The Dual Effect of Mimosine on DNA Replication

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The plant amino acid, mimosine, is an extremely effective inhibitor of DNA replication in mammalian cells, but the mechanism by which this inhibition is achieved is unknown. The drug has been proposed either to inhibit initiation at origins of replication or to inhibit chain elongation by lowering nucleotide pool levels. In an attempt to determine which mode of action is correct, we have analyzed its effects on SV40 DNA replication. Using a two-dimensional gel replicon mapping technique, we show that mimosine completely inhibits incorporation of [3H]thymidine into viral DNA, but only after ~4 h. Qualitative analysis of replication intermediates during this interval suggests that the drug partially inhibits both initiation and elongation, and pulse-chase experiments support this contention. The drug has no effect when added directly to an SV40 in vitro replication extract. However, extracts prepared from cells pretreated with mimosine are compromised in their ability to support replication in vitro in the presence of a full complement of nucleotides. Thus, although mimosine may alter nucleotide pool levels in vivo, it also appears to affect one or more essential replication proteins.

INTRODUCTION

One shortcoming that hampers attempts to identify and characterize origins of replication in mammalian chromosomes is the inability to synchronize cells at the beginning of the S period prior to the actual initiation step. In a typical synchronizing regimen, cells are first arrested in G0 and are then released from the block in the presence of an inhibitor of DNA synthesis (e.g., aphidicolin [1] or hydroxyurea [2, 3]); after the entire population has had time to reach the G2/S boundary, the drug is removed and cells enter the S period in a parasynchronous wave. However, this protocol is not completely satisfactory for studying initiation of replication at the beginning of the S period because of the inefficiency of these inhibitors. For example, the initial rate of replication upon release was shown to increase as the length of the block increases, suggesting that progressively more origins are allowed to fire in the presence of these drugs ([4]; J. L. Hamlin, unpublished observations). In addition, when the amplified CHO dihydrofolate reductase (DHFR) origin was analyzed by a two-dimensional (2-D) gel replicon mapping method, replication intermediates were clearly visible prior to release from aphidicolin or hydroxyurea [5]. Finally, fluorescence-activated cell sorter analysis showed that cells enter and progress slowly through S-phase in the presence of hydroxyurea or aphidicolin after long incubation times [5, 6].

Mimosine was originally reported to inhibit cells in the late G1 period [7–9] and thus represented a potentially superior synchronizing agent. Indeed, when we examined mimosine's effect on the DHFR origin in CHO cells, replication intermediates could not be detected on 2-D gels prior to drug release in synchronized cells [4–6]. Furthermore, the initial rate of replication upon release extrapolated back to 0 regardless of the duration of the block [4]. Both observations suggest that mimosine prevents entry into the S period, which is consistent with the suggestion that it inhibits a late G1 event [7–9]. However, other data made it clear that mimosine also inhibits S phase events. For example, when added to asynchronous CHOC 400 cells with a population doubling time of 20 h, mimosine completely inhibited [3H]-thymidine incorporation within ~2.5 h [6], which cannot be explained by an effect solely in late G1. When the DHFR locus in these mimosine-treated cells was examined on 2-D gels, the small replication bubbles that characterize initiation events were observed to mature in the presence of the drug, but no new bubbles were established [5, 6]. Therefore, we proposed that mimosine might inhibit initiation at origins of replication.

However, replication forks also appeared to move slowly in the presence of mimosine, since the majority did not clear from the DHFR locus for ~4 h after drug addition and a small amount could be detected even 8 h later [5, 6]. Thus, mimosine also appeared to affect...
chain elongation in addition to (or instead of) initiation. Since mimosine is known to chelate iron [10] and to bind pyridoxal phosphate [11, 12], both of which are cofactors for enzymes involved in nucleotide biosynthesis, the possibility arose that mimosine might inhibit elongation by lowering nucleotide pools. In fact, two recent studies have shown that nucleotide levels are depressed in cells treated with mimosine [13, 14]. Because DNA replication could be restored by the addition of iron to the medium [14, 15], it was speculated that the iron-requiring enzyme ribonucleotide reductase might be the target of mimosine. However, we have shown that concentrations of iron or copper that bind equal amounts of mimosine are equally efficient at rescuing replication, even though copper cannot substitute for iron in ribonucleotide reductase [16].

