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Sensitivity of the Origin Decision Point to Specific Inhibitors of Cellular Signaling and Metabolism

Susan M. Keezer and David M. Gilbert[1]

Department of Biochemistry and Molecular Biology, S.U.N.Y. Upstate Medical University, 750 East Adams Street, Syracuse, New York 13210

Chinese hamster ovary (CHO) cells become committed to initiate DNA replication at specific sites within the dihydrofolate reductase (DHFR) locus at a discrete point during G1 phase, the origin decision point (ODP). To better understand the requirements for passage through the ODP, we evaluated the ability of various inhibitors of G1-phase progression to prevent passage through the ODP. Of several protein kinase inhibitors tested, only inhibitors of cyclin-dependent kinase (cdk) activity (roscovitine, olomoucine) prevented passage through the ODP. Inhibitors of MAP kinase (PD98059), PKA (KT5720), PKG (KT5823), as well as inhibition of integrin-mediated signaling by preventing cell adhesion, all arrested cells in the post-ODP stages of G1 phase. Intriguingly, inhibitors of proteasome-dependent proteolysis (MG132, ALLN, lactacystin) and transcription (DRB, α-amanitin, actinomycin D) also inhibited passage through the ODP, whereas inhibition of protein synthesis (cycloheximide) had no effect on the ODP. Cross-checking each inhibitor for its affect on transcription revealed that the ODP could be uncoupled from transcription; MG132 and lactacystin did not inhibit transcription, and KT5720 was a potent inhibitor of transcription. Importantly, cells that were arrested upstream of the ODP with either roscovitine or lactacystin contained functional prereplication complexes (pre-RCs), supporting previous findings that pre-RC formation is not sufficient for origin specification. These results demonstrate that specification of the DHFR origin is independent of growth signaling mechanisms and does not require G1-phase synthesis of a protein regulator such as a cyclin or Dbf4/ASK1, positioning the ODP after pre-RC formation but prior to the activation of the known S-phase promoting kinases.

Key Words: **origin decision point (ODP); cell cycle; protein kinase; DNA replication; transcription.**

INTRODUCTION

Central to each eukaryotic cell cycle is the ability of the cell to replicate its genome once before each division. In the yeast *Saccharomyces cerevisiae,* DNA sequences originally defined as autonomously replicating sequences (ARS elements) have been characterized as the sites where replication initiates. These sites are also the binding sites for the origin recognition complex (ORC), a heterohexameric complex essential for replication in yeast, *Xenopus,* and *Drosophila* [1, 2]. ORC bound to DNA serves as a landing pad for the assembly of prereplication complexes (pre-RCs) during telophase, culminating in the loading of the Mcm2-7 helicase complex (reviewed in [2]. Although metazoan organisms possess homologues to the yeast ORC and pre-RC proteins, it has been difficult to pinpoint a sequence requirement for the initiation of replication [3]. Metazoan origins have been mapped by several laboratories, indicating that replication does not initiate at random sites in the differentiated cells of higher eukaryotes. However, in some cases initiation can be mapped to highly specific sites [4–7], while other loci appear to initiate replication at many sites distributed throughout several tens of kilobases [8–10]. Hence, the means by which specific sites are selected for initiation of replication in higher eukaryotes remains to be determined.

The initiation of replication at the dihydrofolate reductase (DHFR) locus in Chinese hamster ovary (CHO) cells has been extensively studied. Initiation can take place at any of several sites downstream of the DHFR gene [11, 12]. Replication initiates specifically at these same sites when nuclei from CHO cells synchronized in G1-phase are introduced into cell-free extracts from *Xenopus* eggs [13], providing a system with which to examine the requirements for origin specification in mammalian cells. Origin specification requires nuclei from cells synchronized after a discrete point during G1 phase of the cell cycle [13], termed the origin decision point (ODP). With nuclei isolated prior to this point, replication initiates at sites distributed throughout the DHFR locus. The ODP takes place after pre-RC

[1] To whom reprint requests should be addressed. Fax: (315) 464-8750. E-mail: gilbertd@mail.upstate.edu.

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formation [14, 15] but prior to phosphorylation of the retinoblastoma (Rb) tumor suppressor and is independent of serum mitogens [16]. However, cells treated early in G1 phase with the general protein kinase inhibitor 2-aminopurine (2-AP) become arrested in the pre-ODP stages of G1 phase [16], suggesting the requirement for a mitogen-independent protein kinase. Importantly, transformation with SV40 abrogates this arrest point, resulting in the entry of cultured cells into S phase in the presence of 2-AP and initiation at sites dispersed throughout the DHFR locus [17]. These latter experiments, performed without the use of *Xenopus* egg extracts, demonstrate that the ODP is a bona fide regulatory event important for normal G1-phase progression and validate the utility of experiments in *Xenopus* egg extracts to identify inhibitors of origin specification.

Many protein kinases are instrumental in the progression of G1 phase, but most of these are mitogen-dependent. The cyclin-dependent kinase (cdks) family includes at least nine different protein kinases in metazoa, of which cdks 2, 4, and 6 are most instrumental in G1-phase progression [18–20]. Cdk activity is regulated, in part, by the synthesis of cyclins or the degradation of cdk inhibitors, both of which respond to mitogenic cues. Some G1-phase kinases, such as the mitogen-activated (MAP) and focal adhesion (FAK) kinases, directly respond to receptor-mediated external proliferation cues such as growth mitogens or integrins. One interesting candidate for a protein kinase involved in the specification of origins is Cdc7. Cdc7 is necessary for the initiation of DNA replication and its activity requires the expression of a cyclin-like partner, Dbf4/ASK1 [21–23]. Experiments in *Xenopus* egg extracts have identified a critical Cdc7-dependent step taking place after the assembly of pre-RCs but prior to cdk2 activity [24, 25]. However, the synthesis of Dbf4/ASK1 and the activity of Cdc7 appear to be mitogen-dependent in mammalian cells [26, 27]. A mitogen-independent kinase specifically required for an early G1-phase event, such as the ODP, would be novel indeed. Here, we have further investigated the requirement for various signaling and metabolic mechanisms for passage through the ODP. Our results demonstrate that the ODP is independent of cell growth and proliferative signaling mechanisms and does not require the synthesis of a regulatory molecule, such as cyclins or Dbf4/ASK1.

