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The Plant Amino Acid Mimosine May Inhibit Initiation at Origins of Replication in Chinese Hamster Cells

P. J. MOSCA,[1] P. A. DIJKWEL,[2] AND J. L. HAMLIN[2,3]*

Biochemistry Department[2] and Biophysics[1] and Cell and Molecular Biology[3] Programs, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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An understanding of replication initiation in mammalian cells has been hampered by the lack of mutations and/or inhibitors that arrest cells just prior to entry into the S period. The plant amino acid mimosine has recently been suggested to inhibit cells at a regulatory step in late G_1. We have examined the effects of mimosine on cell cycle traverse in the methotrexate-resistant CHO cell line CHOC 400. When administered to cultures for 14 h after reversal of a G_0 block, the drug appears to arrest the population at the G_1/S boundary, and upon its removal cells enter the S phase in a synchronous wave. However, when methotrexate is administered to an actively dividing asynchronous culture, cells are arrested not only at the G_1/S interface but also in early and middle S phase. Most interestingly, two-dimensional gel analysis of replication intermediates in the initiation locus of the amplified dihydrofolate reductase domain suggests that mimosine may actually inhibit initiation. Thus, this drug represents a new class of inhibitors that may open a window on regulatory events occurring at individual origins of replication.

Mammalian origins of replication are positioned at ~100-kb intervals along each chromosomal DNA fiber (9). At present, virtually nothing is known about the nature of the genetic sequences required for origin function or about the *trans*-acting factors that interact with these sequences to effect initiation.

Many approaches to identifying origins of replication have relied on populations of cells that have been induced to enter the S period synchronously, since the beginning of S is the one time during the cell cycle when at least some origins are sure to be firing. A commonly used synchronizing regimen is to arrest cells in G_0 by starvation for serum or isoleucine, followed by release into complete medium containing an inhibitor of DNA replication. The block is enforced for a time long enough to allow most cells to reach the G_1/S boundary, after which the drug is removed and cells enter S in a synchronous wave (e.g., see references 7, 8, and 17).

The inhibitors that have been used in this protocol most frequently are hydroxyurea, which inhibits ribonucleotide reductase (16), and aphidicolin, an inhibitor of DNA polymerases (19). However, neither of these agents is truly satisfactory since both are chain elongation inhibitors that would not be expected to inhibit initiation per se. Thus, most of the events of interest (i.e., initiation at early-firing origins) would have already occurred prior to release from the blocking agent. Unfortunately, drugs that specifically prevent either entry into the S period or initiation at origins have not yet been identified. (Throughout this report, we will use the term "initiation" to mean the setting up of new replication forks, without making a distinction between helix opening and priming of the leading strands of replication at an origin.)

In our laboratory, we are studying the replication pattern of the 240-kb amplified dihydrofolate reductase (DHFR) domain in methotrexate-resistant CHO cells. We have routinely used aphidicolin to arrest cells near the G_1/S boundary after reversal of a G_0 block induced by isoleucine deprivation. By an in vivo labeling method designed to follow fork movement through the DHFR domain after release from an aphidicolin block, it was possible to roughly localize a replication initiation locus lying downstream from the DHFR gene (7). Additional studies have suggested that there actually may be two preferred initiation sites or zones within this region separated by ~22 kb (termed ori-β and ori-γ [1, 6, 12]). A recent in vitro study that analyzed the position at which the leading and lagging strands of replication switch templates near the ori-β locus suggested that most initiation occurs within a single 500-bp fragment (3).

All of these studies are consistent with the presence of at least one, and probably two, fixed origins of replication lying downstream from the DHFR gene. However, the situation became less clear when replication intermediates in the DHFR locus were examined by two complementary two-dimensional (2-D) gel replicon mapping techniques (2, 14). Initiation was found to occur at many random positions throughout a broad zone encompassing both ori-β and ori-γ (4, 5, 18). Because of the difficulty in quantifying the number of initiations per unit length of DNA with these methods, we cannot presently exclude the possibility that more initiations occur in the neighborhood of ori-β and ori-γ than at other regions in the initiation locus, accounting for the preferential labeling of these two regions in previous studies. However, qualitatively speaking, the 2-D gel data argue against a very circumscribed initiation locus analogous, for example, to the *Escherichia coli* or simian virus 40 origin.

To gain a better understanding of initiation in the DHFR domain, it would obviously be advantageous to synchronize cells at a point prior to initiation at early-firing origins. Toward this end, we have been studying the plant amino acid mimosine, which has been reported to inhibit mammalian cells in late G_1 just prior to the S period (11). In a previous report, we showed that this drug is much more effective than aphidicolin at preventing entry into the S period when administered to cells after reversal of a G_0 block (4). Indeed, no initiation appeared to occur in the DHFR locus in the presence of mimosine, and the level of initiation observed after release from this drug was 5 to 10 times higher than in cells released from aphidicolin (4).

* Corresponding author.

However, in this study, we show that mimosine does not arrest cells in late G_1. Rather, mimosine may actually prevent initiation at individual origins of replication.

MATERIALS AND METHODS

Cell culture and synchronization protocols. The CHOC 400 cell line was developed and maintained in minimal essential medium supplemented with nonessential amino acids, 12.5% fetal calf serum, and 400 μg of methotrexate per ml as previously described (7). For experiments, cells were grown in methotrexate-free medium on 10- or 15-cm-diameter plates or in 24-well dishes. Cultures were arrested in G_0 by incubation in isoleucine-free medium for 45 h and were then released into complete medium containing the appropriate inhibitor for 14 h. The cells were washed once with prewarmed serum-free medium and were returned to drug-free complete medium (except where noted in the figure legends). All tissue culture media and sera were obtained from GIBCO.

Radiolabeling protocols. Cells (1×10^5 to 1.8×10^5/16-mm-diameter well) were incubated with 0.2 to 1.0 μCi of [^{3}H]thymidine (85 Ci/mmol; ICN Biomedicals) per ml and 0.2 μg of unlabeled thymidine per ml for the times indicated in the figure legends, and metabolism was stopped by the addition of 1/10 volume 2.3 M citric acid (13). At the end of the experiment, the medium was aspirated, the cells were washed once with phosphate-buffered saline (137 mM NaCl, 5.4 mM KCl, 1.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.2), and DNA was precipitated with 10% trichloroacetic acid at 4°C for at least 2 h. After the wells were aspirated to dryness, the trichloroacetic acid-precipitated material was solubilized in 250 μl of 0.2 M NaOH, and 100 μl was transferred to a vial containing 5 ml of scintillation fluid (Ready-safe; DuPont Chemicals) and 10 μl of concentrated acetic acid. Data were plotted by using Sigmaplot version 4.1; data points represent the means of duplicate determinations (except in Fig. 3A), and error bars indicate the standard deviation (in some cases, the error bars lie within the area of the symbol).