To identify the target of mimosine action, we developed a photochemical cross-linking strategy and were able to demonstrate specific binding of [3H]mimosine to a 50-kDa polypeptide (p50; [15]). p50 was subsequently sequenced and shown to correspond to serine hydroxymethyltransferase (SHMT), an enzyme involved in the penultimate step in thymidine biosynthesis [17]. However, SHMT activity was not inhibited when the drug was added to the enzyme in vitro, under conditions in which mimosine could be cross-linked to SHMT [17; P. J. Mosca, unpublished observations].

Therefore, the binding of mimosine to SHMT and the lowering of deoxynucleotide pools both suggest that mimosine exerts at least some of its inhibitory effect by inhibiting chain elongation. However, inhibition of chain elongation has never been demonstrated directly. Furthermore, these data do not explain why the drug appears to prevent the formation of replication forks (i.e., initiation) when present at the beginning of the S period.

To explore the possibility that mimosine has more than one target in DNA replication, we have examined its effects on SV40 DNA replication. With the exception of the viral T antigen, which is an origin-binding protein and a helicase (reviewed in [18]), viral replication relies solely on host cell proteins, many of which have been purified and characterized (reviewed in [19, 20]). Thus, it should be possible to determine whether mimosine inhibits any host proteins required for initiation and/or chain elongation.

We show that mimosine inhibits SV40 replication in vivo in a manner consistent with effects on both initiation and chain elongation. Although mimosine has no effect on SV40 replication when added directly to an in vitro assay, extracts from cells pretreated with mimosine are compromised in their ability to support viral replication in vitro. The possibility of multiple targets may explain why mimosine is such an efficacious replication inhibitor and suggests that it may be a promising candidate for chemotherapeutic regimens.

### MATERIALS AND METHODS

**Cell culture.** CHOC 400, CV-1, and HeLa cells were maintained as monolayer cultures in minimal essential medium supplemented with nonessential amino acids (MEM; Gibco/BRL) and 10% bovine serum product (Hyclone Laboratories) and were grown in an atmosphere of 5% CO2.

**Viral propagation and preparation of viral replication intermediates.** CV-1 cells were transfected with SV40 viral DNA (Bethesda Research Laboratories, BRL) using Lipofectin (BRL), and a stock was prepared by established procedures [21, 22]. For experiments, ~0.5 PFU/cell was adsorbed to confluent 15-cm plates of CV-1 cells in 10 ml MEM containing 1% serum for 1 h at 37°C, after which additional medium was added. Experiments began 24 h after infection (Time 0). Viral DNA was isolated at the indicated times by the Hirt procedure [23], except that the lysis (Modified Hirt) buffer contained 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, 10 mM NaCl, and 0.6% SDS supplemented with 100 μg/ml Proteinase K (E. M. Laboratories) just prior to use. Replication intermediates were prepared from Hirt supernatants exactly as described [24]. After separation on 2-D gels, the digests were transferred to Hybond-N+, sprayed with ENHANCE (Dupont/NEN), and exposed to X-ray film. The same transfers were then washed with toluene and hybridized with viral DNA labeled with [32P]dCTP by random priming [25]. Hybridization and washing procedures were described previously [4, 26].

**Radiolabeling procedures.** For measuring overall replication rates in cells or in the viral DNA in Hirt supernatants, the labeling cocktail consisted of 5 Ci/ml [3H]thymidine (81.3 Ci/mmol; Dupont/NEN) in normal growth medium supplemented with 0.2 μg/ml unlabeled thymidine. Incorporation into TCA-insoluble material was determined as previously described [6]. For labeling viral replication intermediates to be analyzed on 2-D gels, the medium on each plate was aspirated and replaced with MEM containing 20 μCi/ml [3H]thymidine, 0.2 μg/ml unlabeled thymidine, and 1% serum. Labeling was quenched by aspirating the labeling cocktail, washing with ice-cold phosphate-buffered saline, and adding modified Hirt buffer (see above).