MATERIALS AND METHODS

Cell culture and synchronization. CHOC400 cells, a derivative of CHO cells containing approximately 1000 tandemly integrated copies of a 243-kb segment of the DNA surrounding the DHFR gene [28], were maintained as described [16]. Cells were synchronized in metaphase by incubation with 0.05 μg/ml nocodozole for 4 h followed by isolation of metaphase cells by mitotic shake-off as described [29]. In some cases, metaphase cells were frozen in complete medium plus 10% DMSO at a concentration of 5×10^6/ml until further use. Metaphase-synchronized CHOC400 cells were plated to complete medium (2×10^6 cells/5 ml). For experiments where mitotic cells were allowed to enter G1 phase in suspension, cells were transferred at the same concentration in medium supplemented with 50 mM Hepes, pH 7.4, and cells were incubated at 37°C in a shaking water bath.

Inhibitors. Roscovitine, olomoucine, and staurosporine (Calbiochem) were suspended in DMSO at a 20 mM concentration. PD98059 and apigenin (Alexis Biochemicals) were suspended in DMSO at a 20 mM concentration. Lactacystin, MG132, and ALLN (Biomol Research Labs) were dissolved at 2 mM in water, 50 mM in DMSO, and 25 mg/ml in DMSO, respectively. 2-Aminopurine and cycloheximide (Sigma) were dissolved directly in medium at a 10 mM concentration and 10 mg/ml, respectively. Actinomycin D and α-amanitin (Sigma) were dissolved in 100% EtOH at 5 mg/ml. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sigma) was dissolved in 100% EtOH at a 10 mM concentration. KT5720 and KT5823 (gifts from Kyowa Hakko Kogyo Co. Ltd., Shizuoka, Japan) were dissolved in DMSO, each at a 20 mM concentration.

5′-Bromo-2′-deoxyuridine (BrdU) and 5′-bromouridine (BrU) labeling and staining of cells. Mitotic CHOC400 cells were plated on coverslips in 24-well dishes, 10^5 cells/well, in complete medium. One or two hours after plating (as indicated), medium was replaced with medium containing either drug or vehicle control. For determination of whether inhibitors prevent entry into S phase, 30 μg/ml BrdU (Sigma) was added to each well at 18–20 h after plating, cells were incubated for 30 min at 37°C, and the percentage of BrdU-labeled cells was determined as described [29]. For evaluating the effect of inhibitors on transcription, 5 mM BrU (Sigma) was added 3 h after plating for 90 min. Cells were then washed twice with PBS and fixed at 4°C with 70% EtOH overnight. Cells were then fixed again in 100% MeOH for 10 min at room temperature and washed twice with PBS and a third time with PBS + 0.5% Tween 20. Coverslips were incubated for 2 h at room temperature with mouse monoclonal anti-BrdU (Becton–Dickinson) diluted 1:1 in 0.5% acetylated BSA (sigma)/PBS + 0.05% Tween 20, washed twice with PBS + 0.5% Tween 20, and treated with anti-mouse IgG-FITC (Molecular Probes) diluted 1:50 in 0.5% acetylated BSA/PBS + 0.05% Tween 20 for 1 h at room temperature. Next, coverslips were washed three more times with PBS + 0.5% Tween 20. Nascent RNA was visualized and images were captured using a SPOT CCD camera mounted on a Nikon Labophot-2 microscope and processed using Adobe Photoshop 5.0. For RNA labeling, all solutions were made in diethylpyrocarbonate (DEPC; Sigma)-treated, autoclaved, deionized water. Antibody diluent and wash buffers were supplemented with RNAguard ribonuclease inhibitor (2.9 μ/ml, Amersham Pharmacia Biotech).

Analysis of DNA replication in Xenopus egg extracts. *Xenopus* egg extract preparation, preparation of intact nuclei (introduced at 25,000 nuclei/μl extract), TCA precipitation, and the use of recombinant geminin (40 μM) have been described [15, 29]. The early labeled fragment hybridization (ELFH) assay was performed as described [29]. Briefly, intact nuclei were prepared by permeabilizing the plasma membrane with 80 μg/ml of digitonin for 2 min on ice. CHO nuclei (1.5×10^6) were introduced into aphidicolin-supplemented *Xenopus* egg extract at a concentration of 25,000 nuclei/μl and incubated at 21°C for 1 h. Nuclei were then washed and replication intermediates were labeled with [α-^{32}P]dATP and hybridized to 18 probes corresponding to DNA sequences along a 120-kb region of the CHOC400 DHFR locus as described [29].

Western blotting of extracted cell fractions. Chromatin fractionation with Triton X-100 was performed as described [15]. In brief, synchronized CHOC400 cells were collected by trypsinization and washed twice with ice-cold PBS. Cells (5×10^6) were resuspended in 300 μl of CSK buffer (10 mM Pipes (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml

of leupeptin, 1 μg/ml of pepstatin, 1 μg/ml of aprotinin, and 50 mM NaF, 0.1 mM Navanadate) containing 0.5% Triton X-100 and incubated for 3 min at 0°C. Soluble and insoluble fractions were separated in a microfuge (7500 rpm, 3 min) and the pellet was washed 3 min at 0°C with the same buffer. The insoluble fraction was briefly sonicated for before adding SDS–PAGE loading buffer. Samples were run on a 6.0% SDS–PAGE gel and Mcm7 was detected with a monoclonal anti-Mcm7 antibody (Santa Cruz).