FACS analysis. For fluorescence-activated cell sorter (FACS) analysis, cells were trypsinized, triturated several times, and centrifuged at setting 3 on a tabletop IEC instrument for 3 to 5 min. The supernatant was removed, and a solution containing 4′,6-diamidino-2-phenylindole, a DNA stain, was added (15). The final cell concentration was ~10^6/ml. Samples were analyzed at the University of Virginia FACS facility.

2-D gel electrophoresis. 2-D gel analysis of replication intermediates in the DHFR locus was performed exactly as previously described (5). Each of the samples in the experiments summarized in Fig. 8 and 9 was derived from four 15-cm-diameter plates (~10^8 cells). FACS analysis was performed on control cultures to ascertain that the cells were growing asynchronously at the time that the drugs were added.

RESULTS

Mimosine arrests cells at the G_1/S boundary after reversal of a G_0 block but not when added to asynchronous cultures. Lalande reported that when added to asynchronous cultures, mimosine arrests human lymphoblastoid cells specifically at a point in the late G_1 period ~2 h prior to the onset of S (11). In initial studies, we tested the action of this drug in our standard synchronizing protocol. Cells were arrested in G_0 by isoleucine deprivation and were then released from this block into complete medium containing mimosine for 14 h.

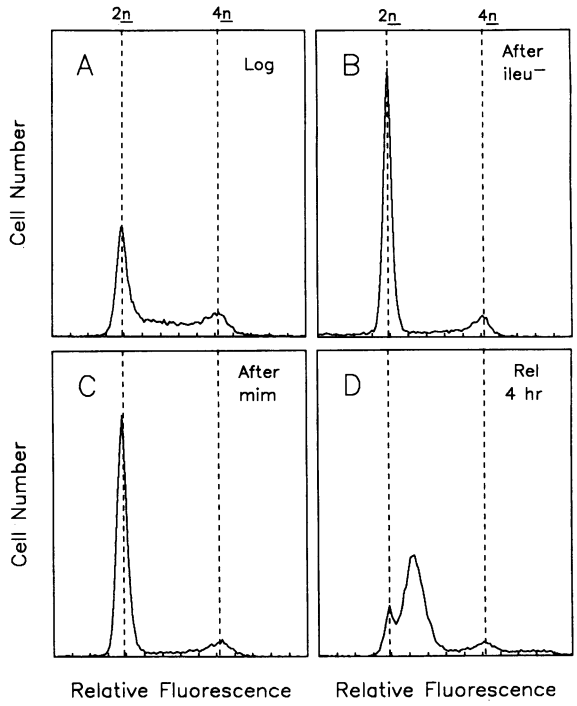

FIG. 1. Reversible arrest of the majority of the population near the G_1/S boundary when mimosine is added to cells released from a G_0 block. A logarithmically growing culture was sampled for FACS analysis at time zero (A), after 45 h of isoleucine starvation (B), after release from starvation into 400 μM mimosine for 14 h (C), and 4 h after release from mimosine (D).

After this protocol, the majority of the population was reproducibly arrested near the G_1/S boundary. As shown in the FACS analysis in Fig. 1C, most of the cells treated with 400 μM mimosine displayed the G_1 ($2n$) DNA content (compare with the log and G_0 populations in Fig. 1A and B); furthermore, 4 h after removal of the drug, about 80% of cells had entered the S period in a synchronous wave (Fig. 1D). (Note that a small but variable number of cells remains indefinitely in the G_1 compartment after this protocol for unknown reasons; compare, e.g., with Fig. 5C and D.)

The kinetics of [^{3}H]thymidine uptake were also monitored after cells were starved for isoleucine, incubated in various concentrations of mimosine for 14 h, and then released from the mimosine block into the S period; a culture blocked with 30 μM aphidicolin was included as a control (Fig. 2A). The population appears to be highly synchronized in mimosine levels as low as 133 μM, traversing the S period in 10 to 11 h (although the shapes of the rate curves are slightly different for each of the three mimosine concentrations tested and for 30 μM aphidicolin).

To examine the kinetics of entry into S more critically, [^{3}H]thymidine uptake was measured in the 15-min interval prior to removal of mimosine, and cumulative incorporation was then measured at 15-min intervals after release (Fig. 2B). Importantly, [^{3}H]thymidine uptake in the presence of mimosine was negligible and did not vary with drug concentration, suggesting that the drug is an effective inhibitor under these circumstances even at 133 μM. In addition, however, a dose-dependent lag is observed after mimosine is

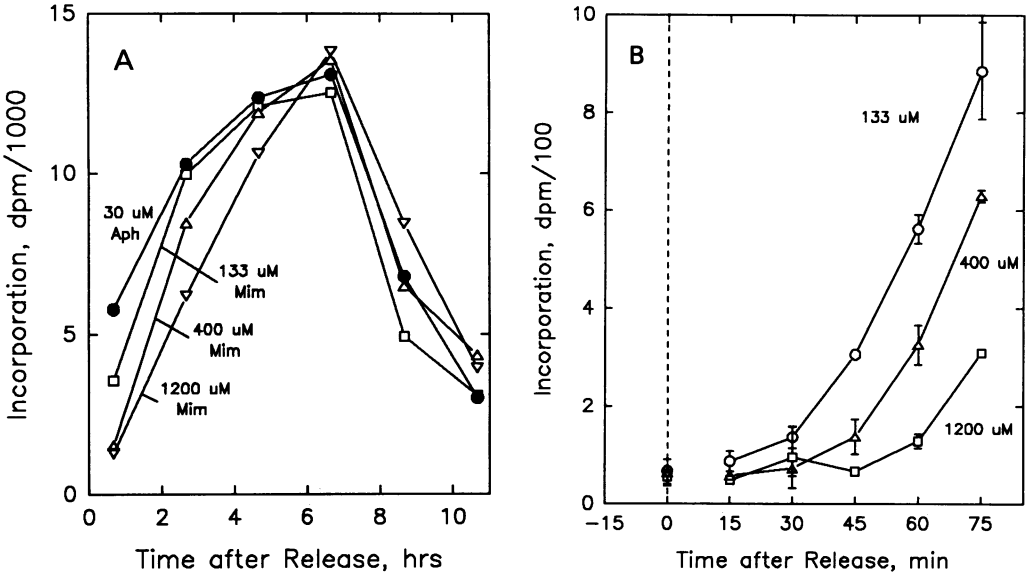

FIG. 2. Evidence from the kinetics of S-phase traverse that mimosine blocks cells at the G_1/S boundary. CHOC 400 cells growing in multiwell dishes were starved for isoleucine for 45 h and were released into complete medium containing the indicated concentrations of either mimosine (Mim) or aphidicolin (Aph) for 14 h. Media were removed and replaced with fresh drug-free media. (A) Individual wells were pulse-labeled for 60 min at 2-h intervals with [^{3}H]thymidine, beginning at time zero. All samples were washed and prepared for counting as described in Materials and Methods. The time coordinate for each point represents the midpoint of the corresponding pulse interval. (B) At 15 min prior to mimosine removal, one set of wells at each drug level was pulse-labeled for 15 min in media containing [^{3}H]thymidine and the appropriate concentration of mimosine. At time zero, mimosine was removed from the remaining wells and replaced with fresh drug-free media containing [^{3}H]thymidine. At 15-min intervals thereafter, citric acid was added to each well, and cumulative incorporation was measured at the end of the experiment as described in Materials and Methods. The data for each time point, including those for the sample that was pulsed in the presence of mimosine, are plotted at the end of the labeling period. The mean of duplicate determinations is shown, and error bars indicate the standard deviations.

removed, probably accounting for the minor differences in the shapes of the rate curves in the early S period in Fig. 2A.