**Pulse-chase experiments.** Twenty-four hours after virus infection, CV-1 cells were treated with mimosine for 2.5 h and were then pulsed for 3 min with 50 μCi/ml [3H]thymidine. Label was then chased for increasing amounts of time in medium containing 1 μg/ml unlabeled thymidine. DNA was prepared as for 2-D gel experiments but the BND–cellulose chromatography step was omitted. After digesting with BamHII (BRL), equal amounts of radioactivity from viral DNA fractions were separated on a 1.5% agarose gel poured in water, equilibrated, and run in 40 mM NaOH/1 mM EDTA at 1 V/cm for 20 h at 4°C. The DNA was transferred to Hybond-N+ by the alkaline method [27] and the transfer was sprayed with ENHANCE and exposed to X-ray film. The resulting autoradiogram was scanned with a Personal Densitometer and Image Quant software from Molecular Dynamics in the University of Virginia Academic Computing Center.

**In vitro replication.** Extracts were prepared from asynchronous subconfluent monolayers of HeLa cells (or from cells treated for various times with 400 μM mimosine) by a modification of a published procedure [28]. Briefly, cells were trypsinized and washed once in complete medium and twice in isotonic buffer. Swelling was effected in a hypotonic buffer, and cells were lysed by forcing the suspension through a 21-gauge needle. After centrifugation to remove nuclei and debris, aliquots were frozen in liquid nitrogen and stored at −70°C. Each assay contained 50 ng supercoiled pUC HSO DNA [29], 100 μg extract protein, 500 ng affinity-purified T antigen, 7 mM MgCl2, 4 mM ATP, 50 μM rNTPs, 2 μCi [32P]dCTP (3000 Ci/mmol; NEN/Du- pont), 25 μM dCTP, 100 μM each dATP, dGTP, and dTTP, 375 μg creatine phosphate, 2.5 μg creatine kinase, and 30 mM Hepes buffer, pH 7.8. Mimosine was added directly to some reactions in concentrations ranging from 1 to 8 mM. Incubations were carried out at 37°C.
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FIG. 1. Kinetics of inhibition of DNA synthesis by mimosine. (A) Uninfected CV-1 cells or cells infected for 24 h were treated with 400 μM mimosine at Time 0. At the indicated times thereafter, individual wells were labeled for 20 min with a solution containing 5 μCi/ml [3H]thymidine and 0.2 μg/ml cold thymidine. At the end of the pulse period, incorporation was quenched with citric acid and incorporation into cellular DNA in uninfected cells was determined as previously described [6]. Incorporation into viral DNA was determined by counting ethanol precipitates of the low-molecular-weight DNA fraction. The resulting values were expressed as a percentage of the incorporation into drug-free controls growing in the same cluster dish. (B) HeLa cells were treated with 400 μM mimosine (open circles) or 1 mM hydroxyurea (filled diamonds) at Time 0, and at the indicated times, wells were pulse-labeled and harvested as in A. In each panel, data points represent the average of duplicate or triplicate samples performed in a single, representative experiment and are plotted at the midpoint of the pulse period. Error bars indicate the standard deviation.

for the indicated times. Reactions were terminated by adding 4 vol of stop buffer (20 mM EDTA, 0.25% SDS), and TCA-precipitable material was recovered on Whatman GF-C filters for scintillation counting. In addition, aliquots from selected samples were prepared for electrophoresis as follows. Samples were treated with 50 μg/ml Proteinase K at 37°C for 30 min prior to extraction with phenol and chloroform/isoamyl alcohol. Ethanol-precipitated DNA was redissolved in TE and digested with EcoRI and DpnI prior to electrophoresis on 1% agarose gels. Visualization and quantitation were carried out with a phosphorimager and Image Quant software (Molecular Dynamics).

RESULTS

Mimosine inhibits the incorporation of radiolabeled thymidine into viral and chromosomal DNA with similar kinetics. We showed previously that when 400 μM mimosine is added to Chinese hamster cells, the rate of [3H]thymidine incorporation into DNA declines gradually to 0 over a period of ~2.5 h [6]. As shown in Fig. 1, 400 μM mimosine has a similar effect when added to either CV-1 African Green Monkey cells (Fig. 1A, open circles) or human HeLa cells (Fig. 1B, open circles). One possible explanation for the slow kinetics of inhibition is that only initiation is inhibited, and thus forks already in progress continue toward their destination. This is the phenotype displayed by temperature-sensitive Escherichia coli initiation mutants [30]. Were this the only mechanism of action of mimosine, then a smaller replicon should display more rapid inhibition kinetics. To test this hypothesis, CV-1 cells were infected with SV40, a eukaryotic DNA tumor virus that completes a single round of replication in about 10 min during lytic infection [31]. Mimosine was added 24 h after infection when viral replication is beginning its exponential phase ([32] and data not shown), and the rate of [3H]thymidine incorporation into the low-molecular-weight DNA fraction was determined. As shown in Fig. 1A (filled circles), incorporation is completely inhibited with kinetics very similar to those observed for cellular replication (open circles). It has also been shown that mitochondrial DNA replication in CHO cells is inhibited by mimosine with similar kinetics [13].

