In Vivo Effects of Cytosine Arabinoside on Deoxyribonucleic Acid Replication in Chinese Hamster Ovary Cells. 1. Resolution of Differential Effects on Mitochondrial and Nuclear Deoxyribonucleic Acid Synthesis†

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ABSTRACT: 1-β-D-Arabinofuranosylcytosine (ara-C) is a clinically useful inhibitor of DNA synthesis, but its mode of action is not well understood. We have examined its effect on the uptake of radiolabeled thymidine in Chinese hamster ovary (CHO) cells entering the S period. The inhibition of thymidine incorporation into DNA by ara-C shows a biphasic dose–response curve. Characterization of DNA synthesized in the presence of the drug by alkaline sucrose gradient sedimentation demonstrates a refractile component at concentrations of the drug greater than 1.0 μg/mL. Restriction digestion of DNA, followed by electrophoresis, Southern transfer, and autoradiography, indicates that as the concentration of ara-C increases, thymidine incorporation is progressively limited to three *Eco*RI fragments whose total length is approximately 15.8 ± 0.2 kilobase pairs. Furthermore, DNA labeled with [^{3}H]thymidine in a high concentration of ara-C was shown to band at a heavier position than main-band DNA in neutral CsCl gradients. Labeling of DNA of CHO cells that lack a functional nuclear thymidine kinase gene suggested that the component whose synthesis is insensitive to the inhibitory action of ara-C is mitochondrial in origin. This suggestion was confirmed by demonstrating that restriction fragments that are labeled in high concentrations of ara-C hybridize to ^{32}P-labeled Chinese hamster mitochondrial DNA (mtDNA). Our results are obtained with nuclear DNA prepared by standard methods and, therefore, indicate that the study of the mode of action of ara-C on DNA synthesis in mammalian cells is complicated by the presence of mtDNA, whose synthesis is at least 50-fold less sensitive to the action of the inhibitor than is nuclear DNA replication.

1-β-D-Arabinofuranosylcytosine (ara-C)[1] is a nucleoside analogue with clinically useful antiviral and antitumor activity. Despite intensive study, the mode of action of ara-C on the inhibition of cellular DNA synthesis has not been definitively established. Although it has been reported that ara-C may inhibit several cellular enzymes, including ribonucleotide reductase (Follmann & Hogenkamp, 1971), adenylate cyclase (Ortiz, 1972), and RNA polymerase (Chuang & Chuang, 1976), the primary effect of the drug appears to be at the level of DNA synthesis. The active form of the analogue is the triphosphate derivative, which constitutes the majority of the intracellular metabolite (Graham & Whitmore, 1970a; Skoog & Nordenskjold, 1971; Cohen & Plunkett, 1975) and is the effective inhibitor in eukaryotic in vitro replication systems (Waqar et al., 1971; Magnusson et al., 1975; Wist & Prydz, 1979).

In cultured mammalian cells, incorporation of ara-C into DNA correlates well with its long-term cytotoxicity (Kufe et al., 1980). Most ara-C residues are found to be incorporated directly into nascent DNA strands in internucleotide linkages (Graham & Whitmore, 1970b; Zahn et al., 1972), although approximately 5% of the incorporated arabinoside is present at the 3′-terminus of nascent DNA (Cohen & Plunkett, 1975; Manteuil et al., 1974). Thus, in cultured cells, ara-C behaves as a leaky chain terminator (Cozzarelli, 1977).

In our laboratory, we have used ara-C in an attempt to limit labeling of DNA in CHO cells entering the DNA synthetic period to those regions containing origins of DNA replication. In the course of these studies, we have found that standard nuclear DNA preparations are contaminated with significant amounts of mitochondrial DNA (mtDNA). The synthesis of mtDNA is at least 50-fold less sensitive to the inhibitory action of ara-C than is nuclear DNA synthesis (Pica Mattoccia & Roberti, 1974), probably because the analogue is not phosphorylated by the mitochondrial nucleoside kinase system (Pica Mattoccia & Sorrentino, 1979). Since mitochondrial DNA (mtDNA) constitutes approximately 1% of the mass of cellular DNA (Bogenhagen & Clayton, 1974), its synthesis contributes significantly to the total cellular incorporation of radiolabeled thymidine under circumstances in which nuclear DNA synthesis is selectively inhibited.

In the present paper, we have used several CHO cell lines to partially resolve the effects of ara-C on mtDNA replication from those specific to nuclear DNA synthesis. In an accompanying paper, we exploit this information in order to examine the mechanism by which ara-C inhibits synthesis of nuclear DNA.

