pre-replication complexes (pre-RCs), which takes place via the recruitment of the hetero-hexameric Mcm helicase complex to chromatin by ORC, Cdc6 and Cdt1. We have shown that Mcm proteins associate with chromatin during telophase (8, 21), within minutes after the destruction of cyclins and geminin by the anaphase promoting complex (APC); cyclin-dependent kinases (Cdk) phosphorylate many pre-RC proteins while geminin sequesters Cdt1 (Figure 2). Association of replication proteins with chromatin is frequently used as the only means to assess their activity, but association does not imply a functional interaction. The *Xenopus* egg extract system allowed us to show functionality of chromatin-associated pre-RCs

because chromatin from metaphase and anaphase cells required *Xenopus* pre-RC proteins for replication while chromatin from telophase cells and later did not (8, 21). To date, this transition pre-RC dependent to pre-RC independent replication is the only demonstration of the functionality of pre-RCs assembled during telophase and early G1 and provides an excellent illustration of the power of this system.

1-2 hours after the assembly of pre-RCs (Timing Decision Point; TDP) and formation of a nuclear envelope, a replication timing program is established that determines the order in which large segments of chromosomes will be coordinately replicated via the synchronous firing of clusters of replication origins (Figure 3). When nuclei isolated from cells 1-2 hours after mitosis (pre-TDP) are introduced into *Xenopus* egg extracts, replication initiates at sites scattered throughout the entire genome at all times during the course of the *in vitro* replication reaction. With nuclei isolated thereafter (post-TDP), replication initiates at the same number of total sites, but the sites are spatially clustered into “domains” and these clusters fire in a specific temporal sequence (7, 16, 17).

The establishment of a replication-timing program restricts initiation to certain chromosome domains at particular times during S-phase but is not sufficient to dictate the precise sites where replication initiates (i.e. the origins of replication). With nuclei isolated within 2-3 hours after the TDP, replication initiates in the proper temporal order but with no apparent site specificity. Thereafter, nuclei experience another transition that specifies origin sites (Origin Decision Point; ODP). The *in vitro* replication program within nuclei isolated post-ODP appears to be indistinguishable from that of cells in

culture (4, 26). These results clearly demonstrate that the mechanisms specifying replication origins are independent of those establishing the time during S-phase at which they will fire (Figure 4).

All of these events take place prior to the Restriction point (R-point), which is the point at which cells become committed to initiate DNA replication and will enter S-phase even when serum mitogens are removed from the medium (15, 27). During G1-phase, intra- and extra-cellular signals dictate whether cells will pass through the R-point or whether they will arrest and enter a state of quiescence termed Go. If the cell enters Go, pre-RCs are disassembled. If conditions are favorable for cell proliferation a set of protein kinases are activated that drive cells through the R-point and initiate replication. The R-point is 2-3 hours after the ODP. Importantly, cells assemble pre-RCs and pass through both the TDP and the ODP on schedule even in the absence of mitogen stimulation.

What is the TDP?

The ability to narrow down the time interval for the TDP and ODP to within 5-10% of the total cell cycle doubling time has allowed us to very precisely correlate these events to other cell cycle regulated events, most of which occur either before (hence are not sufficient) or after (hence are not necessary). Intriguingly, replication domains transition from being highly mobile and randomly located throughout the cell nucleus to being much less mobile (anchored to immobile structures) and localized to defined positions within the nucleus precisely at the TDP (7, 16). Our working hypothesis is that the

anchorage of domains seeds the assembly of sub-nuclear microenvironments with locally high concentrations of chromatin proteins that in turn influence when replication will initiate by setting thresholds for accessibility of S-phase promoting factors to chromatin (Figure 5). Our model, while speculative, can be tested in several ways. Small peptides representing the interacting domains of chromatin proteins can be incubated with nuclei to disrupt their interactions in an attempt to lower the threshold for initiation at specific regions prior to *in vitro* replication. Alternatively, S-phase promoting factors can be targeted to late replicating regions to increase their local concentration, surpassing the hypothetical threshold. Surprisingly, disrupting the well-studied chromatin structure surrounding late replicating centromeres in mouse cells caused only a 15% advance in the replication timing of this chromosomal region (29). However, this treatment did not disrupt the 3D organization of these heterochromatic domains. Moreover, several of the components of the centromeric chromatin either persist throughout the cell cycle or are re-assembled during anaphase, and so cannot be sufficient to dictate timing at the TDP. It is possible that multiple chromatin components contribute incrementally to a timing program, and that the TDP represents the synergistic integration of these components into a sub-nuclear compartment.

There are, of course, alternative models for the TDP. For example, transcription is largely shut down during mitosis. Since early replicating regions are enriched for active genes, it would seem logical to propose that the resumption of transcription after mitosis could set the timing program. However, general transcription resumes prior to the TDP and inhibiting transcription during early G1-phase does not affect the establishment of

timing (unpublished). In addition, we find that mouse centromeric heterochromatin is transcribed, but transcription is silent throughout early G1-phase and is not induced until after the activation of Cdk activity at the restriction point (18). One could also envision the appearance of a post-translational histone modification or the binding of some chromatin protein at the TDP. However, to our knowledge, no early G1-phase molecular change in chromatin structure or composition has been reported. Finally, an event resembling the TDP has been reported in budding yeast (23), which also have a temporal specificity to the firing of their replication origins. One would hope that the power of the yeast system could provide some clues regarding the TDP, but the molecular nature of this event has also remained a mystery.