Mimosine affects the rate of chain elongation. Therefore, both the modes of initiation and the replication times of genomic, mitochondrial, and viral replicons are different, but the inhibition curves obtained with each replicon are similar. Based on these observations, it has been suggested that mimosine effects chain elongation rather than initia-
FIG. 2. Mimosine treatment depresses the rate of chain elongation in virus-infected cells. Twenty-four hours after infection with SV40, CV-1 cells were treated with 400 μM mimosine for 2.5 h. Mimosine-treated cultures and drug-free controls were pulsed for 3 min at 37°C with a solution containing 50 μCi/ml [3H]thymidine. The medium was rapidly aspirated and cells were washed and then incubated with prewarmed medium containing 1 μg/ml thymidine for the indicated times. After isolation of low-molecular-weight DNA and digestion with BamHI (which cuts directly opposite the origin), an equal amount of radioactivity from each sample was separated on an alkaline agarose gel and the DNA was transferred to Hybond-N. The transfer was sprayed with EN3HANCE and exposed to X-ray film (A). Each lane in a shorter exposure of the same blot was scanned with a densitometer. The arbitrary optical density units contained in the bracketed region (representing approximately full-length viral single-stranded DNA) were determined and expressed as a percentage of the label in the entire lane (B). Open circles, no drug; filled circles, mimosine-treated.

The relative slopes of the rate curves obtained from control and mimosine-treated samples (Fig. 2B) show that the rate of chain elongation is inhibited by ~35% after 2.5 h in mimosine, a time when the rate of [3H]-thymidine incorporation into viral DNA is inhibited by 60% and cellular DNA by 80% (Fig. 1A and data not shown). In a similar experiment performed 2 h after the addition of mimosine, the rate of elongation in nascent chains that had already initiated was also diminished by ~35% (data not shown). Thus, mimosine slows the rate of chain elongation, but not to an extent that can explain its overall effect on the rate of [3H]-thymidine incorporation into viral or cellular DNA (as in Fig. 1). Unfortunately, we were not able to conduct this experiment at longer times after adding mimosine, when the degree of inhibition is greater, because of the low level of [3H]-thymidine incorporated in the short pulse time required by this experiment. Similarly, aphidicolin and hydroxyurea could not be used as controls, since the stronger block to elongation almost completely inhibits...
The majority of viral replication intermediates have disappeared by 3 h after mimosine addition, as assessed by either ethidium bromide staining (Fig. 4, top), intrinsic labeling (middle), or by hybridization (bottom). Although the level of intermediates is greatly reduced by 3 h, those that remain are in the form of a bubble arc and, interestingly, a more prominent single fork arc (evident in the probed sample at the bottom). The pulse-labeling patterns (middle) show that the number of initiation events (i.e., the amount of labeled bubble arcs) decreases dramatically from 1 to 3 h in the presence of mimosine, but longer film exposures of the 3-h sample show that a low level of initiation is still occurring (data not shown). The continued presence of a small number of replication intermediates is predicted by the small decrease in the rate of chain elongation observed in cells treated with mimosine (Fig. 2).

Inhibition of replication by aphidicolin and hydroxyurea, but not by mimosine, leads to long-lived broken bubble structures. The appearance of a single fork arc in the presence of mimosine may offer a clue as to the mechanism of action of this inhibitor. One possibility is that the drug induces breakage of replication bubbles, which could then migrate like bona fide single replication forks, as has been suggested previously [35]. This would explain the disappearance of replication bubbles from the DHFR initiation locus when mimosine (but not aphidicolin or hydroxyurea) is added to asynchronous CHOC 400 cells [5, 6]. To investigate this possibility further, the effects of mimosine, aphidicolin, and hydroxyurea on SV40 replication intermediates were compared. The latter two agents provoke an immediate and almost complete inhibition of $[^{3}H]$thymidine incorporation in a variety of cell types [36, 37], as well as in SV40 (Fig. 1). Furthermore, aphidicolin has been shown to destabilize SV40 replication intermediates in vivo [38].