RESULTS AND DISCUSSION

The ODP Is Sensitive to Cyclin-Dependent Kinase Inhibitors

Our previously published data suggest that passage through the ODP requires a mitogen-independent protein kinase activity that acts upstream of the restriction point [16, 17]. To narrow down the range of possible kinases that might be involved, several more specific protein kinase inhibitors were tested for their ability to inhibit the ODP. 2-AP, a protein kinase inhibitor with rather broad specificity that has previously been shown to inhibit passage through the ODP [16], was used as a negative control for origin specification. 2-AP inhibits specificity at the DHFR locus close to that seen with pre-ODP nuclei (Fig. 3) [16]. All the inhibitors chosen target important signaling mechanisms required for G1-phase progression. We first verified that each of these inhibitors was capable of crossing the CHO cell membrane and inhibiting the entry of cells into S phase. Cells were synchronized in metaphase by selective detachment after a brief and completely reversible nocodozole block and then released into G1 phase. At 1–2 h thereafter, cells were treated with each inhibitor and incubated an additional 18–20 h. Cells were then pulse labeled with BrdU, and the percentage of S-phase (BrdU-labeled) cells was determined (Fig. 1). All inhibitors prevented entry into S phase, demonstrating that they are effective inhibitors of G1-phase progression.

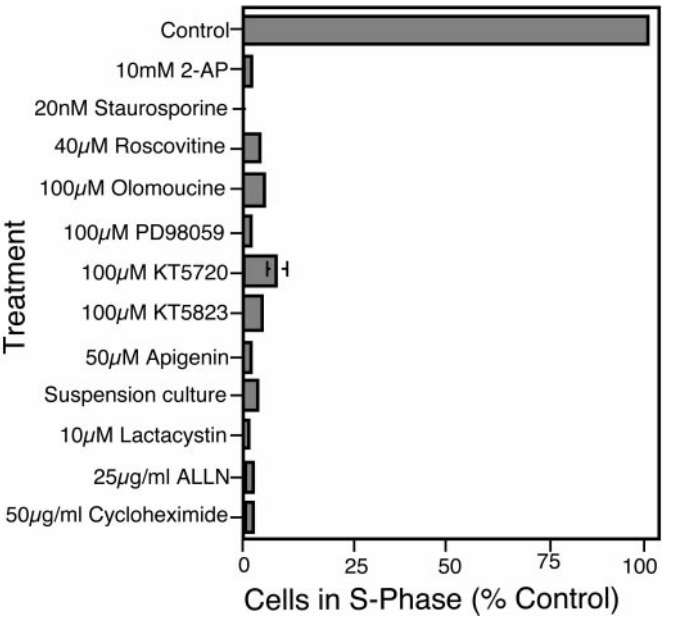

FIG. 1. Various inhibitors of G1 phase progression. CHOC400 cells were synchronized in metaphase and released into G1 phase. "Suspension culture" indicates that cells were prevented from attachment by transferring mitotic cells to flasks in a shaking water bath while all other samples were transferred to dishes and cultured as a monolayer. Inhibitors were added at the indicated concentrations at 1 h (lactacystin, ALLN, and cycloheximide) or 2 h (all others) after mitosis. At 18–20 h after mitosis (a time when 70–90% of untreated cells are in S phase), cells were pulse labeled for 30 min with BrdU and fixed and stained with anti-BrdU antibodies, and the percentage of cells in S phase (BrdU positive), relative to parallel untreated cultures, was calculated. Shown are results using either the lowest concentration of each inhibitor that inhibited the ODP (Fig. 3) or the highest concentration tested that did not inhibit the ODP. For PD98059, KT5720, and KT5823, a 20 μM concentration (the lowest concentration tested) was sufficient for >95% inhibition of entry into S phase (not shown). At least 200 cells were scored per sample. Values denote the mean of two to three experiments and error bars denote the standard deviation about the mean (±SEM), when greater than 2.

Each kinase inhibitor was then tested for its effect on DHFR origin specification. A complete data set using roscovitine as an example is illustrated in Figs. 2A and 2B. CHOC400 cells synchronized in mitosis were released into G1 phase. At 1–2 h after mitosis, various concentrations of each inhibitor were added to aliquots of cells and the cells were collected 4 h later, sufficient time for untreated cells to pass through the ODP but not yet enter S phase [13, 16]. Nuclei from treated and untreated cells were then introduced into *Xenopus* egg extracts and the specificity of initiation within the DHFR locus was evaluated using the ELFH assay [29]. In this assay, replication forks are arrested close to their sites of initiation by supplementing extracts with aphidicolin, which allows the initiation of replication and the synthesis of short (approximately 500 bp) nascent strands, but prevents the processive elongation of those forks. After washing away the aphidicolin, short nascent DNA strands are then labeled with [α-^{32}P]dATP and the ^{32}P-labeled DNA strands are isolated and hybridized to a panel of probes spanning 120 kb of the DHFR locus. With nuclei from cells that have passed through the ODP, the majority of radiolabeled DNA is focused to the intergenic region between the DHFR and 2BE2121 genes. In contrast, nuclei from cells that have been arrested prior to the ODP incorporate radiolabel into nascent strands that hybridize almost uniformly to probes distributed throughout the DHFR locus [16, 17]. As shown in Fig. 2A, roscovitine inhibited passage through the ODP in a concentration-dependent manner, with maximal inhibition at 40 μM or higher concentrations. This treatment had no effect on cell viability as measured by trypan blue exclusion (not shown). Each inhibitor that was found to prevent passage through the ODP was then evaluated for its

G1-phase execution point. An example of this analysis for roscovitine is shown in Fig. 2B. CHOC400 cells were synchronized in mitosis and released into G1 phase as before, and 40 μM roscovitine was added every hour from 1 to 5 h after mitosis. Nuclei were then prepared from these cells and the ELFH assay was performed, as in Fig. 2A. Results demonstrated that the addition of roscovitine at 2–3 h maximally inhibited specification of the DHFR origin.