Whatever model one envisions, it must take into account the fact that replication timing is clearly regulated at the level of domains, not individual origins. The timing program is established prior to the establishment of specific origin sites. Moreover, certain manipulations of post-ODP cell nuclei can disrupt origin specification but not replication timing (unpublished). We find it particularly intriguing that monitoring replication on individual DNA fiber studies revealed that initiation sites are dispersed throughout the genome in pre-TDP nuclei, with most DNA fibers showing only one site of initiation, while the same total number of origins are clustered into groups of several origins per DNA fiber after the TDP (17). This supports models in which domains somehow become organized at the TDP in such a way that a common replication initiation complex elicits the simultaneous firing of clusters of replication origins, without regard for the precise positions of those origin sites. This kind of model (Fig. 6), would imply

that groups of pre-RCs may be gathered into larger “replication factories” at the TDP (14).

What is the ODP?

The ODP is downstream of pre-RC assembly and upstream of the R-point and all known events associated with initiation of replication, placing it in an uncharacterized period of G1-phase. The fact that *Xenopus* egg extracts, incapable of pre-RC assembly, initiate at apparently random sites in pre-ODP nuclei is compelling evidence that pre-RCs are assembed during telophase at many more sites than are chosen for initiation. Somehow then, the ODP must specify which of these many pre-RCs will function as an origin in the upcoming S-phase. It could do this by eliminating/inactivating many pre-RCs or by modifying a subset of these pre-RCs to make them preferred initiation sites (Figure 7A). Unbiased ChIP methods using antibodies to pre-RC proteins could provide direct evidence for their locations in pre- and post-ODP nuclei, but such methods have not yet succeeded in mammalian cells. None of the pre-RC proteins exhibit any changes in apparent molecular weight or association with chromatin at the ODP. Proteins known to be early players in the conversion of pre-RCs to initiation complexes such as Cdc45 and Mcm10 do not associate with chromatin until after the ODP (Figure 1).

We have identified inhibitors of specific cellular processes that prevent passage of cells through the ODP. Interestingly, every inhibitor of transcription inhibits the ODP (Table I and (5, 15) and, since the ODP focuses initiation to an intergenic region (24), it is

reasonable to postulate that transcription eliminates pre-RCs from transcribed regions (Figure 7B). However, transcription is not sufficient for origin specification. Transcription begins prior to the ODP (24) and treatment of pre-ODP cells with proteolysis inhibitors inhibits the ODP without affecting transcription (Table I and (15).

Conclusions and Future Directions

At present, the TDP and ODP are biological phenomena in search of a molecular mechanism. The most obvious candidates for molecular players in replication related processes have been ruled out but there are still some fundamental questions to address. We need to know where all the pre-RCs are and their dynamics during the cell cycle. If their positions are not changing, Is there some fundamental difference between their structure or chromatin environment before and after each of these events, at early vs. late origins, sites chosen for initiation vs. silent sites? It is clear that replication timing is regulated independently of any specific origin site-specificity, and at the level of large chromosome domains. Hence studies of replication timing must address these higher levels of chromosome organization. As for the ODP, we need to know whether we are searching for a global molecular transition during G1 phase, or a series of local, locus-specific events. The only way to do that is to measure the ODP for additional origins. However, mapping even one additional origin requires a full time effort from a talented experimentalist. Finally, it is known that embryonic stem cells have short G1-phases. Since the TDP and ODP are G1-phase decision points, as is the Restriction point, it will

be interesting to study how cells manage to establish a replication program within such a short period of time.

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FIGURE LEGENDS

Figure 1. Establishing a Program for Replication. The establishment of a spatial and temporal program for replication takes place in several discrete steps during early G1-phase. First is the assembly of pre-replication complexes (pre-RCs) consisting of ORC, Cdc6 and Mcm proteins. Pre-RC assembly takes place during telophase but is not sufficient to dictate either the sites where replication will initiate (8, 21), or the temporal order in which replication origins will fire (17). Shortly after the assembly of pre-RCs, and coincident with re-positioning of chromosomes in the newly formed nucleus (7, 16), the replication-timing program becomes established (Timing Decision Point; TDP). Establishment of the order in which chromosome domains will replicate is not sufficient to dictate the sites of initiation within each domain. Origin sites are selected at a distinct point after the TDP (Origin Decision Point; ODP), likely through a mechanism that identifies a subset of pre-RCs as the preferred origin sites(26). Inhibitor studies suggest that the ODP requires transcription, proteolysis, and the activities of one or more protein kinases(15). All of these events are independent of serum mitogens and are upstream of the Restriction point and phosphorylation of the Retinoblastoma (pRB) tumor suppressor protein (27, 28), which leads rapidly to the execution of the replication program established during G1-phase.