Cells infected with SV40 for 24 h were treated with each of these drugs for various time intervals, and replication intermediates were then prepared and analyzed by the 2-D gel replicon mapping method (Fig. 5). In the sample treated with aphidicolin for 2 h, a novel arc is detected below the bubble arc, which migrates below the typical single fork arc (illustrated diagrammatically in Fig. 3). In hydroxyurea, a more complex pattern is observed consisting of the bubble arc, a typical single fork arc, and the novel arc. We recently demonstrated that this novel arc corresponds to a replication bubble that has suffered a single nick at one of the four branch points [24]. In contrast, a 2-h treatment with mimosine does not have a dramatic effect on the pattern of replication intermediates (incorporation of $[^{3}H]$thymidine into viral DNA is inhibited by only ~50% at this time point; Fig. 1A).

By 6 h, samples treated with any of the three drugs
FIG. 4. Effect of mimosine on the pattern of viral replication intermediates. CV-1 cells were infected with SV40 and, 24 h later, 400 μM mimosine was added (Time 0). At the indicated times, samples were pulsed for 10 min with a solution containing 20 μCi/ml [3H]thymidine and 0.2 μg/ml cold thymidine and were harvested immediately thereafter. The low-molecular-weight DNA fraction was prepared and digested with PstI and the samples were separated on a 2-D gel. The ethidium bromide staining pattern was photographed (top), and the gel was then blotted to Hybond-N+ (Amersham). The transfer was sprayed with EN3HANCE (Dupont/NEN) and exposed to X-ray film for 14 days (middle). The fluor was then removed by washing with toluene, and the blots were hybridized with viral DNA labeled with [32P]dCTP (bottom) as previously described [4, 26]. The bubble arc arises from the 4-kb origin-containing fragment, while the signal in the lower right corner represents the top of a termination arc that characterizes the additional 1-kb PstI fragment containing the terminus region of the viral genome.

Display markedly fewer replication intermediates (note longer film exposure times in legend to Fig. 5). The spectrum of forms in aphidicolin- and hydroxyurea-treated cells largely mimics that observed at 2 h. The pattern of replication intermediates in the 6-h mimosone sample (Fig. 5, bottom) is almost identical to that observed 3 h after addition of mimosine (compare to Fig. 4, bottom); i.e., a faint bubble arc and a more prominent single fork arc still persist, but the novel broken bubble arc is undetectable. Note that the 6-h mimosine sample is comparable to the 2-h aphidicolin and hydroxyurea samples, since in all three samples, [3H]-thymidine incorporation has been completely inhibited for 2 h (Figs. 1A and 1B; and data not shown).

The single fork arc could arise because of unidirectional initiation from the SV40 origin, which has been shown to occur in cellular DNA when replication is partially inhibited (e.g., in the presence of protein synthesis inhibitors; [39]). An alternative possibility is that replication intermediates become nicked when DNA synthesis is inhibited; if the intact branch point of a broken bubble continues to replicate by a rolling circle mechanism, then a restriction digest of such a structure would generate a bona fide single fork. The presence of broken bubbles and/or fork arcs in samples treated with each of the three inhibitors shows that, indeed, destabilization of SV40 replication intermediates does occur when chain elongation is inhibited. Therefore, the ratio between broken bubble arcs and fork arcs can be used as a rough indicator of the rate of chain elongation in the presence of these inhibitors. By this argument, aphidicolin is a good elongation inhibitor, since only broken bubbles are observed, whereas mimosine (and to a lesser extent hydroxyurea) allows enough chain elongation to convert a broken bubble into a single fork by rolling circle replication (Fig. 5). Mimosine affects in vitro SV40 replication when extracts are prepared from mimosine-treated cells. We have shown that mimosine inhibits chain elongation to some extent (Fig. 2), which could result from lowered nucleotide pools. However, the magnitude of this effect is less than that of aphidicolin or hydroxyurea (Fig. 5), implying that mimosine should be even more leaky than either of these two agents. Why, then, is mimosine a much more effective inhibitor of DNA synthesis when added to cells crossing the G1/S transition? Since an effect only on elongation cannot explain all of the results obtained in previous studies in CHO C 400 cells, it is likely that mimosine also affects initiation.
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FIG. 5. Inhibition of replication by aphidicolin and hydroxyurea, but not by mimosine, leads to long-lived broken bubble structures. CV-1 cells were infected with SV40 and, 24 h later, 10 μg/ml aphidicolin (APH), 1 mM hydroxyurea (HU), or 400 μM mimosine (MIMO) was added to individual 15-cm plates. Samples were harvested 2 and 6 h after drug addition, and the low-molecular-weight DNA fractions were prepared. After digestion with PstI, separation on a 2-D gel, and transfer to Hybond-N +, viral intermediates were detected by hybridizing with a 32P-labeled viral probe. The 2-h samples (top) were exposed for 6±12 h and the 6-h samples (bottom) for 2–3 days (exposures were adjusted so that the 1n spots were approximately equal).