Roscovitine and olomoucine are both competitive inhibitors of ATP binding that are specific for cdks 1, 2, and 5 (of 25 kinases tested) [30–33]. However, the IC_{50} (concentration to inhibit by 50%) for the inhibition of cdk activity *in vitro* by roscovitine is 10-fold lower than that for olomoucine, and this ratio is similar for the inhibition of all three cdks. To construct a dose–response curve of the ODP to roscovitine and olomoucine, a series of experiments similar to those shown in Fig. 2A was then performed with various concentrations of roscovitine and olomoucine. The *relative specificity of initiation* within the DHFR locus was defined as the average relative early DNA synthesis values for four probes encompassing peak origin activity (highlighted by the shaded vertical line in Figs. 2A and 2B). As shown in Figs. 2C and 2D, the relative IC_{50} for inhibition of passage through the ODP by olomoucine vs roscovitine is approximately 5-fold, similar to the ratio shown to inhibit entry into S phase in normal human fibroblasts [30]. Figures 2C and 2D show that the IC_{50} for inhibition of entry into S phase by these inhibitors, determined as in Fig. 1, is similar to that for ODP inhibition. The ratio of inhibition by these two inhibitors suggests the requirement for a Cdk activity prior to the restriction point. Of the three Cdks that have been demonstrated to be sensitive to these inhibitors, Cdk1 is not activated until late S phase, Cdk5 is neuron specific, and Cdk2 is not activated until the onset of S phase [18, 20] (data not shown). Hence, the early G1-phase target of these inhibitors may be an as yet unidentified target of roscovitine and olomoucine.

Figure 3 summarizes the results of similar experiments performed with all inhibitors described in this report. As discussed above, 2-AP was used as a negative control. Staurosporine is another broad-specificity protein kinase inhibitor. For comparison, results obtained in prior experiments using pre-ODP nuclei as a substrate are also shown. Values represent the relative specificity of initiation within the DHFR locus at the minimum concentration of each drug that showed maximal inhibition of origin specification. In the case of inhibitors that had no effect on the ODP, the relative specificity of initiation was calculated for the maximal concentration tested, often close to the solubility limitations of each inhibitor. We first complete our discussion of the protein kinase inhibitors.

Although it has been shown previously that passage through the ODP is mitogen-independent, it was important to determine whether the MAP kinase pathway is required for passage through the ODP. In particular, roscovitine and olomoucine have been shown to inhibit MAP kinase, albeit at higher concentrations than required for cdk inhibition and at a relative IC_{50} of 2-fold rather than 10-fold [31]. As shown in Fig. 3, PD98059, a specific inhibitor of the MAP kinase pathway [34, 35], had no effect on passage through the ODP. In addition, the staurosporine analogues KT5720 and KT5823, which have been shown to inhibit cyclic AMP and cyclic GMP-dependent protein kinases, also showed no effect on selection of the DHFR origin. Apigenin is a flavinoid that has been shown to inhibit cdk2 activity, possibly through an increase in the levels of the cdk inhibitor protein p21 [36]. At a 50 μM concentration, apigenin prevented cells from passing through the ODP. Although apigenin has also been shown to selectively inhibit the activity of casein kinase II (CKII) [37], neither roscovitine nor olomoucine affect the activity of CKII [31]. These results strengthen the case for the involvement of a cdk-like activity in a prerestriction point step required for passage through the ODP.

Passage through the ODP does not require cell adhesion. In adherent cells, integrin-mediated signal transduction pathways involved in cell cycle control, particularly the MAP kinase pathway, G1 cdks (cdk4/6:cyclin D, cdk2:cyclin E, and cdk2:cyclin A) and phosphorylation of Rb, are inhibited when cells are grown the absence of an extracellular matrix (ECM), preventing entry into S phase [38–40]. To test whether adherence is required for passage through the ODP, CHOC400 cells were released from mitosis and maintained in suspension culture for 6 h. Although aliquots of the same cells were completely prevented from entering S phase (Fig. 1), incubation in suspension culture had no effect on the ODP (Fig. 3). This is consistent with our results indicating that the ODP is independent of Rb phosphorylation and restriction point control [16] and does not require signal transduction through the MAP kinase pathway (above).

Passage through the ODP is inhibited by the proteasome inhibitors lactacystin, ALLN, and MG-132. Cdk activity is regulated by several different mechanisms, including binding to regulatory subunits (cyclins) or to natural inhibitors (CKIs). The presence or absence of cyclins and CKIs has been shown to be regulated in part by proteolysis, particularly through the ubiquitin–proteasome pathway [41–45]. To investigate whether passage through the ODP requires proteolysis, we treated CHOC400 cells with specific inhibitors of the proteasome and examined their effect on selection of the DHFR origin. Three different inhibitors of the pro-