Figure 2. Pre-RC assembly takes place during telophase.

Figure 3. Replication proceeds via the synchronous firing of clustered origins.

Figure 4. Two distinct G1-phase steps restrict the number of potential replication origins to establish a spatial and temporal program for replication. In early G1 phase, many sites distributed throughout the genome have an equal potential to be used as early replication origins. At the TDP, late replicating chromosomal domains become excluded from the pool of potential early replicating origins. At this time, origins within these early replicating domains still have an equal potential for initiation regardless of their position within the domain. At the ODP, a subset of these potential origins are chosen for initiation in the upcoming S phase.

Figure 5. Sub-Nuclear Domains Establish Thresholds for Initiation The Timing Decision Point (TDP), coincident with the re-positioning of sequences within the nucleus (7, 16). In this model, proteins critical for the establishment of higher order chromatin structure are dispersed during mitosis. As domains become anchored during re-positioning, specific chromatin-binding proteins are recruited and accumulate at these sites, creating micro-environments that establish thresholds for the initiation of replication. For simplicity, only a single chromosome territory is shown. Hypothetical heterochromatin proteins are in red and euchromatin proteins in blue. (Modified from (11)

Figure 6. Origins fire in clusters after the TDP. The clustering of active replication origins after the TDP could be due to the formation of multi-replicon complexes.

Figure 7. (A) Working models for the ODP. Our results strongly suggest that the formation of a pre-RC is not sufficient to specify a replication origin. Our current working model is that pre-RCs form during telophase at many sites that initially share an equal potential of being selected as replication origins. Subsequent events taking place at the ODP potentiate some sites and/or inactivate others. These events could include the interaction of specific proteins with pre-RCs directly or epigenetic events that indirectly influence pre-RC potential. **(B) Potential role of transcription in specifying origin sites.** During telophase, pre-RCs assemble at many sites with little or no sequence specificity. At the ODP, the resumption of transcription, which is largely off during mitosis, de-stabilizes pre-RCs that were assembled within transcription units.

Table I. Summary of Inhibitor Experiments.

Experiments similar to those described in Fig. 8 were performed with each inhibitor. Inhibitors were added to cells in the pre-ODP stages of G1-phase, and cells were collected at 5 hours post-mitosis for analysis of origin specificity. For each inhibitor, the relative preference for initiation in the intergenic region vs. the DHFR gene was determined and compared to control untreated cultures. (-) indicates less than 45% and (+) indicates greater than 80% reduction in specificity relative to the control. Transcription inhibition was determined by pulse labeling cells with BrU (bromouridine) at the end of the inhibitor treatment period, and staining cells with anti-BrU antibodies. For inhibition of transcription, (-) indicates that the pattern of BrU incorporation was similar to untreated cells, (+) indicates that little or no BrU staining was detected. Similar results were obtained in at least three independent experiments. N.D.= not determined.