Thus, mimosine is very effective at preventing entry into the S period when delivered to cells after release from a G_0 block. However, when we added mimosine to nonsynchronized cell cultures, we could not reproducibly arrest cells in late G_1. In some experiments, when cells were incubated with mimosine for at least 24 h (about one cell cycle), the majority of the population could be arrested in the G_1 compartment, displaying FACS patterns similar to that shown in Fig. 1C; upon release from the drug, the population entered the S period in a synchronous wave (as in Fig. 1D) (data not shown).

However, in other experiments, mimosine appeared to have little effect when added to log cells. For example, in the FACS analyses summarized in Fig. 3, either 400 μM mimosine or 30 μM aphidicolin was added to asynchronous cultures ~24 h after plating. Samples were taken 24 or 48 h after drug addition, and drug-free controls were included to monitor the growth state at all times during the experiment.

Figure 3A shows that the starting population in this experiment was indeed asynchronous, containing cells in all compartments of the cycle, ranging from a prominent G_1 peak all the way to the G_2 peak. (Note that 30 to 50% of cells in the 4*n* fraction in a log-phase population are probably tetraploids that are in G_1, since some of them can be seen to transit into compartments with greater than 4*n* DNA content in synchronized cells [e.g., Fig. 1D].)

Twenty-four hours later, the drug-free control was essentially unchanged (Fig. 3B). The 24-h aphidicolin and mimosine samples were similar to the drug-free control except for partial depletion of the G_2 peak and a modest enhancement of the G_1 peak (Fig. 3C and D). In the case of aphidicolin, which is a chain elongation inhibitor, this pattern undoubtedly represents cells arrested at various positions in the S period and at the G_1/S boundary.

After 48 h, the cell cycle distribution of the drug-free control was again similar to that of the starting log-phase population, indicating that the cells were still cycling (Fig. 3E). In the aphidicolin-treated sample, however, the G_1 peak had virtually disappeared, and the majority of cells had shifted to various positions in the S period and G_2 (Fig. 3F). This dramatic change relative to the 24-h pattern probably results from the fact that after 12 h in aphidicolin (G_2+M+G_1 in this cell line), all cells will be either at the G_1/S boundary or in S itself and will have 36 h more to leak through S. In the 24-h sample, this interval is only 12 h.

In contrast, the population treated with 400 μM mimosine for 48 h had a distribution similar to that of the drug-free control except for depletion of the G_2 peak and a slight increase in the G_1 compartment (Fig. 3G). This finding suggested either (i) that mimosine is not effective on log-phase cells at 400 μM or (ii) that cells are sensitive to mimosine both at the G_1/S boundary and throughout S.

However, as shown in Fig. 4, when mimosine was added to asynchronous cultures, concentrations as low as 100 μM completely inhibited incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material (albeit after a considerable lag relative to 30 μM aphidicolin, which inhibited uptake almost immediately). Thus, the inability to reproducibly accumulate CHOC 400 cells at the G_1/S boundary by adding 400 μM mimosine to asynchronously growing cultures was not the result of ineffective drug concentrations.

It seemed more likely that mimosine might actually be an

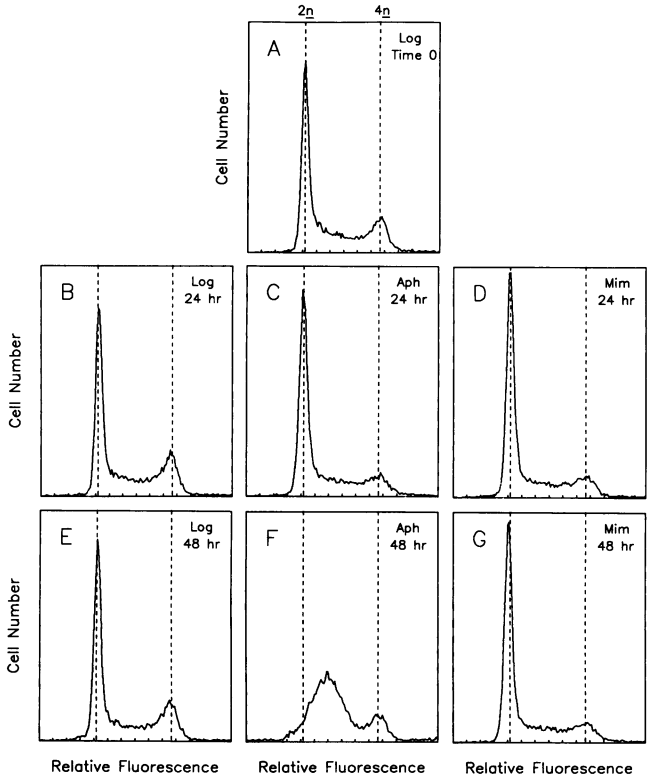

FIG. 3. Evidence that mimosine does not arrest cells quantitatively in late G_1 when added to asynchronous cultures. Twenty-four hours after plating (at ~30% confluence), CHOC 400 cells were treated with fresh medium containing no drug, 400 μM mimosine, or 30 μM aphidicolin. At the time of drug addition (time zero), at 24 h, and at 48 h, individual plates were trypsinized and treated with 4',6-diamidino-2-phenylindole stain (15), and the samples were stored at 4°C until completion of the experiment. Fifteen thousand cells from each sample were then analyzed by FACS analysis, and the resulting data were imported into Sigmaplot version 4.1. Dotted lines were drawn on the basis of the approximate *2n* and *4n* peaks in the time zero sample.