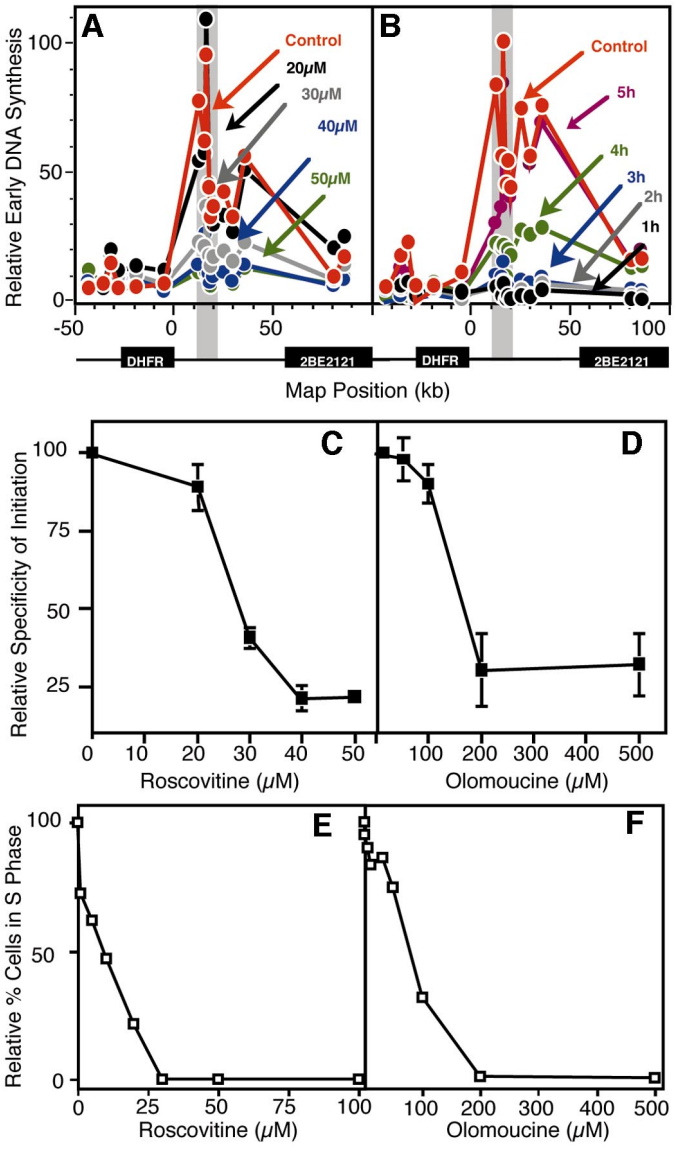

FIG. 2. Inhibition of the ODP by the Cdk inhibitors, roscovitine, and olomoucine. CHOC400 cells were synchronized in metaphase and released into G1 phase as in Fig. 1. In (A), roscovitine was added at the indicated concentrations at 2 h after metaphase and in (B) 40 μM roscovitine was added at the indicated times after metaphase. All cultures were collected at 6 h after metaphase. Nuclei were then prepared and introduced into *Xenopus* egg extracts supplemented with aphidicolin to arrest replication forks close to their sites of initiation. After a 60-min incubation period, nuclei were washed free of aphidicolin, nascent DNA strands were labeled with [^{32}P]dATP, and the distribution of nascent DNA was evaluated by hybridizing radiolabeled nascent DNA to a panel of 18 probes spanning the DHFR locus ("early labeled fragment hybridization" or ELFH assay). Plotted are the relative amounts of hybridization to each probe, denoted as the relative early DNA synthesis, which is expressed as a percentage of that for untreated cultures in each experiment. Shown are the means from three independent experiments. (C and D) Cells were treated with the indicated concentrations of either roscovitine or olomoucine at 2 h and collected at 6 h after metaphase. Nuclei were then introduced into *Xenopus* egg extracts and subjected to the ELFH assay as in (A) and (B). The *relative specificity of initiation* was then defined as the average relative early DNA synthesis values for 4 probes surrounding the peak initiation activity (denoted with a gray line in A and B) and plotted versus the concentration of either roscovitine (C) or olomoucine (D). Shown are the mean values from three independent experiments and the standard deviation about the mean (±SEM), when greater than 3.5. (E and F) Cells were treated with the indicated concentrations of roscovitine or olomoucine at 2 h after metaphase and the percentage of cells that entered S phase was compared to that of untreated control populations as in Fig. 1.

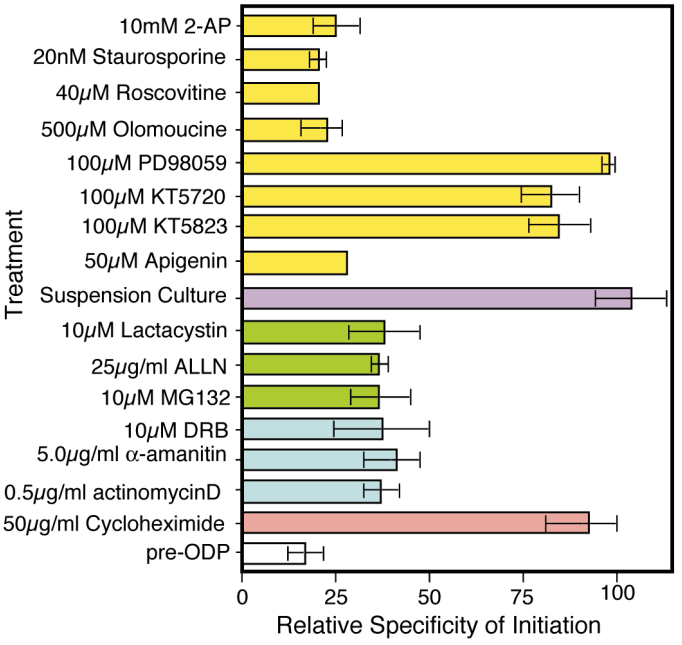

FIG. 3. Inhibition of the ODP by specific inhibitors of cellular signaling and metabolism. CHOC400 cells were synchronized in metaphase and released into G1 phase either in suspension (suspension culture) or as a monolayer, as in Fig. 1. Inhibitors were added at the indicated concentrations at 1 h (lactacystin, MG-132, ALLN, and cycloheximide) or 2 h (all others) after metaphase. At 6 h after metaphase, nuclei were prepared and introduced into *Xenopus* egg extracts, and the specificity of initiation of replication within the DHFR locus was evaluated by the ELFH assay, as in Fig. 2. The relative specificity of initiation for each sample was calculated as in Fig. 2 and expressed as a percentage of that for untreated cultures in each experiment. Shown are the mean values from at least three independent experiments for each inhibitor ±SEM, when greater than 1.0. The concentrations of each inhibitor shown are either the lowest concentration that showed maximal inhibition of the ODP or the maximum concentration tested. For cycloheximide, the concentration shown is 10 times the amount necessary to inhibit 95% of protein synthesis [15]. For PD98059, KT5720, and KT5823, the concentration shown is close to the solubility limit for these inhibitors. Also shown for comparison are the corresponding values for nuclei prepared from pre-ODP cells, derived from data presented in previous publications [29, 66, 67].

teasome [46] prevented cells from passing through the ODP (Fig. 3). Lactacystin, a covalent modifier of the proteasome's active site threonine residues, caused maximal inhibition at a 10 μM concentration. The IC_{50}

for inhibition of entry into S phase by lactacystin was similar to that for inhibition of the ODP, with maximal inhibition at 10 μM (data not shown). MG132 and ALLN, both transition state peptide inhibitors, inhibited passage through the ODP at 10 and 65 μM, respectively. Comparable concentrations of these molecules have been used to inhibit proteasome activity in other cell types [47–49]. No changes in morphology or cell viability (as assayed by trypan blue exclusion) were observed after the 5-h treatment with proteasome inhibitors.

