Magnusson, G., Craig, R., Narkhammar, M., Riechard, P., Staub, M., & Warner, H. R. (1975) *Cold Spring Harbor Symp. Quant. Biol. 34*, 227–233.
Manteuil, S., Kopecka, H., Caroux, J., Drunell, A., & Girard, M. (1974) *J. Mol. Biol. 90*, 751–756.
Ortiz, P. J. (1972) *Biochem. Biophys. Res. Commun. 46*, 1728–1733.
Pica Mattoccia, L., & Roberti, S. (1974) *Biochem. Biophys. Res. Commun. 60*, 882–893.
Pica Mattoccia, L., & Sorrentino, R. (1979) *J. Cell. Physiol. 101*, 17–24.
Rigby, P. W. J., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.
Skoog, L., & Nordenskjold, B. (1971) *Eur. J. Biochem. 19*, 81–89.
Tseng, B. Y., & Goulian, M. (1975) *J. Mol. Biol. 99*, 317–337.
Wahl, G. M., Stern, M., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3683–3687.
Waqar, M. A., Burgoyne, L. A., & Atkinson, M. R. (1971) *Biochem. J. 121*, 803–809.
Wist, E., & Prydz, H. (1979) *Nucleic Acids Res. 6*, 1583–1589.
Woodcock, D. M., & Cooper, I. A. (1979) *Exp. Cell Res. 123*, 157–166.
Zahn, R., Muller, W., Forster, W., Maidhof, A., & Beyer, R. (1972) *Eur. J. Cancer 8*, 391–396.

In Vivo Effects of Cytosine Arabinoside on Deoxyribonucleic Acid Replication in Chinese Hamster Ovary Cells. 2. Cytosine Arabinoside Affects the Rate of Synthesis but Not the Pattern of Labeling of an Amplified Chromosomal Sequence at the Onset of the S Period†

Nicholas H. Heintz and Joyce L. Hamlin*

ABSTRACT: The effect of 1-β-D-arabinofuranosylcytosine (ara-C) on DNA replication in methotrexate-resistant Chinese hamster ovary cells was examined under circumstances in which nuclear DNA synthesis could be distinguished from mitochondrial DNA synthesis. G1-arrested cells were induced to traverse G1 and enter the S phase in the presence of radiolabeled thymidine and various concentrations of the drug. ara-C did not affect the kinetics of G1 traverse and subsequent entry into S after release from isoleucine deprivation, as measured by autoradiography. However, the inhibitor reduced the net rate of thymidine incorporation into nuclear DNA in a dose-dependent fashion. Autoradiography of nuclear matrix–DNA halo structures suggests that the drug inhibits nuclear thymidine incorporation by slowing chain elongation and movement of newly replicated DNA through a matrix-bound replication apparatus. Southern blot analysis of restriction digests of DNA radiolabeled in early S in the presence of ara-C indicates that the synthesis of the early-replicating amplified dihydrofolate reductase domain in these cells begins at sequences identical with those observed in cells synchronized with aphidicolin or hydroxyurea. Progressively lower concentrations of ara-C permit proportionately greater extents of the amplified unit to be replicated. These results suggest that ara-C slows the rate of chain elongation without altering the site at which DNA replication is initiated within individual replicons.

1-β-D-Arabinofuranosylcytosine (ara-C)[1] is a commonly employed inhibitor of DNA replication that has been shown to be incorporated directly into DNA strands in vivo (Graham & Whitmore, 1970; Zahn et al., 1972; Kufe et al., 1980) and has been proposed to act as a leaky chain terminator that slows the rate of chain elongation (Cozzarelli, 1977). It has also been suggested that the drug preferentially inhibits replicon initiation (Fridland, 1977) and may induce rereplication of certain chromosomal segments (Woodcock & Cooper, 1979).

In our laboratory we have shown that ara-C effectively prevents incorporation of radiolabeled thymidine into nuclear DNA but has negligible effect on the replication of mitochondrial DNA (mtDNA) in either Chinese hamster ovary (CHO) cells or CHO cells lacking nuclear thymidine kinase activity (CHO TK$^-$). The preferential labeling of mtDNA in the presence of ara-C is of considerable importance, since we and others have found that nuclear DNA preparations are invariably contaminated with significant quantities of mtDNA. We consider it likely, therefore, that inadvertent contamination with mtDNA has caused misinterpretation of experimental observations concerning the mechanism of action of ara-C. In order to help clarify the action of this drug on DNA synthesis, we have examined its effects under two circumstances in which nuclear DNA synthesis can be distinguished from mtDNA synthesis.

In the first approach, we examined the effect of the drug on the grain distribution over individual nuclear matrix–DNA halo structures prepared from cells labeled during entry into the S period. Several studies have shown that DNA replication occurs at a central salt-resistant nuclear matrix in a variety of eukaryotic cell types (Dijkwell et al., 1979; McCready et al., 1980; Pardoll et al., 1980). The matrix and its associated DNA can be visualized by relaxing supercoiled DNA with

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received August 11, 1982; revised manuscript received February 9, 1983.* Supported by National Institutes of Health Grant GM26108 and a Basil O'Connor grant from the March of Dimes. J.L.H. is a recipient of an American Cancer Society Faculty Research Award, and N.H.H. was supported by an NIH postdoctoral fellowship.