Materials and Methods

Reagents. 1-β-D-Arabinofuranosylcytosine was obtained from Sigma. Restriction enzymes were from Bethesda Research Labs and were used as recommended by the supplier. [*methyl*-^{14}C]Thymidine (50–60 mCi/mmol) was obtained from Amersham; [*methyl*-^{3}H]thymidine (77 Ci/mmol) was from New England Nuclear. Chinese hamster mitochondrial

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[1] Abbreviations: CHO, Chinese hamster ovary; TK, thymidine kinase; ara-C, 1-β-D-arabinofuranosylcytosine; mtDNA, mitochondrial DNA; DBM, diazobenzoxymethyl; kb, kilobase pairs; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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DNA was generously provided by Ruth Sager, Harvard University.

Cells and Cell Culture. CHO cells were originally obtained from D. F. Peterson (Los Alamos, NM) and were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids, 10% donor calf serum, and 50 μg/mL gentamicin in an 8% CO_2 water-saturated environment. CHO cells deficient in nuclear thymidine kinase (CHO TK^-) were obtained from L. Siminovitch (Toronto, Canada). CHO TK^- cells were transformed to the TK^+ phenotype by transfection with a recombinant plasmid containing the SV40 origin of replication, the herpes simplex thymidine kinase gene, and pBR322 as the vector (J. D. Milbrandt and J. L. Hamlin, unpublished results). Eagle's medium lacking isoleucine was reconstituted from Selectamine kits (Gibco) and was supplemented with 10% dialyzed fetal bovine serum. Media and other tissue culture supplies were from Gibco.

Determination of Percent Labeled Nuclei. Nuclei were prepared and autoradiographed in Kodak NTB-2 emulsion as previously described (Hamlin & Biedler, 1981). Nuclei containing at least a 20-fold grain count over an equivalent area of background were considered labeled.

Preparation of DNA. Cells were trypsinized, pelleted by centrifugation, and resuspended in RSB (0.01 M Tris-HCl, pH 7.6, 0.01 M NaCl, 1.5 mM $MgCl_2$) for 5 min prior to the addition of Nonidet P-40 (NP-40, Shell) to 1%. After an incubation on ice for 5 min, the cell suspension was triturated gently several times, and the samples were then centrifuged at approximately 500*g* for 5 min. The cytoplasmic supernatant was aspirated, and the nuclear pellet was washed with 2 mL of RSB/NP-40 prior to lysis in 1% lauroylsarcosine in 10 mM Tris-HCl (pH 7.9), 0.1 M NaCl, and 75 mM EDTA. After exhaustive digestion with Proteinase K (EM Biochemicals) and extraction with phenol and chloroform, DNA was precipitated with ethanol and was resuspended in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The 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.

Alkaline Sucrose Gradient Sedimentation Analysis of Nascent DNA. Radiolabeled DNA was sedimented on 5–20% linear alkaline sucrose gradients prepared in 0.3 M KOH, 0.7 M KCl, and 1 mM EDTA as described by Tseng & Goulian (1975). Samples were sedimented at 45 000 rpm for 3 h in a SW56 rotor (Spinco). Fractions were collected and neutralized with HCl, and the radioactivity in each fraction was measured. Linearity of the gradients was determined by the refractive index of selected fractions.

Density Gradient Sedimentation. CsCl solutions were made up in 10 mM Tris-HCl, pH 7.4, and 6 mM EDTA to a density of 1.712 g/cm^3. Each 4.5-mL tube contained 625 ng of DNA, and samples were run for 72 h at 35 000 rpm in a SW50.1 rotor (Spinco). Twelve-drop fractions were collected and diluted with 0.5 mL of H_2O and were then counted in 3 mL of scintillation cocktail. The linearity of the gradients was determined by refractive index measurements. A ^{14}C-labeled *Drosophila melanogaster* DNA preparation (main-band density = 1.712 g/cm^3) was included as a standard (5000 dpm/gradient).

Gel Electrophoresis, Southern Blotting, and Hybridization. Restriction digests of DNA were separated on agarose gels in 40 mM Tris-HCl (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA containing 1.0 μg/mL ethidium bromide. *Hin*dIII or *Eco*RI digests of λ phage DNA were used as molecular weight markers where appropriate. Restriction digests were transferred from agarose gels to activated ABM paper (Schliechter and Schuell) by the method of Wahl et al. (1979). Autoradiograms of [^{14}C]thymidine-labeled DNA on these blots were developed after 4–6 weeks exposure of Kodak X-Omat XR-5 film at −70 °C. Chinese hamster mitochondrial DNA was nick translated (Rigby et al., 1977) with [^{32}P]dCTP to a specific activity of 10^8 dpm/μg. Hybridization was performed as described by Wahl et al. (1979).