Figure 1

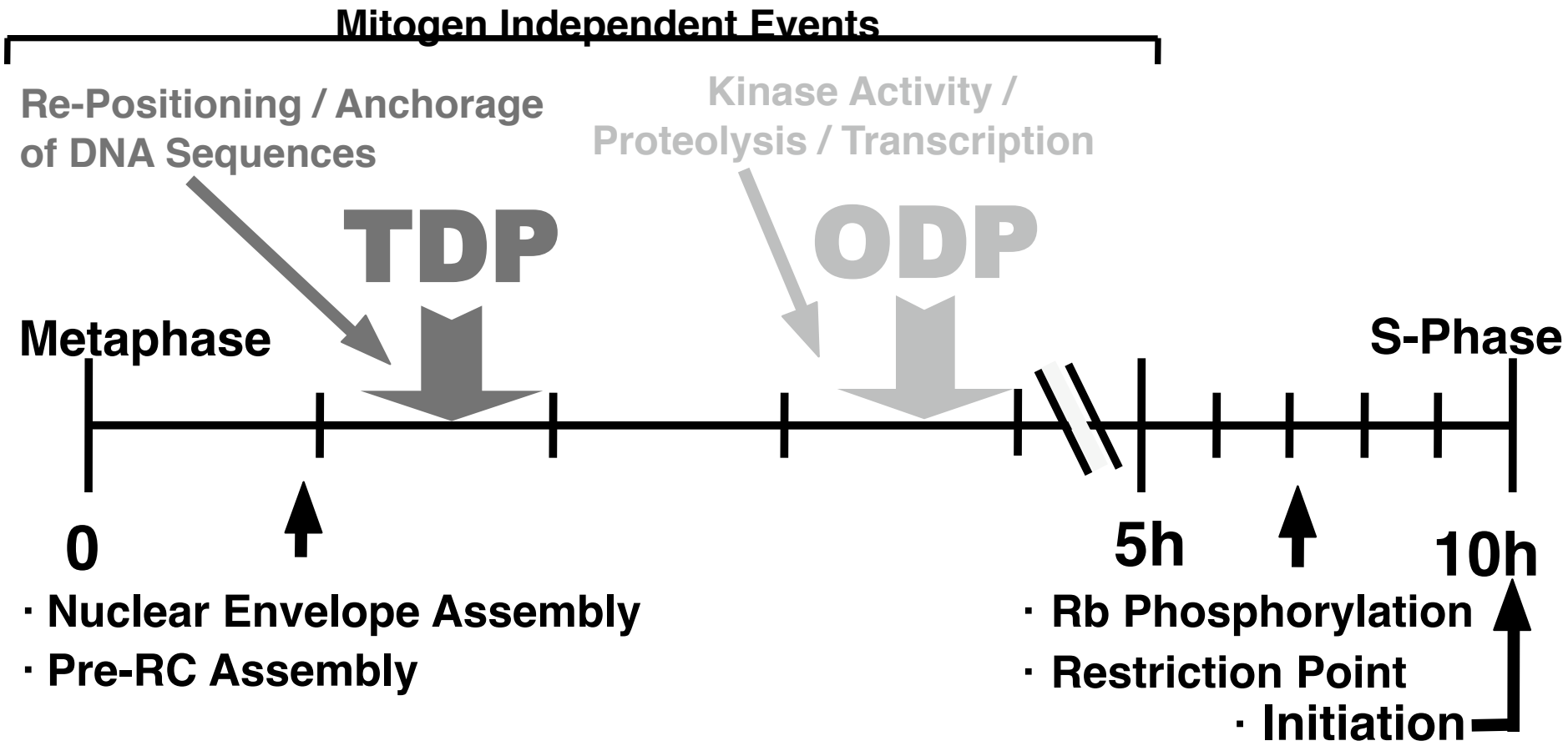