[1] Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; MTX, methotrexate; DHFR, dihydrofolate reductase; DBM, diazobenzoxymethyl; mtDNA, mitochondrial DNA; ELF, early-labeled fragment; NP-40, Nonidet P-40; kb, kilobase pairs.

0006-2960/83/0422-3557$01.50/0

ethidium bromide and ultraviolet light, thereby releasing nuclear DNA into a halo surrounding the central matrix (Vogelstein et al., 1980). Autoradiography of matrix–halo structures after pulse-labeling experiments suggests that DNA moves away from the matrix as it is replicated (McCready et al., 1980; Vogelstein et al., 1980). We have therefore examined the effect of ara-C on the distribution of grains over individual matrix–halo structures. An advantage to this approach is that it permits the examination of the effects of ara-C on individual cells.

In the second approach, we have determined the action of ara-C on the pattern of labeling of specific early-replicating restriction fragments arising from an amplified chromosomal sequence. We have established a methotrexate-resistant CHO cell line, CHOC 400, that contains approximately 1000 copies of a 135-kb early-replicating sequence that includes the gene for dihydrofolate reductase (DHFR) (Milbrandt et al., 1981). This amplified sequence can be visualized on ethidium bromide stained gels as a series of 25–30 over-molar restriction fragments superimposed against the background smear of unique-sequence DNA. The amplified DHFR genes present in CHOC 400 cells have been located in several homogeneously staining chromosomal regions by in situ hybridization (Milbrandt et al., 1981). These regions have been shown to be early replicating in retroactive labeling studies (Hamlin & Biedler, 1981; Milbrandt et al., 1981). Restriction endonuclease analysis of DNA labeled in synchronized cells indicates that initiation of replication of the DHFR domains occurs within a specific subset of the restriction fragments derived from this region and that replication of the entire 135-kb repeat appears to proceed in a predetermined order (Heintz & Hamlin, 1982). Molecular cloning and mapping experiments have shown that these early-labeled fragments are colinear in the genome (Heintz et al., 1983) and are derived from a single region 20-kb from the 3′-end of the DHFR gene (N. H. Heintz and J. L. Hamlin, unpublished results). Thus, CHOC 400 cells provide a useful model system in which the effects of various inhibitors on the synthesis of a defined chromosomal sequence can be analyzed.

In the present study, the effects of ara-C on the pattern of labeling of both nuclear matrix–DNA halo structures and the amplified sequences in DHFR domains were examined by inducing CHOC 400 cells to enter the S phase in the presence of radiolabeled thymidine and various concentrations of the drug. By examining the action of ara-C at a time when cells would be in the early S period, we hoped to detect effects on initiation and/or chain elongation.

Materials and Methods

Reagents. ara-C was obtained from Sigma. [*methyl*-^{14}C]Thymidine (50–60 mCi/mmol) was from Amersham; [*methyl*-^{3}H]thymidine (77 Ci/mmol) was from New England Nuclear. Aphidicolin was a gift of Boots Pure Drug, Ltd. (Buckingham, England).

Cells and Cell Culture. CHOC 400 cells resistant to 0.8 mM methotrexate were obtained by stepwise selection as previously described (Milbrandt et al., 1981) and were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids, 10% donor calf serum, 400 μg of MTX/mL, and 50 μg of gentamycin sulfate/mL. Eagle's medium lacking isoleucine was reconstituted from Selectamine kits (Gibco) and was supplemented with 10% dialyzed fetal bovine serum. Media and other supplies were from Gibco. Synchronization of CHOC 400 cells with aphidicolin or hydroxyurea was as previously described (Heintz & Hamlin, 1982).

DNA Preparation, Restriction Digestion, Gel Electrophoresis, and Southern Transfer. DNA was prepared from nuclei by hypotonic lysis in Nonidet P-40 as previously described (Heintz & Hamlin, 1983). DNA samples were digested with restriction enzymes under the conditions suggested by the supplier (Bethesda Research Laboratories). DNA concentration was determined fluorometrically with Hoechst 33258 (American Hoechst Corp.) by the method of Labarca & Paigen (1980). Aliquots of radioactive samples were dissolved in Ready-Solv EP (Beckman) prior to scintillation counting.

Restriction digests of DNA were separated on agarose gels and were transferred to activated ABM paper (Schliechter and Schuell) by the method of Wahl et al. (1980). Autoradiograms of ^{14}C-labeled DNA on these blots were developed after 4–6 weeks exposure of Kodak X-Omat XR-5 film at −70 °C.

Preparation and Autoradiography of Nuclear Matrices. Nuclear matrices with surrounding halos of DNA were prepared from experimental cultures by using graded salt extractions and ethidium bromide–UV nicking, exactly as described (Vogelstein et al., 1980). To facilitate handling of labeled cells, cultures were plated on 1-cm diameter circular coverslips in Falcon P60 dishes.