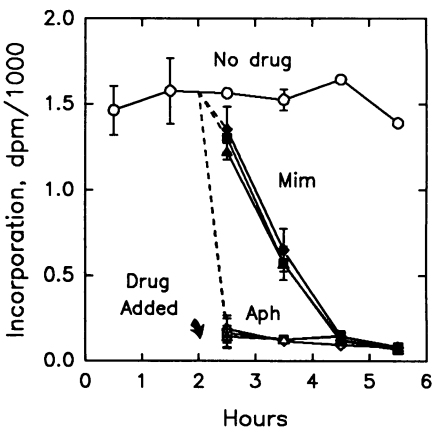

FIG. 4. Complete inhibition of [^{3}H]thymidine uptake into asynchronous cultures by mimosine concentrations as low as 100 μM. Asynchronous cultures of CHOC 400 cells were grown in 24-well cluster dishes to ~1.5 × 10^5 cells per well. At the start of the experiment, one sample was prepared for FACS analysis to ascertain that the cells were still growing asynchronously, and the remaining wells received 1 ml of prewarmed medium. In the drug-free control, individual wells were pulse-labeled with [^{3}H]thymidine for 60 min at 1-h intervals starting at time zero. Beginning 2 h later, the remaining wells were pulse-labeled for 1 h with radioactive medium additionally containing aphidicolin (Aph; 7.5, 15, or 30 μM; open symbols) or mimosine (Mim; 100, 200, or 400 μM; closed symbols). Samples were processed and analyzed as described in the legend to Fig. 2. The mean of two determinations is plotted at the midpoint of each pulse period.

S-phase inhibitor and that in some of our early experiments in which the growth state of the cells was not carefully monitored, the presumably asynchronous cultures to which we added mimosine might actually have been arrested (or close to arrest) in G_0 at the beginning of the experiment. This condition could result from partial synchronization at the time of plating or medium depletion during the course of drug treatment. The former possibility seemed particularly likely, since we were in the habit of replating cells for experiments from confluent stocks that could have been in G_0 at the time of trypsinization. Upon addition of mimosine in fresh medium several hours later, the cells would then advance across G_1 just as if they had been released from a G_0 block caused by isoleucine deprivation.

Mimosine affects S-phase cells. Lalande (11) concluded that mimosine acted in late G_1, a conclusion based partly on the results of experiments in which nonsynchronized human lymphoblastoid cells were treated with mimosine for 16 h and were then released from mimosine into aphidicolin. He found that these cells were unable to progress any further in the cell cycle, whereas cells released from an aphidicolin block into mimosine were able to transit into the S period (at least when monitored in the subsequent 4 h [11]). Since aphidicolin is a chain elongation inhibitor, this result led to the conclusion that mimosine acts before DNA replication commences.

We performed a similar experiment on CHOC 400 cells by using similar drug levels; in this case, however, the cultures were arrested in G_0 by isoleucine deprivation and were then synchronized near the G_1/S boundary by a 14-h incubation in either 200 μM mimosine or 15 μM aphidicolin. The drugs were removed and replaced either with drug-free medium or with medium containing the same or the other drug.

The FACS distributions in Fig. 5A and B show that the majority of CHOC 400 cells were arrested near the G_1/S boundary after a 14-h incubation in either aphidicolin or mimosine after reversal of a G_0 block. Furthermore, upon removal of either drug, almost the entire population advanced into the S period in a relatively synchronous wave, although the mimosine samples appear to be somewhat behind those released from aphidicolin (Fig. 5C and D, sampled 6 h after drug removal; all subsequent samples were also sampled at 6 h). As expected, when the original drug was replaced with a fresh aliquot of the same drug, cells remained at or near the G_1/S interface (Fig. 5E and F).

Most importantly, however, while aphidicolin appears to have inhibited further progression of cells released from mimosine (Fig. 5H), mimosine did not prevent aphidicolin-released cells from progressing into the S phase, albeit only into early S (Fig. 5G).

Studies on the uptake of [^{3}H]thymidine corroborated the results of the FACS analyses (Fig. 6). When cells were released from either aphidicolin or mimosine into drug-free medium, incorporation of [^{3}H]thymidine increased at similar rates over the 6-h interval examined in this experiment, although there was a 30- to 40-min lag before cells released from 400 μM mimosine actually entered the S period (Fig.

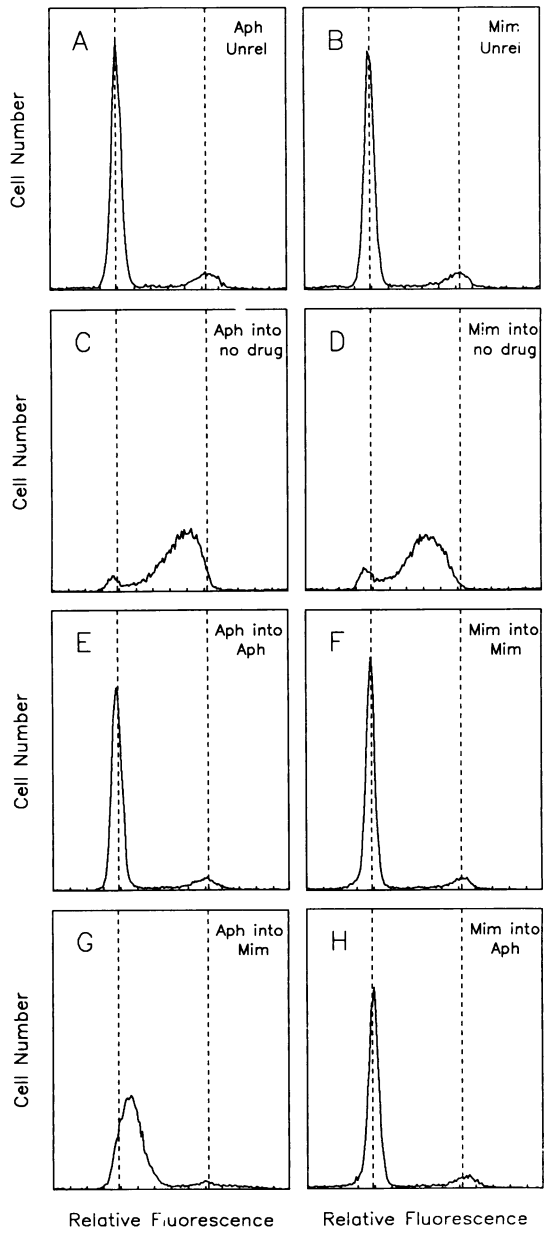

FIG. 5. Inhibition by mimosine of progression of cells released from an aphidicolin block. CHOC 400 cells growing in multiwell dishes were deprived of isoleucine for 45 h, after which the medium was replaced with fresh medium containing either 200 μM mimosine or 15 μM aphidicolin. After 14 h, the drugs were washed out and replaced with fresh medium containing no drug, the same drug, or the other drug. Samples were prepared for FACS analysis at time zero, prior to drug removal (A and B), or at 6 h after media were changed (C to H).