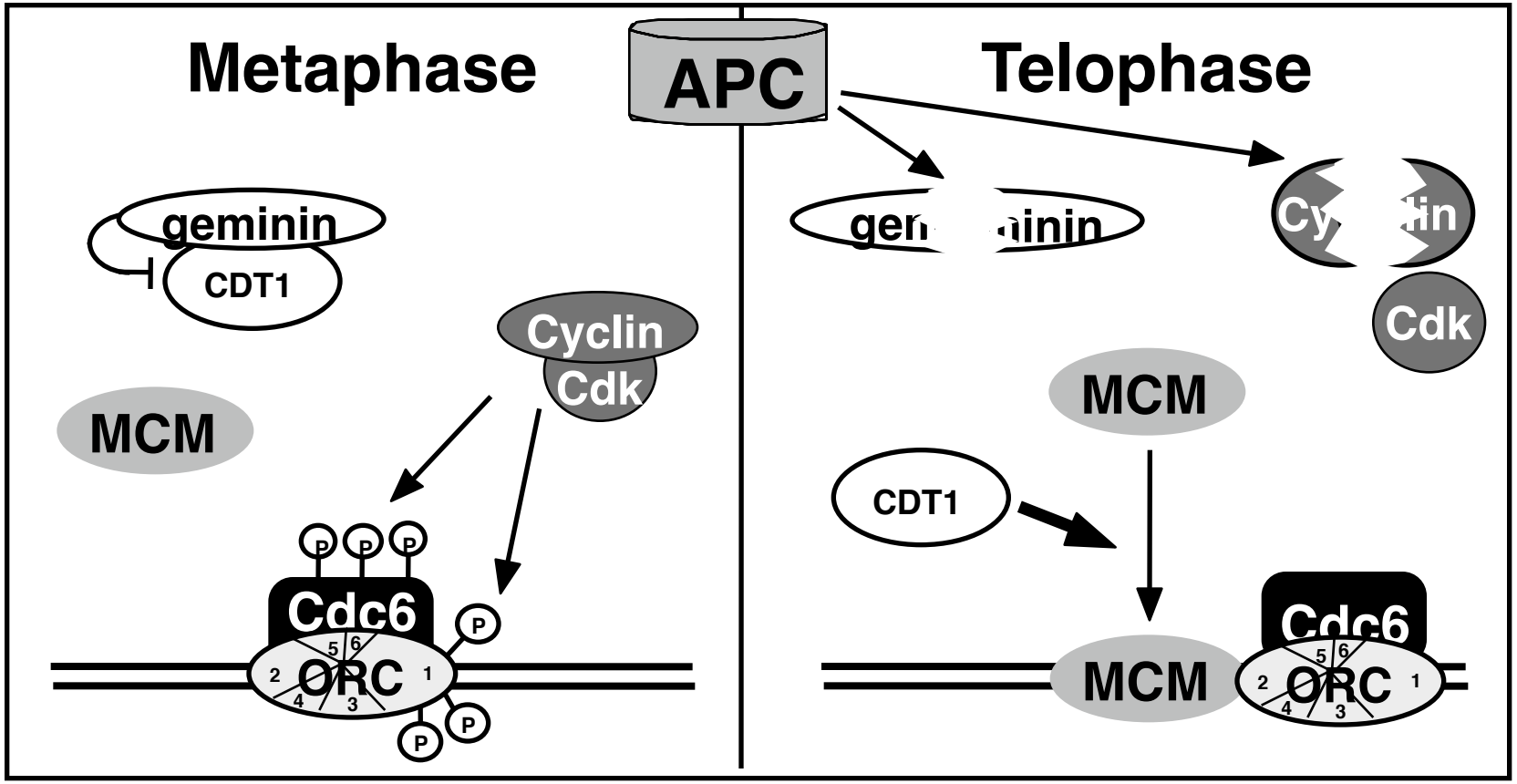