Autoradiography of air-dried matrices was performed by dipping coverslips mounted on glass slides in NTB2 liquid emulsion (Kodak) diluted with an equal volume of 0.6 M sodium acetate. After an air-drying, the emulsion was exposed for 3–7 days at 4 °C. The autoradiographs were developed in D19 (Kodak) for 3 min, were rinsed in deionized water, and were fixed for 5 min with Kodafix. Slides were rinsed again for 10 min prior to staining for 15 min in a 1:50 dilution of Giemsa. Photomicrographs of matrix autoradiographs were taken with a Model BH Olympus microscope equipped with a Polaroid camera.

Statistical Analysis of Grain Distribution over Matrix–Halo Structures. The area delimited by silver grains over individual matrix–halo structures was determined by planimetry with equivalently enlarged photomicrographs of the original autoradiographs. The areas of control and ara-C-treated structures were compared by using a multirange Student's two-tailed t test as described by Duncan (1955).

Results

Effect of ara-C on G1 Traverse and Entry into S Period. CHOC 400 cells were collected in early G1 by starvation for the essential amino acid isoleucine as previously reported (Heintz & Hamlin, 1982). After 50 h in medium lacking isoleucine, less than 0.5% of CHOC 400 cell nuclei are labeled by a 2-h pulse with [^{3}H]thymidine. When the G1 block is reversed by the addition of complete medium containing isoleucine, cells enter the S period in a semisynchronous wave with kinetics identical with those of CHO cells (Heintz & Hamlin, 1983). As in CHO cells, when ara-C is present in the restoration medium at the time of release from the G1 block, the rate at which CHOC 400 cells traverse G1 is not affected by any dose of drug tested up to 10 μg/mL. Although the drug does not affect the rate at which cells enter S (measured as percent labeled nuclei), the grain density over each nucleus is reduced with increasing ara-C concentration (data not shown). In experiments described below, cultures were harvested at 12 h after the restoration of complete medium, at which time 90% of the cells have entered S in the absence or presence of the drug.

Effect of ara-C on Incorporation of [^{14}C]Thymidine into CHOC 400 DNA. Figure 1 depicts the effect of ara-C on [^{14}C]thymidine incorporation into CHOC 400 DNA labeled for 12 h after release from the G1 block. As we have discussed

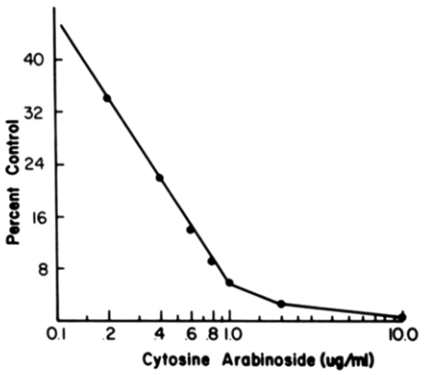

FIGURE 1: Effect of ara-C on incorporation of [^{14}C]thymidine into DNA of CHOC 400 cells. Cultures were arrested in G1, as described under Materials and Methods, and were released from the block by adding complete medium containing 1 μCi of [^{14}C]thymidine/mL and the indicated concentrations of ara-C. Samples were processed for scintillation counting as described and are expressed as percent of label incorporated per microgram of DNA relative to a culture labeled in the absence of ara-C.

in the preceding paper (Heintz & Hamlin, 1983), this inhibition curve represents a composite effect on both nuclear and mitochondrial DNA replication. As in CHO cells, nuclear DNA synthesis in CHOC 400 cells is inhibited by ara-C in a dose-dependent fashion at concentrations up to approximately 1.0 μg/mL, although CHOC 400 cells are somewhat less sensitive to ara-C than are CHO cells [see Figure 1B in Heintz & Hamlin (1983)]. At higher doses, a refractile component is detected that we have identified as mtDNA synthesis (Heintz & Hamlin, 1983). We have identified the specific restriction fragments arising from the 15.8-kb circular mitochondrial genome whose synthesis is refractile to ara-C inhibition and thus can distinguish the labeling of nuclear and mitochondrial restriction fragments in experiments described below.

Effect of ara-C on Labeling of Nuclear Matrix–DNA Halo Structures. In the previous paper, DNA labeled in CHO cells upon entry into S in the presence of ara-C was characterized by alkaline sucrose gradient sedimentation. ara-C has complex effects on the sedimentation profile of nascent DNA, but a primary effect appeared to be inhibition of chain elongation (i.e., net reduction in all size categories). Sedimentation of CHOC 400 DNA labeled under identical conditions yielded similar results (data not shown).