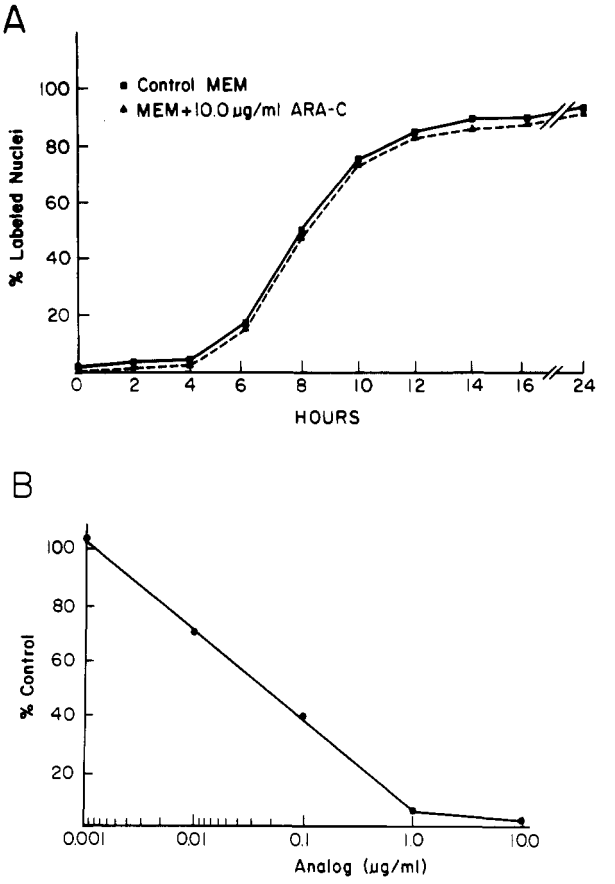

FIGURE 1: Effect of ara-C on G1 traverse and on [^3H]thymidine incorporation. CHO cells were plated in 60-mm dishes and were deprived of isoleucine; 46 h later, cultures were refed complete medium containing the indicated concentrations of ara-C and 1 μCi of [^3H]thymidine/mL. (A) At intervals thereafter, cells were trypsinized and prepared for autoradiography as described; at least 200 cells were scored for each sample. (B) At 12 h after being refed complete medium, cultures were trypsinized, and TCA-insoluble radioactivity in duplicate samples at each drug dose was determined by washing cells twice with phosphate-buffered saline followed by precipitation in 10% trichloroacetic acid at 4 °C for 60 min. Pellets were redissolved in 200 μL of 1 N NaOH, neutralized with an equal volume of 40% acetic acid, and counted in 4 mL of scintillation cocktail.

Results

Effect of ara-C Incorporation of [^{14}C]Thymidine in CHO Cells Entering S Phase. In the studies to be described, the effect of ara-C on DNA synthesis was examined as the cell population entered the S period in a semisynchronous wave. We assume that under these conditions, both initiation of replicons and chain elongation are occurring.

The population was arrested in early G1 by deprivation of the essential amino acid isoleucine (Ley & Tobey, 1970). This block was reversed by addition of complete medium. The kinetics of G1 traverse and entry into the S period were determined by pulse-labeling cells with [^3H]thymidine at various intervals after reversal of the G1 block. It can be seen in Figure 1A that by 12 h after restoration of complete medium,