Protein extracts from primate cells support semi-conservative replication on SV40 origin-containing plasmid templates in vitro, provided that T antigen is added to the reaction mixture. This system allows an examination of any direct effect of mimosine on enzyme activity (by adding the drug to the in vitro reaction) or indirectly by preparing extracts from cells treated with mimosine. In these studies, extracts were prepared from asynchronous HeLa cells, which are a more potent source of replication proteins than CV-1 cells ([40]; and R. F. Kalejta, unpublished observations). Mimosine inhibits DNA synthesis in HeLa cells (Fig. 1B) with the same slow-stop phenotype that characterizes its effect on CV-1 cells (Fig. 1A) and on CHOC 400 cells [6].

When 1 mM mimosine is added directly to an in vitro replication extract prepared from HeLa cells, the rate of SV40 replication is not detectably slowed even 2 h after drug addition (Fig. 6A). The same result was obtained when longer reaction times and higher mimosine concentrations (up to 8 mM mimosine) were tested (data not shown). Thus, it appears that the drug does not directly inhibit any of the replication proteins that are limiting in these extracts.

However, when extracts are prepared from cells that are treated with mimosine for various time intervals in vivo, replication potential is depressed (Fig. 6B). After only 1 h in mimosine, when the in vivo replication rate is reduced by ~40% (Fig. 1B), the amount of total viral DNA synthesized in vitro is depressed by ~25%; after a 2-h treatment, when the in vivo rate is depressed by ~85%, both the initial rate and the extent of replication are inhibited by ~60%. After 6 h, when the in vivo replication rate has been completely depressed for ~2 h, the amount of SV40 replicated in vitro is inhibited by 70%. As with controls, extracts prepared from cells pretreated with mimosine in vivo are not further inhibited by mimosine in vitro (Fig. 6A, triangles). An effect on nucleotide pools can explain the inability of mimosine to inhibit replication when added directly to an in vitro reaction ([14]; and Fig. 6), but does not explain why extracts prepared from cells pretreated with mimosine are compromised for replication in vitro in the presence of a full complement of nucleotides. To illustrate this point, we tested extracts prepared from cells treated with hydroxyurea, an agent known to inhibit replication by nucleotide depletion.

As shown in Fig. 6C, pretreatment with 1 mM hydroxyurea in vivo for either 1 or 3 h had only a small effect (~15%) on SV40 replication in vitro, even though DNA synthesis was completely inhibited for virtually the entire pretreatment interval (Fig. 1B). This finding suggests that when replication is inhibited for lengthy intervals with any drug, replication proteins may be degraded, leading to a decrease in replication potential.
The replication potential of extracts is inhibited when cells are pretreated with mimosine in vivo. (A) HeLa cells were treated for 4 h in vivo either with drug-free medium or with 400 μM mimosine. Extracts were then prepared and tested in an in vitro replication reaction with an SV40 origin-containing plasmid, T antigen, and [32P]dCTP (open symbols). A subset of the reactions additionally received 1 mM mimosine (filled symbols). The amount of DNA synthesized was determined as described under Materials and Methods. (B) HeLa cells were treated with mimosine in vivo for the indicated times, and extracts were prepared and used in in vitro replication reactions. Samples were taken at 0.5, 0.75, 1, 2, and 4 h in vitro. (C) Extracts were prepared from HeLa cells treated either with no drug (circles) or with hydroxyurea for 1 h (squares) or 3 h (diamonds) in vivo.