6A). This result agrees with the [^{3}H]thymidine uptake data in Fig. 2B, demonstrating a dose-dependent lag before replication begins.

When cells were returned to the same drug in which they were originally blocked, the subsequent rate of [^{3}H]thymidine uptake remained at very low values (Fig. 6A). Furthermore, when cells were released from mimosine into aphidicolin, replication was almost completely inhibited for at least the next 6 hr (Fig. 6A), in agreement with the results of Lalande for human cells (11). Thus, by this criterion, aphidicolin acts either at the same or at a later step than does mimosine.

In contrast, when CHOC 400 cells were released from aphidicolin into mimosine (Fig. 6A), DNA replication initially proceeded unabated, but sometime after 3 h, [^{3}H]thymidine uptake into DNA leveled off. These data argue that mimosine affects DNA replication per se, but only after a considerable lag.

To determine the length of this lag more precisely, the [^{3}H]thymidine uptake experiment was repeated, taking time points at 1-h intervals after addition of mimosine to cells released from an aphidicolin block. As seen in Fig. 6B, the rate of [^{3}H]thymidine uptake does not begin to decline until after the second hour and reaches zero by 4 h. This lag cannot be explained solely by inefficient drug entry, since a noticeable reduction in the rate of [^{3}H]thymidine uptake was observed within about 1 h after addition of mimosine to asynchronous cultures (Fig. 4). Furthermore, if the lag were due solely to inefficient drug entry, one would expect to observe a more rapid effect at higher mimosine concentrations; however, such a dose effect was not observed (Fig. 4). In addition, we show below that the effects of mimosine can be detected at the level of replication forks within ~2 h after addition to log cells.

Coupled with the FACS analyses in Fig. 5, these results therefore indicate that even after cells have synthesized considerable amounts of DNA subsequent to release from aphidicolin, they are again sensitive to the effects of mimosine at a later time point(s).

Mimosine inhibits subsequent DNA replication when added in early and middle S phase but probably not in very late S phase. The previous experiments suggested that mimosine arrests cells not only at the G_1/S boundary but also at a later point(s) in the S period itself. To address this question directly, CHOC 400 cells were treated with 400 μM mimosine for 14 h after reversal of a G_0 block, the drug was washed out, and at intervals thereafter, mimosine was readministered. The subsequent effects on cumulative [^{3}H]thymidine uptake were then monitored.

In the experiment shown in Fig. 7, 400 μM mimosine inhibited subsequent DNA replication almost completely when readministered to cells at any time prior to 5 h after entry into the S period, in each case with a lag period of 2 to 3 h. Addition of mimosine at 5 h had only a modest effect, and addition at 8 h had no measurable effect on subsequent DNA replication. Given the lag observed with the other time points, it is not possible to determine from this experiment alone whether there is a refractile period in late S phase. However, in an independent experiment, mimosine appeared to have no effect when added at 5 h or any time thereafter (12a), suggesting that by the sixth or seventh hour of the S period, the cells may indeed become insensitive to mimosine.

Thus, it appears that mimosine inhibits DNA replication at multiple times during the S period (with the possible exception of late S), but only after about a 2-h lag. In contrast, aphidicolin inhibits replication almost immediately regardless of how late in S the drug is administered (5a; also see data on log-phase cells in Fig. 4).

Mimosine and aphidicolin have very different effects on replication fork movement. When aphidicolin was added to asynchronous cultures of CHOC 400 cells (Fig. 4), [^{3}H]thymidine uptake into DNA was completely inhibited within the first hour, whereas complete inhibition did not occur for ~2.5 h after the addition of mimosine. While some of this lag

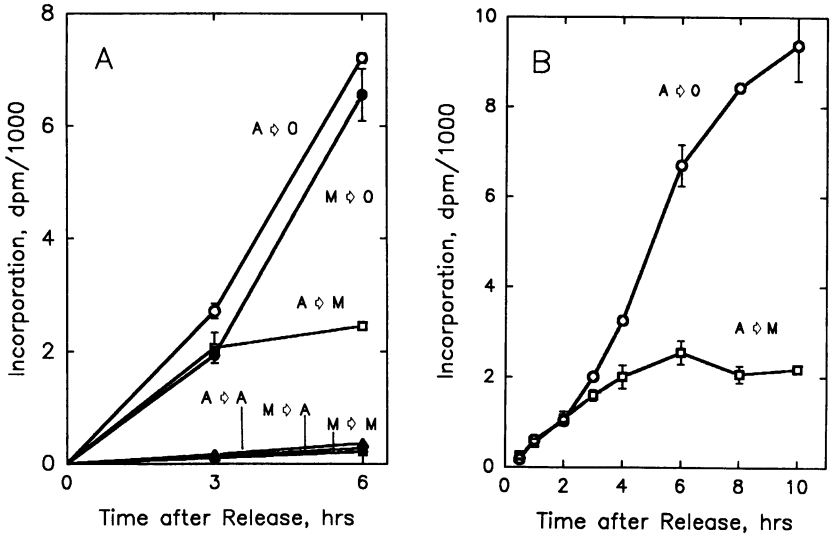

FIG. 6. Detection of the effects of mimosine on progression through S only after the third hour of S. (A) Cells treated as described in the legend to Fig. 5 were released from the blocking agent into medium containing 1.0 μCi of [^{3}H]thymidine per ml, 0.2 μg of thymidine per ml, and no drug, 200 μM mimosine (M), or 15 μM aphidicolin (A), as indicated. Duplicate wells were assessed for [^{3}H]thymidine uptake after 3 and 6 h. Note that this experiment was performed at the same time as the experiment in Fig. 5. (B) Cells were treated as for panel A except that cells were released from 15 μM aphidicolin into radioactive medium with or without 200 μM mimosine and were sampled at the times indicated.

might be attributed to the slow influx of mimosine, it was also important to determine whether the effects of mimosine and aphidicolin on replication forks were qualitatively different. We therefore used a 2-D gel electrophoretic method that affords a comprehensive picture of the replication intermediates in a fragment at the time of sampling. The method takes advantage of the different mobilities of fragments containing either a replication fork or a replication bubble (2). The restriction fragment of interest can be analyzed by separating a genomic DNA digest on a 2-D gel, transferring the digest to a membrane, and hybridizing it with a probe specific for that fragment.