In order to further examine the action of ara-C on chain elongation in CHOC 400 cells, we studied the effect of the drug on the labeling pattern of individual nuclear matrix–DNA halo structures. Fluorescence micrographs of salt-washed nuclei and of matrix–halo structures are pictured in Figure 2. It can be seen that the area of fluorescent DNA increases approximately 5-fold when the supercoiled nuclear DNA is relaxed by ethidium bromide staining and nicking with ultraviolet light (compare Figure 2A to Figure 2B), but the size of the central nuclear core remains the same. The central salt-resistant portion of these cellular structures is referred to as the nuclear matrix or scaffold (Berezney & Coffey, 1977; Comings & Okada, 1976; Cook et al., 1976; Hodge et al., 1977). ara-C has no effect on the size or shape of the fluorescent DNA halos or on the matrix itself (data not shown).

In order to examine the effect of ara-C on the labeling of DNA associated with these structures, cells were arrested in G1 by isoleucine deprivation and were then released from this block by restoration of complete medium with or without ara-C. When matrices are prepared from cells at 12 h after

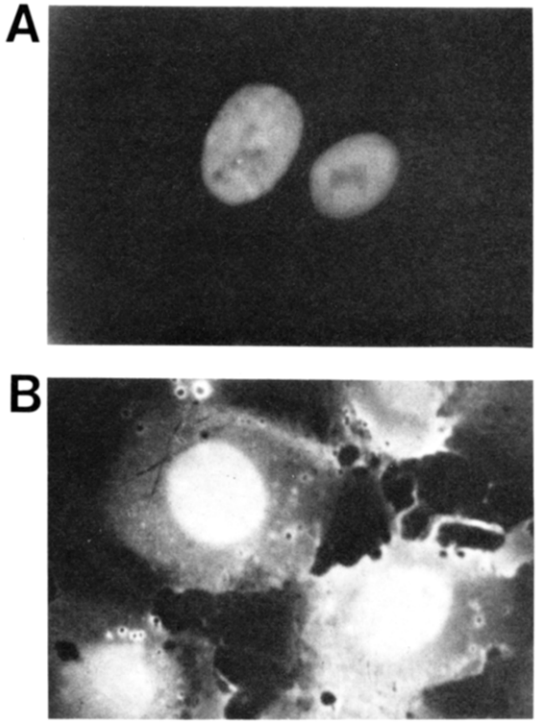

FIGURE 2: Fluorescence micrographs of nuclei and nuclear matrix–DNA halo structures. (A) CHOC 400 cells were trypsinized, and nuclei were prepared by hypotonic NP-40 treatment. Nuclear preparations were spread and air-dried on microslides and were stained for 10 min with 1 μg of ethidium bromide/mL of water. (B) CHOC 400 cells growing on circular coverslips were subjected to NP-40, followed by increasing salt concentrations (to 2 M NaCl). The preparations were then stained with 100 μg of ethidium bromide/mL of high salt buffer (Vogelstein et al., 1980) for 1 min and were then irradiated for 2 min by inverting the coverslip over a U.V. Products C-61 subilluminator. The coverslips were then subjected to decreasing concentrations of salt and were finally washed with water and air-dried. The preparations were then restained with 1 μg of ethidium bromide/mL of water. The above procedure is described in detail in the paper by Vogelstein et al. (1980). Micrographs were taken at 400-fold magnification under ultraviolet epi-illumination with exciter filter UG-1 and barrier filter 530 on an Olympus Model BH research microscope.

reversal of the G1 block, a time at which 90% of cells have entered S, a high density of silver grains covers the entire area of each matrix–halo structure (Figure 3A). As indicated in Table I, the area delimited by grains in the absence of ara-C is equivalent to that of matrix–halo structures visualized by fluorescent microscopy. Under these conditions, greater than 50% of the cells have been in S for at least 4 h (Figure 1A), and all of the restriction fragments derived from the amplified DHFR sequence are labeled in addition to unique-sequence DNA (see below).

If ara-C is added to the culture medium at the time of release from the G1 block, CHOC 400 cells traverse G1 and enter S with the same kinetics (data not shown). Autoradiographs of matrix–halo structures from cells subjected to this treatment show a progressive reduction in the area covered by silver grains as the concentration of ara-C increases (Figure 3). We have measured the areas covered by silver grains in these autoradiographs by planimetry, including only the area of relatively uniform grain density over each structure. Statistical analysis of these autoradiographs (Table I) shows that concentrations of ara-C greater than or equal to 0.5 μg/mL significantly reduce labeling of DNA released into the peripheral halo. This is not simply due to reduction in the specific activity of the DNA. For example, structures labeled with 100-fold less [^{3}H]thymidine do not show a significant reduction in the area delimited by grains, even though the specific activity