This phenomenon is observed to occur in mimosine, where the 4- and 6-h samples (Fig. 6B) show less replication than the 3-h sample, even though mimosine reduces replication in tissue culture cells by >95% by 3 h. However, the magnitude of the inhibition by mimosine during the first 3 h of pretreatment suggests that this agent has a specific effect on replication proteins, as opposed to a general time-dependent degradation.

We also determined the relative distributions among semi-conservative and repair DNA synthesis in the in vitro replication assays. A time-course study was performed using extracts from untreated or mimosine-treated cells and [32P]dCTP incorporation was determined for each time point (data not shown). Additional aliquots from the 4-h sample were analyzed by gel electrophoresis after linearization with EcoRI and digestion with DpnI. The latter enzyme does not digest hemi-methylated or unmethylated DNA that would result from one or two rounds of semi-conservative replication in the in vitro assay; however, DpnI would cleave template molecules that have undergone repair synthesis but that still maintain doubly methylated, unreplicated patches. The distributions of DpnI-resistant and -sensitive DNA in these samples were determined from autoradiographs with a phosphorimager, and total incorporation after 4 h in vitro was normalized to the total number of counts in the drug-free control.

Table 1 shows that after a 4-h incubation in vitro, overall incorporation was decreased by 45% in extracts from mimosine-treated cells. In addition, there was a slight decrease in the relative percentage of semi-conservative DNA replication (as opposed to repair). One conclusion from this data is that semi-conservative replication is more sensitive to mimosine than repair. However, it is possible that the nicking activity observed in vivo in cells treated with any of the three drugs (Fig. 5) is also present in the extracts. Thus, increased repair might result from the presence of more nicks or gaps in the template DNA.

DISCUSSION

The effects of mimosine have been studied in several different contexts. Mimosine was originally proposed to inhibit cells in the late G1 period [7–9], based on fluorescence-activated cell sorter analysis of cell cycle distributions after administration to an asynchronous cell population.

### TABLE 1

<table>
<thead>
<tr>
<th>% Incorporation</th>
<th>% Semi-conservative</th>
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<tbody>
<tr>
<td>Control (no drug)</td>
<td>100</td>
</tr>
<tr>
<td>Mimosine-treated</td>
<td>43</td>
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Note. In vitro reactions were carried out with extracts from non-treated cells or from cells treated in vivo with 400 μM mimosine for 2.5 h. Aliquots were removed from the in vitro reactions for scintillation counting. Total incorporation after 4 h in vitro is expressed as the percentage of one of the 4-h control samples. Additional aliquots were processed, digested with EcoRI and DpnI, run on a gel, blotted, and visualized with a phosphorimager. The signal from the full-length (DpnI-resistant, semi-conservatively replicated) band was determined as a percentage of the total signal in the lane.
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Mimosine has also been shown to have no effect when added directly to a variety of other in vitro replication systems in which nucleotides are supplied in excess, prompting the suggestion that it may inhibit some aspect of nucleotide biosynthesis [13, 14, 43]. Furthermore, we have shown that mimosine inhibits replication after a lag period when administered to cells that are well into the S period [6].

Thus, while it is difficult to exclude the possibility that mimosine may have an effect in late G1, there is little doubt that mimosine is an S phase inhibitor as well. The question is whether it affects elongation, initiation, or both. We believe that mimosine cannot simply be an inhibitor of chain elongation analogous to hydroxyurea or aphidicolin by the following arguments.

When either aphidicolin or hydroxyurea is delivered to cells traversing the G1/S boundary, neither drug is able to prevent initiation and a significant amount of chain elongation from occurring, as assessed on 2-D gels, [3H]thymidine incorporation, or fluorescence-activated cell sorter analysis [4; J. L. Hamlin, unpublished observations].

In contrast, when cells are released from a G0 block and are collected at the beginning of the S period with mimosine, replication forks cannot be detected on 2-D gels prior to release [4–6]. Furthermore, the initial rate of replication after removal of mimosine extrapolates back to 0 regardless of the length of the block [4]. When mimosine is added to an asynchronous cell population, bubble arcs disappear from the DHFR locus before fork arcs, which eventually clear (albeit very slowly) [5, 6]. Based on these properties, we have suggested that mimosine acts before the hydroxyurea- and/or aphidicolin-sensitive steps and may inhibit initiation of DNA replication.