Mitogen Independent Events
Re-Positioning / Anchorage of DNA Sequences
Kinase Activity / Proteolysis / Transcription
TDP
ODP
Metaphase
S-Phase
0
5h
10h
· Nuclear Envelope Assembly
· Pre-RC Assembly
· Rb Phosphorylation
· Restriction Point
· Initiation

Figure 2


Metaphase
APC
Telophase
geminin
CDT1
Cyclin
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MCM
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ORC
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Cdc6
MCM
ORC

Figure 3

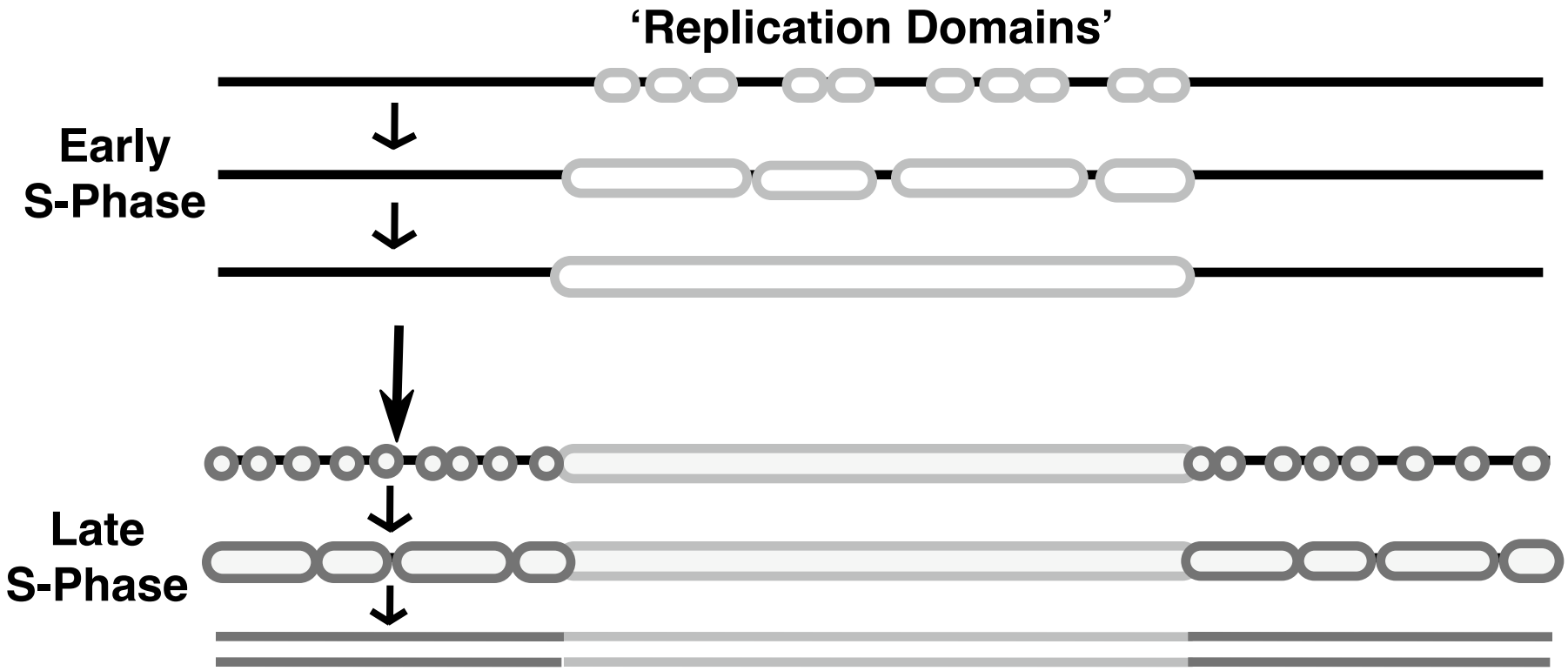

Figure 4

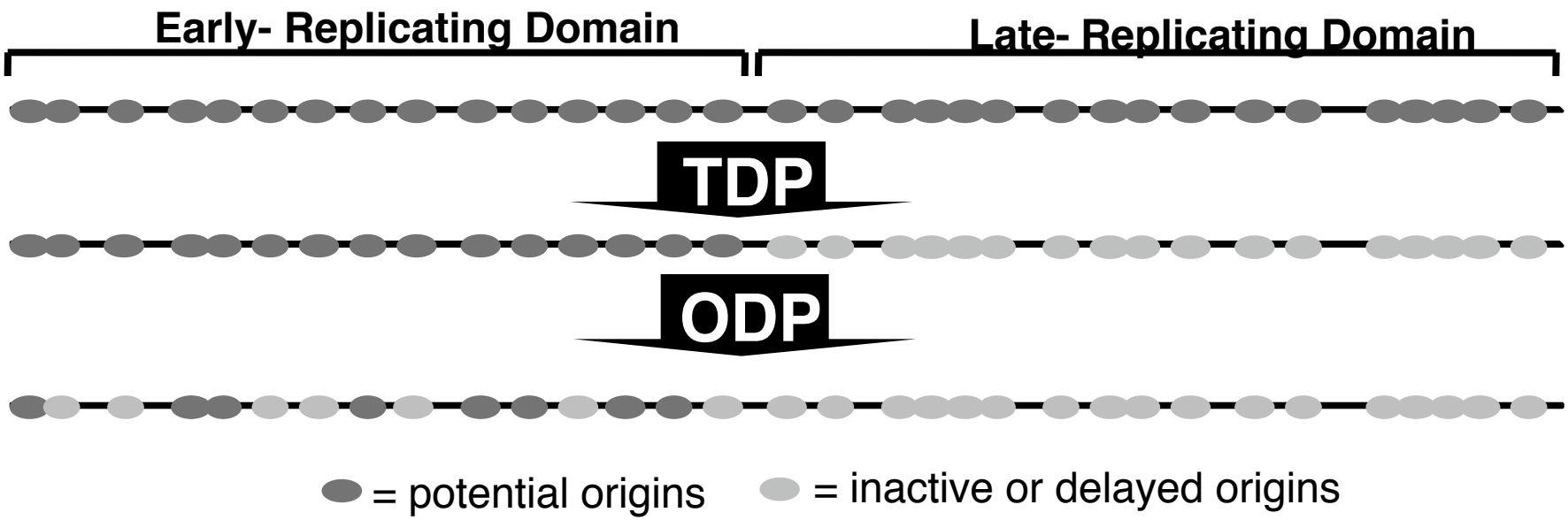
Early- Replicating Domain
Late- Replicating Domain
TDP
ODP
= potential origins
= inactive or delayed origins