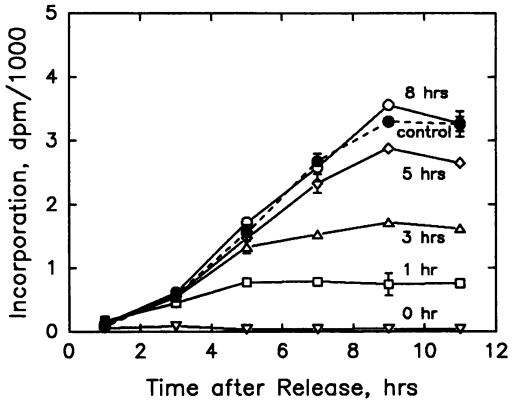

FIG. 7. Effect of mimosine on cells throughout most of the S period. CHOC 400 cells growing in multiwell dishes were deprived of isoleucine for 45 h and were then incubated in complete medium containing 400 μM mimosine for 14 h. At time zero, the wells were washed, and 1 ml of medium containing 0.2 μCi of [^{3}H]thymidine per ml and 0.2 μg of thymidine per ml was added to each. At the indicated times, the drug-free radioactive medium was replaced with radioactive medium additionally containing 400 μM mimosine. The control received no drug. Incorporation was arrested with citric acid at 2-h intervals beginning at 1 h.

As shown in Fig. 8, a given restriction fragment will trace a different arc in the gel depending upon whether it (i) is nonreplicating (represented by the 1*n* spot, as in Fig. 8A and B), (ii) contains an active origin (a bubble arc, as in Fig. 8B), or (iii) is replicated passively from an outside origin (resulting in a single fork arc, as in Fig. 8A).

Figure 8 shows autoradiographs from the DNA of CHOC 400 cells that were arrested near the G_1/S boundary with either 400 μM mimosine or 30 μM aphidicolin for 14 h after reversal of a G_0 block (Fig. 8C and E) and were subsequently released from the block for 90 min (Fig. 8D and F). The transfers have been hybridized with a probe specific for a 6.2-kb *Eco*RI fragment from the ori-β region of the DHFR domain.

In agreement with earlier studies (4), only the 1*n* spot is prominent in cells arrested with mimosine (although after very long exposures, some small forked structures can be discerned) (Fig. 8C). Thus, it appears that mimosine is very effective at preventing initiation when it is delivered during the G_1 period. In contrast, significant escape synthesis occurs when cells are maintained in 30 μM aphidicolin for 14 h after reversal of a G_0 block, as evidenced by the presence of a discernible single fork arc (Fig. 8E; Fig. 8E and F are reproduced from reference 4).

By 90 min after removal of mimosine, when maximum initiation is occurring in the DHFR locus (4), a composite pattern of a single fork arc and a bubble arc is observed (Fig. 8D). We have argued that this result would be obtained if initiation occurs at random positions throughout the initiation locus (4, 18). Thus, any given fragment in this locus would be replicated sometimes from an internal initiation site and sometimes by forks emanating from initiation sites outside of the fragment. Note that the amount of replication intermediates detected in the initiation locus is 5- to 10-fold higher in mimosine-synchronized cells (Fig. 8D) than it is in

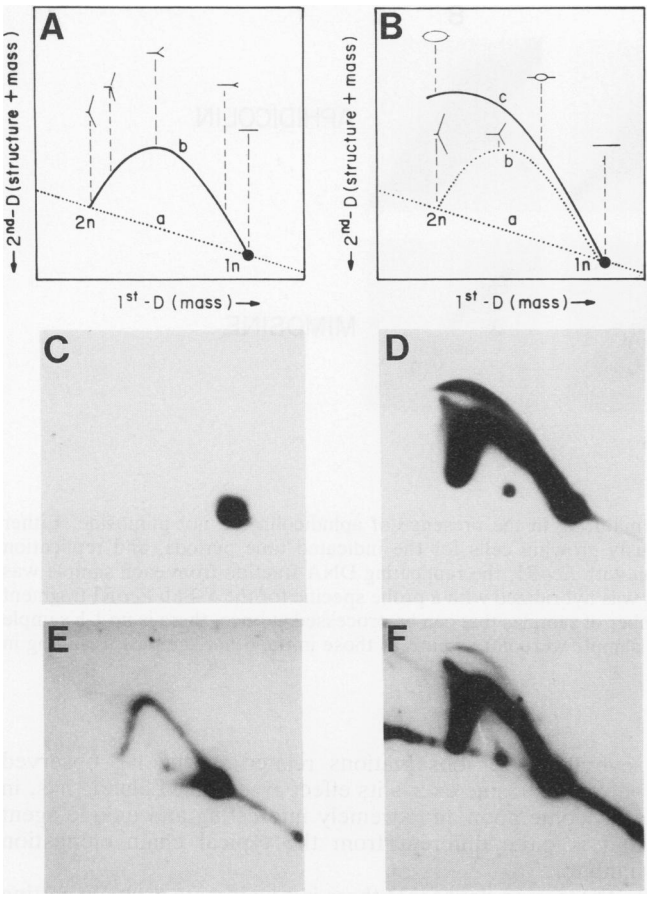

FIG. 8. 2-D gel analysis of replication intermediates in the ori-β region after release from a G_1/S block induced by either aphidicolin or mimosine. (A and B) Patterns that would be obtained on a 2-D gel when a fragment containing either single forks or centered bubbles is analyzed with an appropriate probe. (C to F) Assays on cells that were starved for isoleucine and then arrested near the G_1/S boundary with either 400 μM mimosine (C and D) or 30 μM aphidicolin (E and F). At time zero, prior to drug removal (C and E), or 90 min after release into drug-free medium (D and F), samples were taken and replication intermediates were prepared by the matrix enrichment procedure described previously (5), using *Eco*RI to digest the DNA loops from the matrices. After separation on 2-D gels and transfer to a membrane, the digests were analyzed with a probe specific for the 6.2-kb *Eco*RI fragment that is centered over the ori-β region. Note that in panels D to F, the digests were slightly incomplete, resulting in the appearance of an additional faint 1*n* spot at a higher molecular weight on the diagonal.

cells 90 min after release from an aphidicolin block (Fig. 8F), strengthening the argument that significant initiation has already occurred in the presence of aphidicolin (4).

When we analyzed the effects of mimosine and aphidicolin at the level of replication forks after addition to actively dividing asynchronous cultures, the results obtained with the two drugs were also quite different (Fig. 9).

In the control DNA from log-phase cells, the composite pattern of bubbles and fork arcs was again observed in the 6.2-kb *Eco*RI fragment that is centered in the ori-β locus (Fig. 9). By 2, 4, and 8 h after addition of aphidicolin, this pattern did not change significantly, arguing that aphidicolin simply arrests or slows replication forks and has no other gross effects. However, on the basis of the FACS analyses in Fig. 3, in which aphidicolin was shown to be a very leaky inhibitor, it is likely that aphidicolin lowers the flux of replication forks through the fragment but does not prevent new initiations from occurring within the fragment or new forks from entering the fragment to replace those that slowly transit and exit. (Note also that continual incubation with aphidicolin provokes the appearance of a large amount of material extending leftward from the peak of the fork arc. We do not understand the origin of this material but have consistently observed it when aphidicolin is added to asynchronous cultures. Note also that the material on the diagonal of nonreplicating DNA represents partial digestion products.)