If, indeed, mimosine affects replication differently than do aphidicolin and hydroxyurea, then what is its mechanism of action? Since mimosine is a much more efficient inhibitor of replication overall, and if its only activity were to lower nucleotide pools, it should inhibit elongation much more effectively than hydroxyurea. However, we show here that both hydroxyurea and aphidicolin are superior chain elongation inhibitors, reducing the rate of chain elongation by more than 95%, while mimosine slows chain elongation by only 35% at a time when the overall rate is inhibited by more than 60–80% (Figs. 1 and 2B). Thus, mimosine must have additional effects in vivo, which could be mediated by a protein involved in DNA replication. Effects of mimosine on SV40 in vitro reactions support this contention: although the drug has no effect when added directly to an in vitro reaction, extracts prepared from cells treated with the drug have a markedly reduced replication potential in vitro. Since inhibition is only partial, however, mimosine must have more than one inhibitory activity in vivo, only one of which is recapitulated in the SV40 replication assay in vitro. The existence of multiple cellular targets could explain why mimosine is such an effective inhibitor of cellular DNA replication.

A very recent study also supports the contention that mimosine has multiple targets in vivo. p21 is an inhibitor of cyclin-dependent kinases, which are required to stimulate cell cycle progression (see [44] for recent review). Cyclin-dependent kinase inhibitors such as p21 block these signaling pathways and their overproduction can result in cell cycle arrest [45]. It was shown that mimosine treatment leads to an increase in p21 mRNA and protein levels [46]. Furthermore, there was a concomitant decrease in cyclin-E-associated kinase activity, which is usually elevated at the G1/S boundary. Interestingly, however, in cells lacking p21, DNA replication was still inhibited by mimosine to 75% of the level in p21-containing cells, arguing that different cyclin-dependent kinases or other factors may also be targeted by mimosine.

Several other potential mediators of mimosine's effects on mammalian cells have been identified. In our own laboratory, we have demonstrated that mimosine can be photochemically cross-linked both in vivo and in vitro to serine hydroxymethyltransferase (SHMT), the enzyme that catalyzes the penultimate step in thymidine biosynthesis [17]. Furthermore, in extracts from cell lines that are deficient in SHMT, cross-linking to the 50-kDa polypeptide cannot be detected [15, 47]. These data support the suggestion that mimosine may function solely by lowering nucleotide pools [13, 14]. However, this straightforward interpretation is complicated by the fact that mimosine has no effect on the activity of SHMT when added directly to the enzyme in vitro in circumstances in which binding to mimosine is clearly detectable [17]. Thus, it is conceivable that...
mimosine could affect allosteric interactions between or among SHMT and other enzymes intimately involved in establishing and/or propagating the replication fork. In this scenario, one effect of mimosine could be to prevent replication forks from being established once the helix is melted at an origin.

Interestingly, the gene product of a thermosensitive E. coli mutant (dnaR) with an initiation defect has also been shown to be involved in nucleotide metabolism [48–50]. The mutation was shown to map in the prs gene, which encodes phosphoribosylpyrophosphate (PRPP) synthetase, an enzyme involved in the biosynthesis of purine and pyrimidine nucleotides, NAD, histidine, and tryptophan [51]. The synthesis of chromosomal, but not λ phage, DNA is inhibited at the restrictive temperature, showing that a lack of nucleotides per se is not sufficient to explain the defect in initiation at oriC [48]. Initiation of chromosomal replication in the dnaR mutant resumes after a return to the permissive temperature, but only after a substantial lag. Well before DNA synthesis resumes, PRPP synthetase activity reaches its normal cellular level, again suggesting that lowered nucleotide pool levels alone cannot explain the defect. This lag could conceivably result from the requirement to reestablish a multi-enzyme replication complex prior to initiating nascent chains.

Finally, in addition to its effect on DNA replication, mimosine has been shown to inhibit deoxyhypusyl hydroxylase and thus to prevent the formation of hypusine, a rare amino acid found in the eukaryotic initiation factor eIF-5a [9]. This protein is involved in the translation of messenger RNAs and thus could be involved in the regulation of progression through G1. If all of the many potential targets of mimosine turn out to be real ones, it might explain why mimosine is such an efficacious inhibitor of passage into and progression through the S period.

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