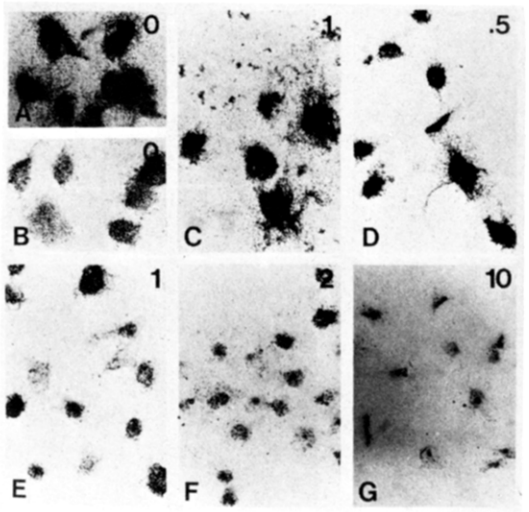

FIGURE 3: Effect of ara-C on labeling of nuclear matrices. CHOC 400 cells were collected in G1 by isoleucine deprivation. Twelve hours after release from the G1 block, matrix–halo structures were prepared and autoradiographed as described. (A) The distribution of silver grains over matrix–halo structures labeled with 1.0 μCi/mL [^{3}H]-thymidine (50 Ci/mmol) with no ara-C added. (B) Same as (A) except that cells were labeled with only 0.01 μCi/mL [^{3}H]thymidine. (C–G) Effect of ara-C at the indicated concentrations (μg/mL) on grain distribution over matrix–halo structures labeled with [^{3}H]-thymidine for 12 h after release from the G1 block. All figures were photographed at 200-fold magnification.

Table I: Statistical Analysis of Effect of ara-C on Labeling of Matrix-Halo Structures[a]

sample	mean radius	standard deviation	n	significance
fluorescent halos	0.510	±0.023	10	NS
control A (1.0 μCi/mL)	0.545	±0.041	15	NS
control B (0.01 μCi/mL)	0.478	±0.062	16	NS
0.1 μg of ara-C/mL	0.451	±0.049	14	NS
0.5 μg of ara-C/mL	0.300	±0.025	23	$P < 0.01$
2.0 μg of ara-C/mL	0.245	±0.017	17	$P < 0.01$
10.0 μg of ara-C/mL	0.183	±0.006	25	$P < 0.01$

[a] CHOC 400 cells entering the S phase were labeled with [^{3}H]thymidine in the presence or absence of ara-C, and matrix–halo structures were prepared and autoradiographed as described in the legend to Figure 3. The area delimited by silver grains in photomicrographs of autoradiographs of matrix–halo structures (as depicted in Figure 3) was determined by planimetry as described in the text. The areas of control structures labeled in the presence of 1.0 μCi/mL [^{3}H]thymidine (control A) were compared to those of ethidium bromide stained halos visualized by fluorescence microscopy (fluorescent halos) and to structures labeled in 0.01 μCi/mL [^{3}H]thymidine (control B) by using a Student's two-tailed t test (Duncan, 1955). The controls were found not to differ significantly (P greater than 0.1), and the pooled average (510 ± 47) was used for comparison to each group of ara-C-treated samples by using the multirange comparison t test of Duncan (1955). Areas are expressed in arbitrary units; n indicates the number of structures analyzed in each group.

of the DNA is reduced to 7% of the control (Table I, control B, and Figure 3, panel B). In contrast, DNA labeled in the presence of 0.5 μg/mL ara-C has a higher specific activity (nearly 13% of the control), and yet the area delimited by grains over matrix–halo structures at this concentration of ara-C is significantly reduced (Table I).

In addition to an effect on the area delimited by silver grains, ara-C also appears to reduce the number of grains per unit area as the concentration of drug increases (Figure 3). It is not possible to quantitate the number of grains over matrix–halo structures at all drug doses in a single autoradiographic exposure. However, it is clear that the grain densities at high

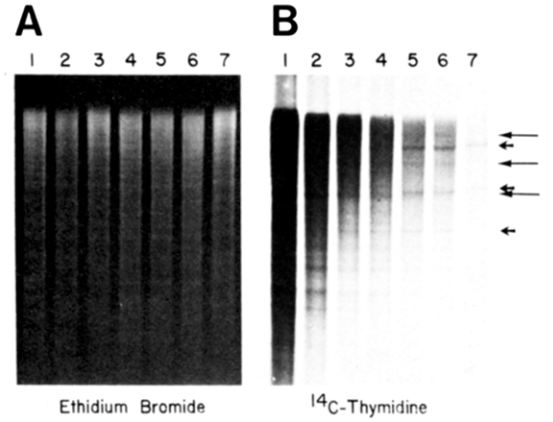

FIGURE 4: Effect of ara-C on pattern of labeling of amplified sequence in CHOC 400 cells. Cultures were labeled for 12 h after release from the G1 block in the presence of 1 μCi of [^{14}C]thymidine/mL and various concentrations of ara-C. High molecular weight DNA was prepared and was digested with *Eco*RI, and the digests were separated on a 0.8% agarose gel. After staining with ethidium bromide, the gel was photographed by ultraviolet subillumination (A). The DNA was then transferred to DBM paper and was autoradiographed on XR-5 film for 3 weeks (B). 2.5 μg of DNA was loaded in each well. Large arrows indicate ELFs at 11.5, 6.1, and 4.2 kb, as discussed in the text. Small arrows indicate the position of the mtDNA *Eco*RI fragments at 8.4, 4.4, and 3.0 kb. ara-C concentrations for lanes 1–7 in both photographs are 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 μg/mL of medium, respectively.