One difference between the results with proteolysis inhibitors and each of the protein kinase inhibitors is that, in experiments similar to those described in Fig. 2B, proteasome inhibitors had to be added to cells no later than 1 h after mitosis for maximal inhibition of the ODP (not shown). This is unlikely to be due to slow penetration of cells by these inhibitors, since addition of lactacystin or MG132 to metaphase cells prevented exit from mitosis (not shown), which takes place less than 40 min after metaphase [15]. It could, however, be due to the gradual accumulation of a protein product whose steady state levels are kept in check by proteolysis or to an inhibitor whose destruction several hours prior to the ODP is necessary for continued progression through G1 phase.

We have previously shown that the β-lactone ring form of lovastatin, which inhibits proteasome activity [50], does not inhibit the ODP [51]. These seemingly contradictory results can be reconciled by the fact that proteasome inhibition requires exposure of cells to high concentrations of lovastatin (40 μM) for long periods of time (36 h) [50]. At these high concentrations, CHO cells are not viable (J.-W. Wu and D.M.G., unpublished observations), although a 32-h exposure to 5 μM lovastatin is sufficient to arrest CHO cells in the post-ODP stages of G1 phase [51]. Furthermore, we found that 10 μM lactacystin or 10 μM MG132 inhibited exit from metaphase (Keezer and Gilbert, unpublished observations), most likely through inhibition of the proteasome activity of the anaphase promoting complex (APC). However, 5 μM lovastatin had no effect on the exit of cells from mitosis. These observations suggest that higher concentrations of lovastatin are necessary to inhibit the proteasome relative to that required to arrest cells in the post-ODP stages of G1 phase.

Inhibition of the ODP is not due to the disruption of pre-RCs. Pre-RCs form during telophase [14, 15], so their assembly is completed prior to addition of inhibitors in this report. However, it remained possible that inhibitors of the ODP were causing the disassembly of pre-RCs. This seemed unlikely, since the sum total for the hybridization of early replication intermediates to all DHFR probes after initiation in *Xenopus* egg extract was similar regardless of whether cells were treated with inhibitors of the ODP (Fig. 4A). This implies that the same number of replication forks were assembled within the DHFR locus in the presence or the absence of inhibitors. To verify that Mcm proteins were still associated with chromatin after drug treatment, we extracted soluble proteins from cells treated in G1 phase with roscovitine or lactacystin and analyzed

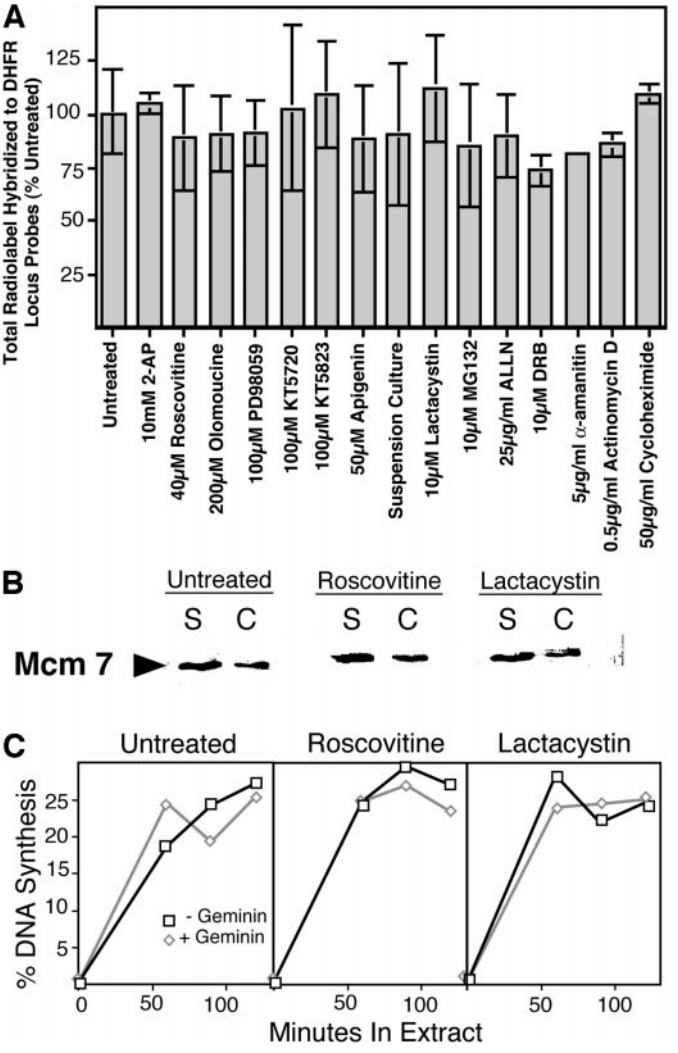

FIG. 4. The ODP inhibitors do not disrupt pre-RCs. (A) The sum total cpm hybridized to all 18 DHFR probes for the origin mapping experiments shown in Fig. 3, as well as untreated cells, is shown. Since the assay measures extension of preexisting forks, equivalent amounts of label indicate that a similar number of replication forks were assembled within the DHFR locus under all conditions. (B) Cells were synchronized and treated from 1 to 6 h after mitosis with either 40 μM roscovitine or 10 μM lactacystin or without any inhibitors. Soluble (S) and chromatin-containing (C) protein fractions were prepared at 6 h after mitosis and analyzed for the presence of Mcm7 by immunoblotting. (C.) Nuclei from cells synchronized and treated as in B were incubated in *Xenopus* egg extract with or without 40 nM geminin and supplemented with [^{32}P]dATP. At the indicated times, aliquots were removed and the percentage of input DNA replicated was evaluated by acid precipitation.