Cells treated with 400 μM mimosine gave an entirely different picture. One hour after drug addition, prominent bubble and single fork arcs could still be observed, but by 2 h, the bubble arc had virtually disappeared. By 4 h, the lower-molecular-weight part of the single fork arc had diminished, and by 8 h, almost the entire fork arc was gone except for a narrow zone around the peak of the arc (Fig. 9).

It appears, therefore, that mimosine allows both bubbles and forks to mature out of the fragment but prevents new internal initiations from occurring. Furthermore, in the presence of mimosine, no new forks enter from initiations occurring at sites outside of the fragment. This is the result that would be expected if mimosine prevents initiation of replication but not chain elongation per se.

DISCUSSION

A compound that inhibits cells at a regulatory step in late G_1 would be extremely useful in studies on the G_1/S transition and subsequent initiation at origins of replication. Although the plant amino acid mimosine has been reported to have these properties (11), we show here that it does not arrest the entire population of CHOC 400 cells in late G_1 when added to actively dividing asynchronous cultures. Instead, the distribution of cells treated with mimosine for 48 h differs little from the distribution of the asynchronous drug-free control except for a diminution in the G_2 peak and a slight enrichment in the G_1 compartment (Fig. 3). However, the same drug concentration noticeably inhibits uptake of [^{3}H]thymidine when added to asynchronous cultures after a lag of less than 1 h and does so completely by 3 h (Fig. 4).

The additional observation that mimosine routinely arrests the population at the G_1/S boundary when administered to cells released from a G_0 block argues strongly that, in fact, mimosine is an inhibitor of DNA replication (Fig. 1 and 2). This effect was demonstrated directly in uptake studies on synchronized S-phase cultures; DNA replication was inhibited when the drug was added at any time prior to and including the fifth hour of S but only after a lag period of 1 to 2 h (Fig. 7).

The question arises as to why Lalande's experiments suggested that mimosine might be an inhibitor of a late G_1 event in human lymphoblastoid cells (11). One possible explanation is that Chinese hamster ovary cells might be peculiarly sensitive to the drug throughout the S period in addition to an effect in late G_1. However, we were also unable to arrest murine lymphoid cells in G_1 when mimosine was added to asynchronous cultures (12a). Thus, we consider this explanation unlikely.

We consider it more likely that the presumably asynchronous cultures to which Lalande added mimosine were not actually cycling; rather, they may have been partially or completely arrested in G_0 as a result of nutritional or serum

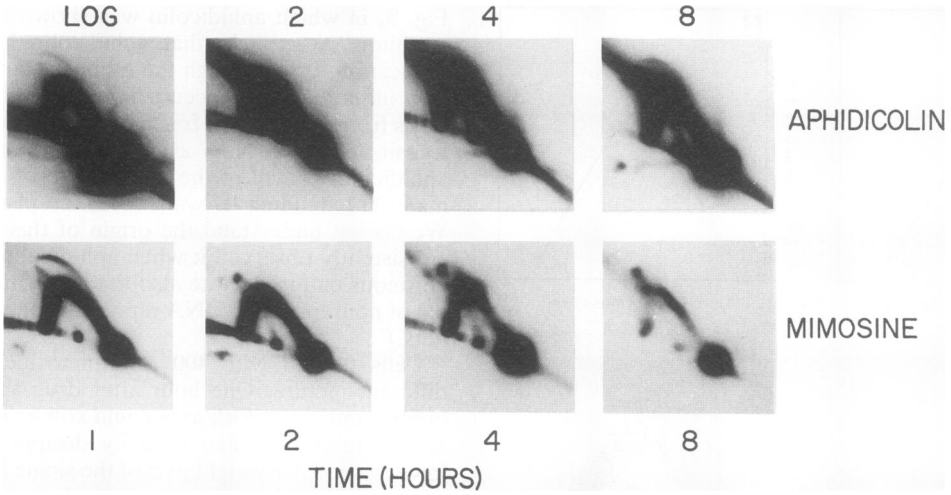

FIG. 9. Evidence that replication intermediates are prevented from maturing in the presence of aphidicolin but not mimosine. Either aphidicolin (30 μM) or mimosine (400 μM) was added to asynchronously growing cells for the indicated time periods, and replication intermediates were then isolated as previously described. After digestion with *Eco*RI, the replicating DNA fraction from each sample was separated on a 2-D gel, the digest was transferred to a filter, and the filter was hybridized with a probe specific for the 6.2-kb *Eco*RI fragment that contains the ori-β region. Note that because of limitations on the number of samples that can be processed at once, there is no 1-h sample for aphidicolin. Note also that replication intermediates in the log-phase sample were not as pure as those in the other samples, resulting in a much larger contribution to the 1*n* nonreplicating spot.

deprivation either prior to or during incubation with mimosine (the FACS analyses presented in Lalande's report indeed suggest that very few cells were in the S phase in cultures that were supposed to be asynchronous [11]). After addition of mimosine in fresh medium, the cells would then have advanced across G_1 and collected at the beginning of S. In initial experiments in our own laboratory, we occasionally experienced a similar phenomenon, but when measures were taken to ensure that cells were not inadvertently presynchronized by nutritional deprivation or plating from confluent stocks, we were not able to quantitatively arrest cells in G_1 with mimosine (e.g., Fig. 3).

Lalande further concluded that mimosine acts at a point about 2 h prior to the S period, since there was a 2-h lag after mimosine was removed before cells entered S. However, given the arguments presented above and the uptake studies presented in Fig. 2B, the lag that he observed after removal of mimosine probably represents slow efflux of the drug rather than a legitimate interval between a mimosine-sensitive step and the onset of the S period. At the lowest drug level tested in Fig. 2B (133 μM), replication is completely inhibited, yet when the drug is removed, the population enters the S period without a significant delay. It therefore appears that mimosine acts at a step very close to the beginning of the S period, possibly at initiation per se.

Finally, mimosine was suggested to act prior to aphidicolin (a known chain elongation inhibitor), since when aphidicolin-blocked cells were released into medium containing mimosine, they proceeded into the S period, as monitored by FACS analysis during the next 4 h (11). However, in a similar experiment on CHOC 400 cells, we observed that in cells released from aphidicolin into mimosine, DNA replication was completely inhibited by mimosine, but only after ~3 h (Fig. 6B).

At this juncture, therefore, one could conclude that mimosine is just another drug to be added to an already lengthy list of S-phase inhibitors, with the disadvantages of being slow to equilibrate intracellularly and slow to wash out. But several further observations related to the lag observed before mimosine exerts its effect argue that Lalande has, in fact, come upon an extremely interesting and unique agent that is quite different from the typical chain elongation inhibitor.