levels of ara-C (i.e., 1, 2, and 10 μg/mL, Figure 3) are significantly reduced from those observed at 0.1 and 0.5 μg of ara-C/mL. This result suggests that ara-C may also have an effect on replicon initiation, as indicated by alkaline sucrose density gradient studies (Heintz & Hamlin, 1983). The pattern of synthesis under these conditions is consistent with the notion that DNA is synthesized at the matrix and moves away from the matrix as synthesis proceeds, in agreement with the observations of Vogelstein et al. (1980). Our data further suggest that ara-C acts by limiting the rate of polymerization of nascent DNA at a matrix-bound replication apparatus, thus preventing movement of DNA (grains) into the peripheral halo. Although the decreasing grain density per unit area with increasing ara-C concentration suggests that the number of replicons in operation may be decreased by ara-C, it is not possible to determine if this effect is secondary to an inhibition of chain elongation.

Effect of ara-C on Replication Pattern of Amplified DHFR Domains. From studies on individual matrix–DNA halo structures, ara-C appears to affect chain elongation and possibly initiation of replicons. To further examine these possibilities, we studied the effect of ara-C on the synthesis of the amplified DHFR domains in methotrexate-resistant CHOC 400 cells by allowing cultures to enter S in the presence of the drug and [^{14}C]thymidine. The distribution of label in CHOC 400 DNA was assessed by Southern blotting of restriction digests followed by autoradiography.

Figure 4A shows the ethidium bromide staining pattern of CHOC 400 DNA synthesized in the presence or absence of ara-C. Digestion of these samples with *Eco*RI yields approximately 30 distinct over-molar bands that arise from the amplified DHFR domain (Milbrandt et al., 1981). We have estimated that the sum of the lengths of the 30 fragments is approximately 135 kb. We have shown that initiation of replication in this region occurs within a subset of these bands, which we have termed early-labeled fragments (ELFs) (Heintz & Hamlin, 1982). These fragments are derived from a single region 30 kb from the 3′-end of the DHFR gene and represent sequences including and surrounding the origin of replication

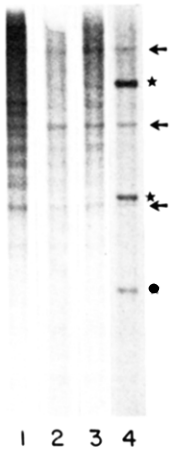

FIGURE 5: Comparison of fragments synthesized in the presence of a high level of ara-C with ELFs detected in early S in synchronized cells. CHOC 400 cells growing in 60-mm dishes were labeled with 1 μCi of [^{14}C]thymidine/mL as follows: (Lane 1) An exponential culture was labeled for 60 min, and DNA was prepared. (2) Cells were arrested in G1 as described and were released into complete medium containing 5 μg of aphidicolin/mL of medium for 12 h. The medium was then replaced with fresh medium containing 1 μCi of [^{14}C]thymidine/mL, and cells were harvested 40 min later. (3) This is the same as in (2) above except that 1 mM hydroxyurea replaces aphidicolin. (4) Cells were labeled with 1 μCi of [^{14}C]thymidine and 2.5 μg of ara-C/mL for 12 h after release from the G1 block. DNA was prepared as described and was digested with *Eco*RI. Digests were separated on a 0.8% agarose gel, transferred to DBM paper, and autoradiographed. Each well contained 2.5 μg of DNA. Arrows indicate position of ELFs at 11.5, 6.1, and 4.2 kb. Asterisks designate mtDNA fragments.

associated with each amplified domain (Heintz et al., 1983). The positions of the early-labeled restriction fragments generated by *Eco*RI digestion are indicated by large arrows in Figure 4B.

ara-C has no obvious effect on the ethidium bromide staining pattern of the amplified DHFR domain at any concentration examined (Figure 4A). In contrast, ara-C affects the distribution of [^{14}C]thymidine throughout the spectrum of restriction fragments derived from the amplified sequence (Figure 4B). At low doses, the entire spectrum of fragments is labeled (Figure 4B, lane 2). As the dose of ara-C increases, however, labeling becomes progressively limited to a set of discrete fragments derived from both nuclear and mitochondrial sequences. Synthesis of fragments previously identified as mitochondrial in origin (indicated by small arrows) does not appear to be significantly affected by increasing concentrations of ara-C (compare lanes 4–7 in Figure 5B). However, synthesis of the fragments derived from the amplified chromosomal DHFR domains shows a dose-dependent sensitivity to the drug, and labeling of these sequences becomes simplified and eventually appears to be predominantly restricted to the early-labeled fragments as the concentration of ara-C increases (large arrows, Figure 4B).