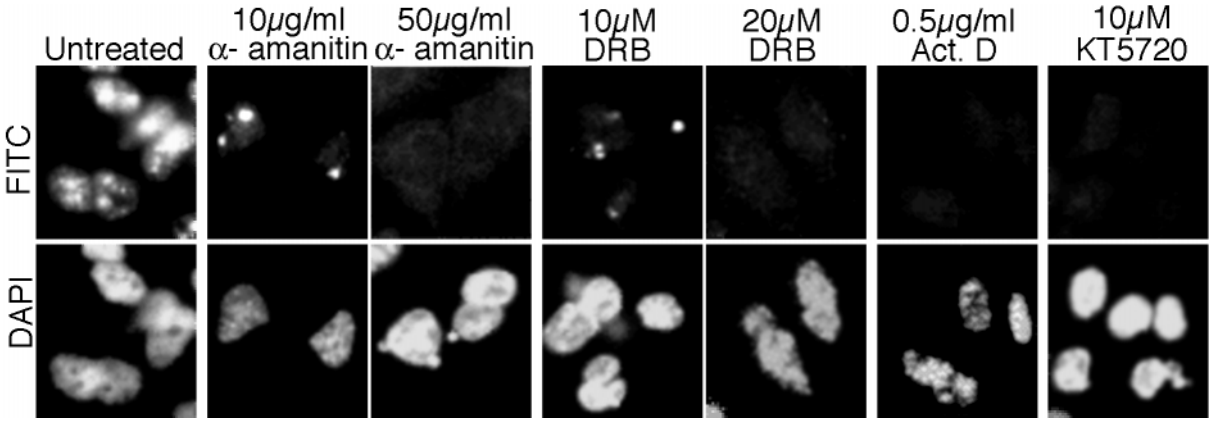

FIG. 5. Inhibition of transcription. CHOC400 cells were synchronized in metaphase and released into G1 phase as in Fig. 1. Inhibitors were added at the indicated concentrations at 2 h after metaphase and BrU was added 1 h thereafter. At 4.5 h after metaphase, cells were fixed and stained with anti-BrU antibodies as described under Materials and Methods. Untreated nuclei incorporated BrU into both nucleoli and sites of RNA PolII synthesis distributed throughout the nucleus. Both α-amanitin and DRB inhibit RNA PolII but not nucleolar RNA synthesis at lower concentrations and inhibited all RNA synthesis at higher concentrations, as expected [68, 69]. Actinomycin D (Act. D) and KT5720 inhibited all detectable RNA synthesis at the lowest concentrations tested.

both the soluble and chromatin-containing insoluble fractions for the presence of Mcm7 (Fig. 4B). Inhibition of the ODP had no detectable effect on the amount of Mcm7 associated with chromatin. To confirm that roscovitine- and lactacycstin-treated cells still contained functionally active pre-RCs, nuclei were introduced into *Xenopus* egg extracts supplemented with a nondegradable form of geminin [52], a potent inhibitor of the Mcm loading reaction. Under these conditions, geminin completely prevents the association of *Xenopus* Mcm proteins with CHO chromatin and completely inhibits the replication of chromatin that does not contain functional pre-RCs [14, 15]. As shown in Fig. 4C, the efficiency of replication within nuclei from cells treated with roscovitine or lactacycstin from 1 h after mitosis was unaffected by the presence of geminin. These results confirm our previous findings that pre-RC formation is not sufficient for DHFR origin specification and demonstrate that roscovitine and lactacystin do not inhibit the ODP through a disruption of pre-RCs.

The ODP is sensitive to the transcriptional inhibitors DRB, α-amanitin, and actinomycin D, but does not require protein synthesis. Cyclin-dependent kinases are often regulated by the synthesis of their cyclin partners [53]. In addition, transcription of Dbf4 is induced during G1 phase [26, 27]. Hence, we examined whether either transcription or protein synthesis is required for passage through the ODP. Three transcriptional inhibitors were tested. DRB has been shown to inhibit phosphorylation of the C-terminal domain (CTD) of the large subunit of RNA polymerase II [54]. α-Amanitin interacts with the largest subunit of RNA polymerase, inhibiting its activity by blocking the incorporation of ribonucleotides [55]. Actinomycin D, a DNA intercalater, is thought to inhibit transcription by preventing the advancement of the RNA polymerase [56]. We first tested the concentrations of these drugs that inhibit transcription in CHO cells. Cells in the pre-ODP stages of G1 phase were treated with transcription inhibitors at various concentrations. Three hours later, nascent RNA was labeled with BrU. Cells were then fixed and nascent RNA was visualized with an anti-BrdU monoclonal antibody. As shown in Fig. 5, DRB inhibits RNA PolII transcription but not nucleolar RNA synthesis at 10 μM. At 20 μM, DRB also inhibits nucleolar transcription. α-Amanitin inhibited RNA PolII and total transcription at 10 and 50 μg/ml, respectively. Actinomycin D inhibited all transcription at 0.5 μg/ml. These results define the concentrations of DRB, α-amanitin, and actinomycin D that are required to inhibit transcription in CHO cells.

Next, each of these drugs was tested for its ability to inhibit passage through the ODP. As shown in Fig. 3, all three of these transcriptional inhibitors are potent inhibitors of origin specification at or close to concentrations required for inhibition of RNA PolII transcription in these cells. These results suggest that transcription is required for origin specification. Inhibition of the ODP could be due to the requirement to synthesize a cyclin-like protein kinase activator. Alternatively, transcription per se could be required for origin specification either by displacing pre-RCs from transcription units or by creating superhelical tension upstream and downstream of targets [3]. To distinguish between these possibilities, we examined the requirement for protein synthesis. We have previously shown that 5 μg/ml of cycloheximide is sufficient to inhibit greater than 90% of protein synthesis in CHOC400 cells [15]. As shown in Fig. 3, even 10 times this con-

centration of cycloheximide has no effect on passage of cells through the ODP. Hence, either it is the act of transcription per se that is necessary to specify origins at the DHFR locus or all three transcriptional inhibitors have as yet undefined pleiotropic effects that have in common the ability to inhibit origin specification.