Figure 5

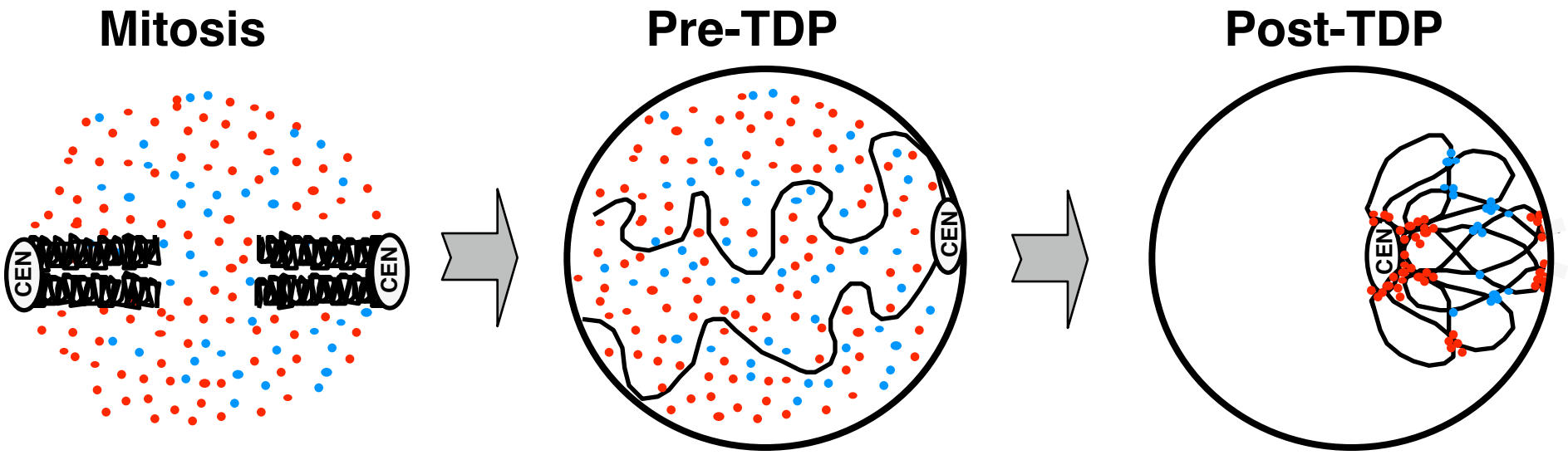

Figure 6

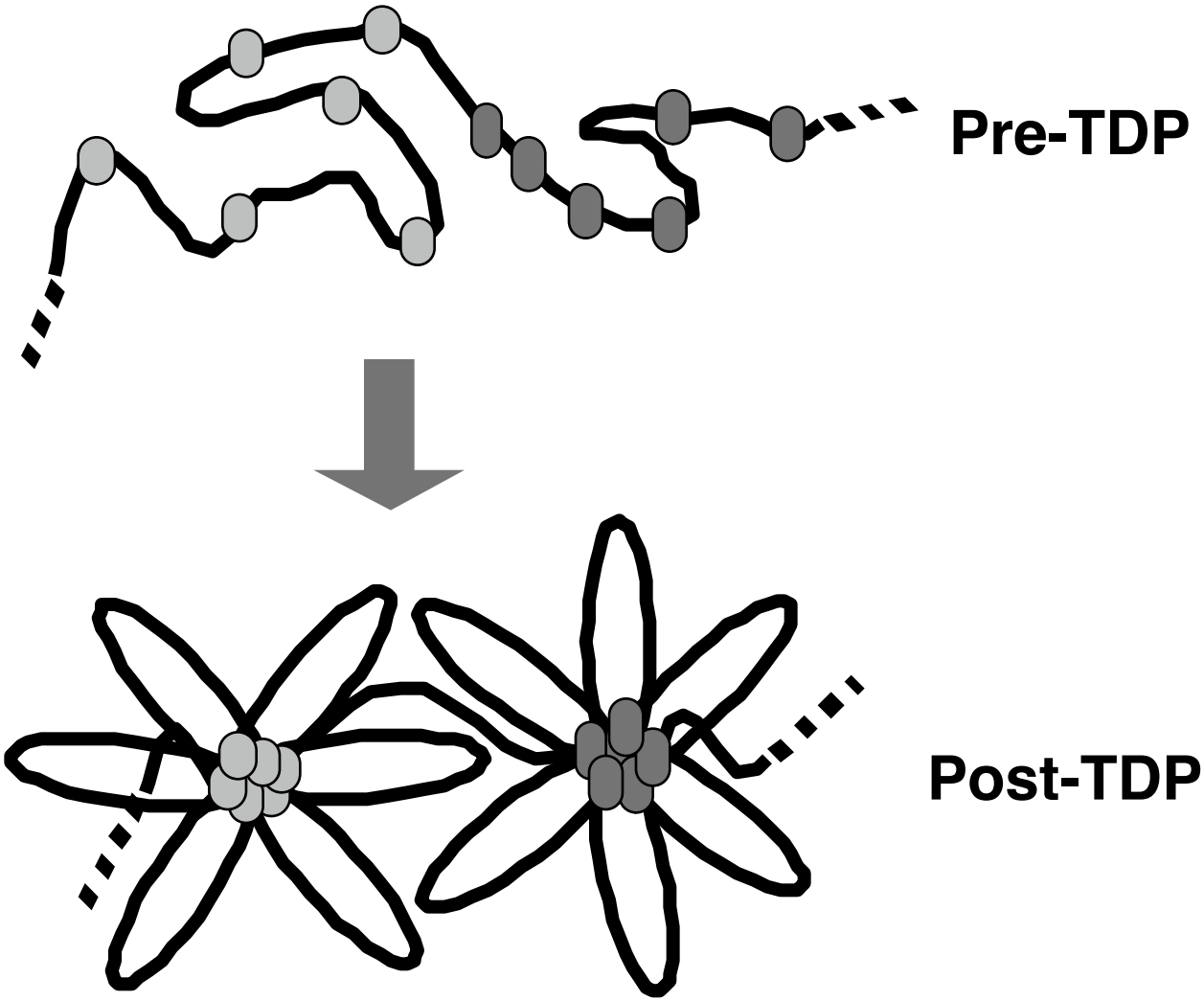

Figure 7A

A.

Pre-ODP:

- Pre-RCs form at many degenerate ORC recognition sites
- All sites are equi-potent at this time

Direct Molecular Activation of the Site(s) to be Utilized

Epigenetic Event Potentiates Some Sites and Represses Others

Post-ODP:

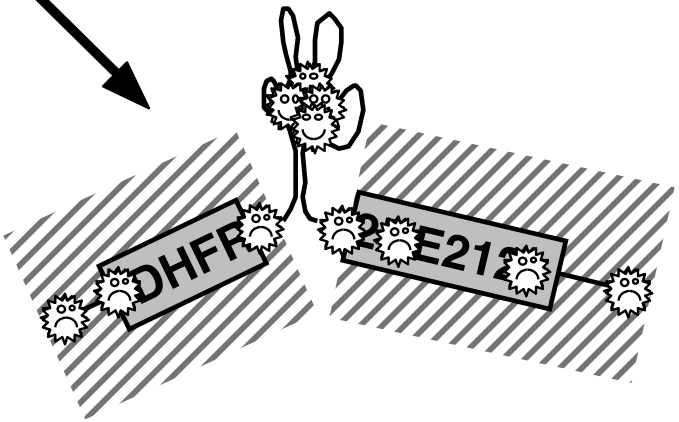

Figure 7B

B.