There is no doubt that there is a legitimate problem getting mimosine into cells rapidly; transport studies with [^{3}H]mimosine demonstrate that it takes more than 30 min for the drug to reach steady-state intracellular levels (12a). In addition, there could be a lengthy rate-limiting processing step or steps that might be required to convert mimosine to an active form.

Nevertheless, the [^{3}H]thymidine uptake data obtained when mimosine is added to log-phase cells show that the drug does reduce uptake significantly after only 1 h (although uptake is reduced completely only after ~3 h; Fig. 4). This finding raises the question of why it takes almost 3 h for mimosine to show a measurable effect when it is administered to cells released into the S period from an aphidicolin block (Fig. 6). This slow-stop phenotype is a characteristic of bacterial mutations in genes affecting initiation at ori*C* (10), and it suggested to us that mimosine might actually inhibit initiation at mammalian origins.

Some support for this suggestion was obtained in the experiment in which mimosine was added back to cells at different times in the S period to determine whether there was a time beyond which it had no effect (i.e., after the last initiations had occurred in the mid- or late S period) (Fig. 7). Although this experiment is somewhat indeterminate because of the 30- to 60-min lag before mimosine equilibrates in cells and exerts a measurable effect, the data in Fig. 7 (as well as those obtained in other experiments in our laboratory) suggest that mimosine has little effect on [^{3}H]thymidine uptake after the sixth or seventh hour of the S period in the CHOC 400 cell line. This coincides roughly with the time that the rate of DNA replication begins to fall off (Fig. 2A), undoubtedly because the number of replication forks in operation begins to decrease. This, in turn, must be due

largely to a decrease in the rate at which new initiations occur at origins.

The strongest argument that mimosine is not simply a chain elongation inhibitor comes from the 2-D gel analyses on synchronized and on log-phase cells (Fig. 8 and 9). When CHOC 400 cells are arrested in G_0 and then released into complete medium containing mimosine for 14 h, virtually no initiation can be detected in the DHFR initiation locus by 2-D gel analysis (Fig. 8) (4). In contrast, cells released from a G_0 block into high levels of aphidicolin undergo significant initiation and escape synthesis (Fig. 8) (4), as would be expected of a leaky chain elongation inhibitor.

Furthermore, when mimosine is added to asynchronous cultures, replication bubbles present in a fragment from the DHFR initiation locus prior to drug addition are allowed to mature out of the fragment, as are single forks passing through the fragment from outside origins (Fig. 9). However, no new initiations occur within the fragment in the presence of mimosine and fewer and fewer forks enter the fragment from outside, suggesting that all initiation is effectively prevented by the drug. Intriguingly, there appears to be a narrow zone in the approximate center of the 4.3-kb *Xba*I fragment at which forks are slowed or stopped for a lengthy interval in the presence of mimosine, but we do not understand the significance of this pause site.

In contrast, aphidicolin causes little change in the pattern of replication intermediates detected in the DHFR initiation locus (Fig. 9). As pointed out earlier, however, there must be a slow flux of replication forks through this locus in the presence of aphidicolin, since it is clearly a very leaky chain elongation inhibitor (Fig. 3).

At present, we have no idea what mimosine may actually do at the molecular level. One can speculate that the drug binds to a protein that directly interacts with origins of replication, locking the protein into a conformation that prevents initiation. Alternatively, mimosine could have a secondary or higher-order effect, inhibiting the activity of a protein that ultimately modulates the activity of an origin-binding protein.

Regardless of its mode of action, mimosine is clearly superior to aphidicolin for obtaining cells that enter the S period synchronously, and it should prove very useful in a variety of studies related to events occurring at the G_1/S boundary as well as in early S phase.

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REFERENCES

1. **Anachkova, B., and J. L. Hamlin.** 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. **9**:532–540.
2. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins in ARS plasmids in S. cerevisiae. Cell **51**: 463–471.
3. **Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis.** 1990. Identification of an origin of bidirectional replication in mammalian chromosomes. Cell **62**:955–965.
4. **Dijkwel, P. A., and J. L. Hamlin.** 1992. Initiation of DNA replication in the dihydrofolate reductase locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. Mol. Cell. Biol. **12**:3715–3722.
5. **Dijkwel, P. A., J. P. Vaughn, and J. L. Hamlin.** 1991. Mapping replication initiation sites in mammalian genomes by two-dimensional gel analysis: stabilization and enrichment of replication intermediates by isolation on the nuclear matrix. Mol. Cell. Biol. **11**:3850–3859.

5a. **Hamlin, J. L.** Unpublished data.

6. **Handeli, S., A. Klar, M. Meuth, and H. Cedar.** 1989. Mapping replication units in animal cells. Cell **57**:909–918.
7. **Heintz, N. H., and J. L. Hamlin.** 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. USA **79**:4083–4087.
8. **Heintz, N. H., J. D. Milbrandt, K. S. Greisen, and J. L. Hamlin.** 1983. Cloning of the initiation region of a mammalian chromosomal replicon. Nature (London) **302**:439–441.
9. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. **32**:327–337.
10. **Kornberg, A.** 1980. DNA replication, p. 374–375. W. H. Freeman and Co., San Francisco.
11. **Lalande, M.** 1990. A reversible arrest point in the late G_1 phase of the mammalian cell cycle. Exp. Cell Res. **186**:332–339.
12. **Leu, T.-H., and J. L. Hamlin.** 1989. High resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation. Mol. Cell. Biol. **9**:523–531.

12a. **Mosca, P. J.** Unpublished data.

13. **Moscovitis, G., and A. B. Pardee.** 1980. Citric acid arrest and stabilization of nucleoside incorporation into cultured cells. Anal. Biochem. **101**:221–224.
14. **Nawotka, K. A., and J. A. Huberman.** 1988. Two-dimensional gel electrophoretic method for mapping DNA replicons. Mol. Cell. Biol. **8**:1408–1413.
15. **Owens, G. K., P. S. Rabinovitch, and S. M. Schwartz.** 1981. Smooth muscle cell hypertrophy versus hyperplasia in hypertension. Proc. Natl. Acad. Sci. USA **78**:7759–7763.
16. **Skoog, L., and B. Nordenskjold.** 1971. Effect of hydroxyurea and 1-β-D-arabinofuranosyl-cytosine on deoxyribonucleotide pools in mouse embryo cells. Eur. J. Biochem. **19**:81–89.
17. **Tobey, R. A., and H. A. Crissman.** 1972. Preparation of large quantities of synchronized mammalian cells in late G_1 in the pre-DNA replicative phase of the cell cycle. Exp. Cell Res. **75**:460–464.
18. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell **61**:1075–1087.
19. **Wang, T.-F.** 1991. Eukaryotic DNA polymerases. Annu. Rev. Biochem. **60**:513–552.