In order to confirm this suggestion, we compared DNA labeled in the presence of a high concentration of ara-C (2.5 μg/mL) to that labeled during the first 40 min of S in synchronized CHOC 400 cells (Figure 5). When CHOC 400 cells are labeled during the first 40 min of the S period after release from either aphidicolin or hydroxyurea, a characteristic subset of the amplified bands (arrows) is labeled more intensely than the remainder of the fragments derived from the amplified domain (compare lanes 2 and 3 to the log-labeled DNA in lane 1, Figure 5). When CHOC 400 cells enter S in the presence of 2.5 μg of ara-C/mL, labeling is limited to this same set of fragments, in addition to those derived from mitochondrial DNA (Figure 5, lane 4). This result supports the notion that ara-C acts to slow chain elongation and, therefore, progressively restricts labeling to fragments containing or flanking origins of DNA synthesis. Furthermore, ara-C does not appear to effect the position at which DNA synthesis initiates in this region. Note that in the aphidicolin and hydroxyurea samples, the prominently labeled ELFs are accompanied by fainter bands that represent sequences flanking the initiation locus. These bands are not significantly labeled in the presence of ara-C, however, indicating that the drug has effectively limited replication to regions very close to and including the initiation locus. If restriction endonuclease analysis under these labeling conditions were not complicated by the presence of mitochondrial DNA, the use of ara-C for labeling early-replicating sequences would be superior to either aphidicolin or hydroxyurea.

Discussion

Cytosine arabinoside is known to inhibit nuclear DNA replication in mammalian cells but has little effect on mtDNA synthesis. We have found the presence of mtDNA in standard nuclear DNA preparations to complicate studies on the action of ara-C on nuclear DNA synthesis. In order to obviate this problem, we have examined effects of the drug under two conditions in which nuclear DNA synthesis can be unambiguously monitored. We examined the effect of ara-C on the rate of movement of DNA away from its site of synthesis on the nuclear matrix. In addition, we studied the effect of ara-C on the synthesis of the highly amplified chromosomal sequence that includes the DHFR gene in MTX-resistant CHOC 400 cells.

In recent years, a number of studies have examined the residual structures obtained from nuclei by extraction with nonionic detergents and 2 M NaCl. The matrix has been identified as the site of DNA replication by a variety of means (McCready et al., 1980; Vogelstein et al., 1980; Dijkwel et al., 1979). These studies have suggested models in which DNA is replicated by moving through a matrix-associated replication complex (Pardoll et al., 1980; McCready et al., 1980; Dijkwel et al., 1979). If ara-C acts as a chain terminator, one would predict that as cells enter the S phase, the drug should inhibit labeling of the peripheral DNA halo in matrix–halo structures. Indeed, autoradiography of individual matrix–halo structures prepared from cells entering S in the presence of both radiolabeled thymidine and ara-C clearly shows that the drug slows the rate of movement of labeled DNA away from the central core (Figure 3). This is consistent with the suggestion that ara-C acts directly on the process of chain elongation. At high doses of the drug, replication of DNA appears to be limited to sequences close to or associated with the matrix. It is under these conditions that synthesis of the amplified DHFR domains is limited to early-replicating fragments, implying that origins of DNA synthesis are associated with specific sites on the matrix during the onset of S, as we have previously proposed (Hamlin et al., 1982).

Although mitochondrial DNA has not been located in these experiments, we have previously shown the ara-C-sensitive portion of cellular DNA synthesis to be nuclear in origin. Thus, the changes in patterns of grain distribution generated under these conditions are undoubtedly due to chromosomal DNA synthesis. In addition, we have observed no effect of ara-C on the physical appearance of matrix–halo structures, as judged by fluorescent microscopy. This agrees with the observation that ara-C does not affect association of DNA with the salt-resistant nuclear core in sedimentation studies (Dijkwel et al., 1979).

Although autoradiography of matrix–halo structures permits examination of the effect of ara-C on DNA replication in individual cells, studies of the effects of the drug on the replication of the amplified DHFR domains in CHOC 400 cells permits examination of its effect on specific nuclear sequences. Analysis of the synthesis of the restriction fragments derived from the DHFR domains shows that ara-C does not alter the site at which DNA replication is initiated (Figures 4 and 5). At high doses of drug, synthesis of only those regions known to replicate in the earliest part of S is permitted. This suggests that, as chain elongation is inhibited, synthesis proceeds slowly away from origins into flanking sequences. Lower doses of the drug, which permit greater extents of chain elongation to occur, allow sequences distal to initiation sites to be replicated. The order of synthesis under these conditions appears to be the same as that observed in synchronized cells, in that later-replicating portions of the amplified repeats are only synthesized in low doses of ara-C (data not shown). Although we do not as yet have a complete restriction map of the amplified unit, preliminary mapping studies are consistent with this observation.

The differential effect of ara-C on the synthesis of early- and late-replicating portions of the amplified units implies that initiation of replication is not the primary event sensitive to ara-C. If initiation were especially susceptible to the action of the drug, one would expect the synthesis of ELFs to be preferentially inhibited. In fact, at high doses of drug, these sequences are the *only* portions of the amplified units to be labeled. However, one component of the action of ara-C may be at the level of initiation, since the signals obtained from ELFs in these experiments are reduced, as is the grain density per unit area over matrix–halo structures in high doses of the analogue.