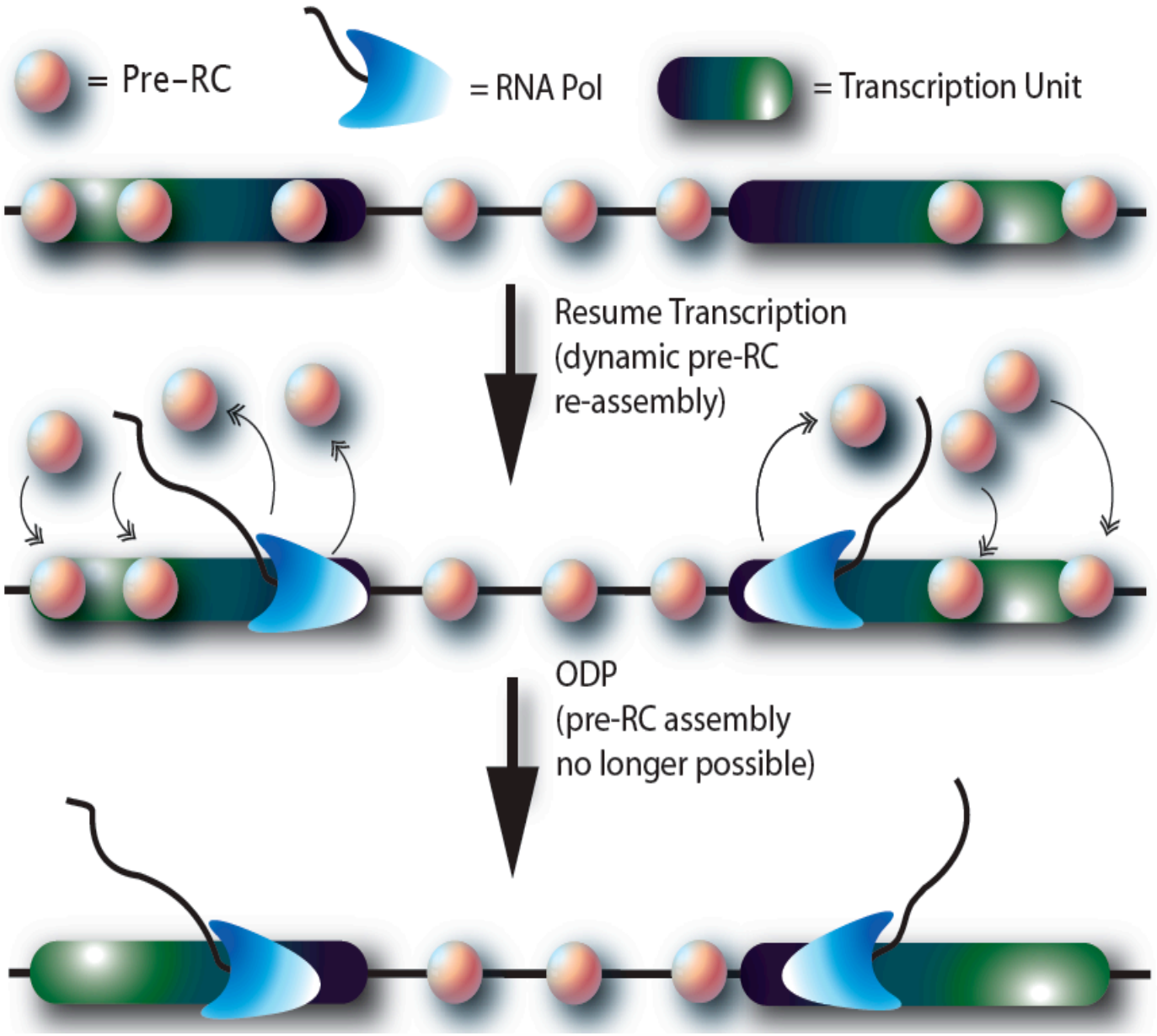
= Pre-RC
= RNA Pol
= Transcription Unit
Resume Transcription
(dynamic pre-RC
re-assembly)
ODP
(pre-RC assembly
no longer possible)

Table I

Inhibitor	Known Target(s)	Inhibits ODP	Inhibits Transcription
Roscovitine	cdk1,2,5,7	+	+
Olomoucine	cdk1,2,5,7	+	+
PD98059	MAP kinase pathway	-	-
KT5823	PKG	-	-
KT5720	PKA	-	+
Apigenin	CKII, MAP kinase pathway, cdk2	+	+
Suspension culture	Integrin signaling	-	N.D.
Lactacystin	Proteasome	+	-
ALLN	Proteasome	+	-
MG132	Proteasome	+	-
DRB	Transcription	+	+
α -amanitin	Transcription	+	+
Actinomycin D	Transcription	+	+
Cycloheximide	Translation	-	-
ICRF-187	Topoisomerase II	-	-
VM26	Topoisomerase II	+	+
VP16	Topoisomerase II	-	-
TSA	De-acetylase	-	N.D.
Distamycin	AT-minor groove	-	N.D.
Chromomycin	GC-minor groove	+	N.D.