KT5720 uncouples transcription from passage through the ODP. If transcriptional activity is necessary and sufficient to allow cells to pass through the ODP then, in principle, all drugs shown to inhibit the ODP should also inhibit transcription and all drugs that inhibit transcription should inhibit the ODP. To investigate this hypothesis, CHOC400 cells were treated with each of the drugs used in this study at 1–2 h after mitosis, and BrU was added at 3 h after mitosis. Cells were incubated for an additional 1.5 h in the presence of BrU and in the continued presence of the appropriate inhibitor. At 4.5 h after mitosis, cells were fixed and stained for incorporated BrU. Although the ODP inhibitors roscovitine, olomoucine, and apigenin also inhibited transcription, KT5720, a drug that has no effect on the ODP at a 100 μM concentration, completely inhibited detectable transcription at 10 μM, indicating that transcription per se is not required for passage through the ODP (Fig. 5 and Table 1). Furthermore, the proteasome inhibitors lactacystin and MG132, which inhibit passage through the ODP, did not have a detectable effect on transcription in this system. Cycloheximide and PD98059 inhibited approximately 50% of transcription at concentrations at which they have no effect on the ODP. KT5823 has no effect on the ODP or transcription at 100 μM. Together, these results suggest that transcription, at least the amount of transcription necessary for detection by BrU labeling, is neither necessary nor sufficient for origin specification. Thus, the nature of the inhibition of the ODP by DRB, α-amanitin, and actinomycin D remains to be defined.

TABLE 1

Comparison of the Effects of Inhibitors on the ODP vs Transcription

Drug	Inhibits ODP	Inhibits transcription	Known target(s)
Roscovitine	+	+	cdk1,2,5
Olomoucine	+	+	cdk1,2,5
PD98059	−	±	MAP kinase pathway
KT5720	−	+	PKA
KT5823	−	−	PKG
Apigenin	+	+	CKII, MAP kinase pathway, cdk2
Suspension culture	−	ND	Integrin signaling
Lactacystin	+	−	Proteasome
ALLN	+	ND	Proteasome
MG132	+	−	Proteasome
DRB	+	+	Transcription, CKII, cdk7
α-Amanitin	+	+	Transcription
Actinomycin D	+	+	Transcription
Cycloheximide	−	±	Translation

Note. Experiments similar to those described in Figs. 2–4 were performed with each inhibitor. For inhibition of the ODP, (−) indicates less than 45% and (+) indicates greater than 80% reduction in the relative specificity of initiation, as compared to controls (see Fig. 3). For inhibition of transcription, (−) indicates that the pattern of BrU incorporation (see Fig. 5) was indistinguishable from untreated cells, (+) indicates that no BrU staining was detected, and (±) indicates that approximately 60% of cells incorporated BrU, although labeled cells were visibly fainter than untreated cells. Similar results were obtained in at least three independent experiments.

Insight into the Molecular Basis of the ODP

Table 1 summarizes the results presented in this report. There are two definitive conclusions one can draw from these results. First, specification of the DHFR origin at the ODP is clearly independent of the known signaling mechanisms for growth and proliferation of cells during G1 phase. Inhibition of the MAP kinase pathway, integrin-mediated cell signaling, and PKG and PKA kinases have no effect on passage through the ODP. Inhibition of protein synthesis has no effect on the ODP. These results effectively rule out the involvement of such molecules as MAP kinase, cdk2/cyclin E, and cdk2/cyclin A in the ODP. They also rule out cdk4 and cdk6, which are activated by cyclin D in response to cell growth control signals and are not inhibited by either roscovitine or olomoucine [31]. Cells pass through the ODP on schedule in serum-free medium, isoleucine-free medium [16], in the presence of protein synthesis inhibitors (Fig. 3, Table 1), or when prevented from attachment (grown in suspension) (Fig. 3, Table 1), all conditions that prevent cdk 4,6 activation [57]. Together, these results indicate that the ODP is responding to internal cell-cycle cues that are independent of growth signals and restriction point control.

The second definitive conclusion from these results is that passage through the ODP does not require the *de novo* synthesis of a specific protein kinase regulatory subunit. In fact, we found that cells could pass through the ODP on schedule even when high concentrations of cycloheximide were added as early as metaphase (S.M.K., Y. Okuno, and D.M.G., unpublished). This result not only rules out a requirement for synthesis of a cyclin partner but also makes it highly unlikely that origin specification requires Cdc7 since the activity of Cdc7 requires the *de novo* G1-phase synthesis of its cyclin-like partner, Dbf4/ASK1 [26, 27]. Also, *in vitro* kinase activity of Cdc7 is not inhibited by roscovitine, even at 400 μM (H. Masai, personal communication). Cdc7 was an attractive candidate for the ODP kinase,

since studies in *Xenopus* egg extracts indicate that Cdc7 is required after pre-RC formation but prior to cdk2 to elicit the initiation of DNA replication [24, 25].

Inhibitors that prevent passage through the ODP provide useful tools with which to test hypotheses about the nature of the ODP, but our results demonstrate that the consortium of these inhibitors does not converge upon an obvious common denominator (Table 1). Either several of these inhibitors have more pleiotropic effects than previously anticipated or there are many different aspects of cellular metabolism that can impinge upon the passage of cells through the ODP. Inhibitor studies are inherently flawed by the potential of pleiotropic effects. For example, DRB inhibits CKII [58], cdk7, and cdk8 [59, 60]. It is possible that the ODP-inhibiting effect of DRB lies in its inhibition of a kinase activity, especially since Cdk7 functions both in the capacity of transcriptional regulator and cdk activating kinase (CAK) [61]. Actinomycin D binds to both double-stranded and single-stranded nucleic acids and intercalates into DNA [56, 62, 63]. This drug could exert its effect on the ODP by changing the physical environment of the DNA at and around the origin, allowing alternative initiation sites to be utilized. Actinomycin D also inhibits DNA primase [64]. Since some evidence suggests that association of polα-primase with chromatin during G1 phase may be independent of pre-RC formation [65], it is possible that inhibition of the ODP by transcriptional inhibitors is mediated through inhibition of primase. However, it is equally possible that each of these drugs is inhibiting a step upstream of the ODP, necessary for passage through the ODP but not directly involved in origin specification. Further experiments utilizing alternative approaches to identify molecular events leading to origin specification will be necessary to interpret the ODP inhibiting effects of these drugs. Nonetheless, these inhibitors provide useful tools with which to test hypotheses regarding the molecular basis of the ODP, to uncouple events, and to establish an order of events. Here, we have used these inhibitors to position the ODP downstream of pre-RC formation and upstream of S-phase promoting kinase activities.

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