The results of these studies support the notion that ara-C acts as an inefficient chain terminator whose presence at the 3′-terminus of nascent DNA inhibits the polymerization of the subsequent nucleotide, as suggested by Cozzarelli (1977). The extent of inhibition of the rate of nuclear DNA synthesis could therefore depend on the number of residues of ara-C incorporated per unit length of DNA synthesized. The apparent effect of ara-C on initiation may be explained in two ways: (1) complete synthesis of certain replicons may be required in order for initiation of subsequent replicons to occur; or (2) unidirectional synthesis of a portion of one strand at the origin may be required prior to the onset of bidirectional replication. Precedents for both possibilities exist. First, a number of studies have shown that a temporal order of synthesis occurs in eukaryotes [for review, see Hand (1978)]. Second, unidirectional synthesis has been shown to precede bidirectional replication in both SV40 viral (Hay & DePhamphilis, 1982) and mitochondrial (Clayton, 1982) DNA replication. If ara-C inhibits elongation of the strand whose synthesis is necessary for bidirectional replication to proceed, then an effect on initiation, albeit secondary to chain elongation, would be observed.

In summary, we have distinguished the effects of ara-C on nuclear DNA replication from its effects on mtDNA synthesis. Our results are consistent with the suggestion that the primary mode of action of the drug is at the level of chain elongation, and we believe its effects on initiation of replication may be secondary to inhibition of chain elongation. ara-C does not alter the sites at which DNA replication is initiated within the amplified DHFR domains in CHOC 400 cells, nor does it seem to change the order in which DNA within individual replicons is synthesized.

Acknowledgments

We thank Boots Pure Drug, Ltd., for its gift of aphidicolin, Steve Rothman for help with photography, and Michael Johnson for statistical analysis of data.

Registry No. ara-C, 147-94-4.

References

Berezney, R., & Coffey, D. A. (1977) *J. Cell Biol. 73*, 616–637.

Clayton, D. A. (1982) *Cell (Cambridge, Mass.) 28*, 693–705.

Comings, D. E., & Okada, T. A. (1976) *Exp. Cell Res. 103*, 341–360.

Cook, P. R., Brazell, I. A., & Jost, E. (1976) *J. Cell Sci. 22*, 303–324.

Cozzarelli, N. R. (1977) *Annu. Rev. Biochem. 46*, 641–648.

Dijkwel, P. A., Mullenders, L. H. F., & Wanka, F. (1979) *Nucleic Acids Res. 6*, 219–230.

Duncan, D. B. (1955) *Biometrics 11*, 1–42.

Fridland, A. (1977) *Biochem. Biophys. Res. Commun. 74*, 72–78.

Graham, F. L., & Whitmore, G. F. (1970) *Cancer Res. 30*, 2627–2635.

Hamlin, J. L., & Biedler, J. L. (1981) *J. Cell. Physiol. 107*, 101–107.

Hamlin, J. L., Montoya-Zavala, M., Heintz, N. H., Milbrandt, J. D., & Azizkhan, J. C. (1982) in *Gene Amplification* (Schimke, R. T., Ed.) pp 154–159, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hand, R. (1978) *Cell (Cambridge, Mass.) 15*, 317–325.

Hay, R. T., & DePhamphilis, M. L. (1982) *Cell (Cambridge, Mass.) 28*, 767–779.

Heintz, N. H., & Hamlin, J. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4083–4087.

Heintz, N. H., & Hamlin, J. L. (1983) *Biochemistry* (preceding paper in this issue).

Heintz, N. H., Milbrandt, J. D., Greisen, K. S., & Hamlin, J. L. (1983) *Nature (London) 302*, 439–441.

Hodge, L. D., Mancini, P., Davis, F. M., & Heywood, P. (1977) *J. Cell Biol. 72*, 192–208.

Kufe, D. W., Major, P. P., Egan, E. M., & Beardsley, G. P. (1980) *J. Biol. Chem. 255*, 8997–9000.

Labarca, C., & Paigen, K. (1970) *Anal. Biochem. 102*, 344–352.

McCready, S. J., Godwin, J., Mason, D. W., Brazell, I. A., & Cook, P. R. (1980) *J. Cell Sci. 46*, 365–386.

Milbrandt, J. D., Heintz, N. H., White, W. C., Rothman, S. M., & Hamlin, J. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6043–6047.

Pardoll, D. M., Vogelstein, B., & Coffey, D. S. (1980) *Cell (Cambridge, Mass.) 19*, 527–536.

Vogelstein, B., Pardoll, D. M., & Coffey, D. S. (1980) *Cell (Cambridge, Mass.) 22*, 79–85.

Wahl, G. M., Stern, M., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3683–3687.

Woodcock, D. M., & Cooper, I. A. (1979) *Exp. Cell Res. 123*, 157–166.

Zahn, R., Muller, W., Forster, W., Maidhof, A., & Beyer, R. (1972) *Eur. J. Cancer 8*, 391–396.