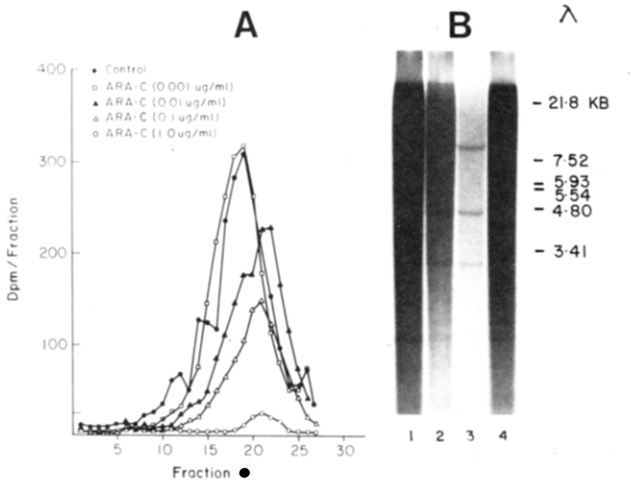

FIGURE 2: Effect of ara-C on size distribution and restriction pattern of replicated DNA. CHO cells plated in 100-mm dishes were arrested in G1 and were released into complete medium containing the indicated concentrations of ara-C and 1 μCi/mL [^{14}C]thymidine. High molecular weight DNA was prepared from each sample (Materials and Methods). (A) 1.0 μg of DNA from each plate was carefully layered onto individual 4.5-mL alkaline 5–20% sucrose gradients, using a wide-bore pipet. Gradients were run for 3 h at 45000 rpm in an SW56 rotor at 4 °C. Fractions were collected and counted for radioactivity (Materials and Methods). Fraction 1 represents the bottom of the gradient. (B) 2.5 μg of DNA from each sample was digested with 10 units of *Eco*RI and was run on a 0.8% nondenaturing agarose gel. DNA was transferred to DBM paper and was autoradiographed for 21 days at −70 °C. The lengths of the *Eco*RI fragments of λ phage DNA included as a standard are indicated in kilobases. Concentrations of ara-C for each sample in lanes 1–4 are 0.01, 0.1, 1.0, and 0.0, respectively. Note that the photograph in (B) is underexposed to bring out details in lanes 1, 2, and 4. See Figure 4 for a more accurate representation of background labeling at high ara-C doses.

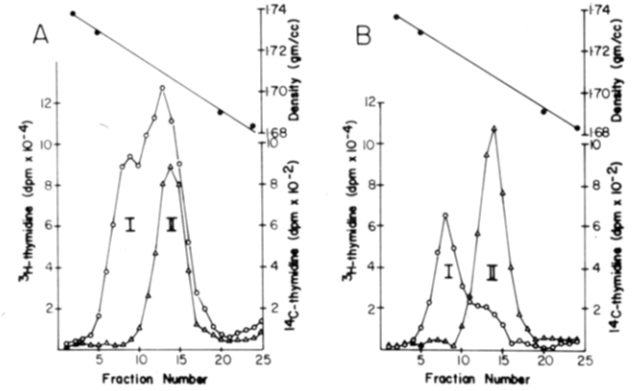

FIGURE 3: Density gradient analysis of DNA synthesized in the presence of ara-C. CHO cells were labeled for 12 h after release from the G1 block in 10 μCi/mL [^{3}H]thymidine with or without 2.5 μg of ara-C/mL. DNA was prepared as described, and 625 ng of each sample was diluted in 10 mM Tris-HCl, pH 7.4, 6 mM EDTA, and CsCl to a final density of 1.712 g/cm^3; 5000 dpm of [^{14}C]thymidine-labeled *Drosophila melanogaster* DNA was included as a standard (main-band density 1.712 g/cm^3). Samples were run for 72 h at 35000 rpm in an SW50.1 rotor. Each gradient was fractionated, and the radioactivity for both labels was determined by scintillation counting. The density of the indicated fractions was determined by refractometry. (A) The density gradient profile of CHO DNA labeled for 12 h after release from G1. (B) The density gradient profile of CHO DNA labeled for 12 h after release from G1 in the presence of 2.5 μg of ara-C/mL. Open circles indicate [^{3}H]thymidine dpm; open triangles indicate [^{14}C]thymidine dpm.

90% of the cells have traversed G1 and are synthesizing DNA. If ara-C is included in the restoration medium at a high dose (10 μg/mL), the rate at which cells enter the S period (measured as percent labeled nuclei) is not affected (Figure 1A), although the grain density over individual nuclei is much reduced (not shown). This result indicates that ara-C has little effect on G1 events required for entry into S. In subsequent experiments, ara-C was included in the restoration medium along with radiolabeled thymidine, and cells were harvested at 12 h.

Although ara-C has no apparent effect on the rate that cells enter S, it displays a biphasic dose-dependent inhibition of [^{14}C]thymidine incorporation into cellular DNA (Figure 1B). At concentrations ranging from 0.001 to 1.0 μg/mL, inhibition of thymidine incorporation into cells entering the S period shows a linear correlation with the logarithm of ara-C concentration. However, at doses exceeding 1.0 μg/mL, inhibition of the remaining 5–7% of uptake occurs with a slope approximately 50-fold less than that observed at lower doses. This biphasic dose–response curve suggests that there are two components of nuclear DNA preparations that are differentially sensitive to the action of ara-C.

Physical Characterization of DNA Synthesized in the Presence of ara-C. DNA synthesized in cells entering the S period in the presence of ara-C was characterized by sedimentation on alkaline sucrose gradients, by equilibrium sedimentation in neutral cesium chloride, and by restriction digestion, electrophoresis, and autoradiography. Figure 2A shows the effect of ara-C on the alkaline sedimentation profile of DNA synthesized in CHO cells by 12 h after release from the G1 block. As the concentration of inhibitor is increased, there is a general trend toward decreasing size of nascent single-stranded DNA and an overall inhibition of synthesis of all fragment sizes, as noted by others (Fridland, 1977; Bell & Fridland, 1980). As the dose of ara-C is increased from 0.01 μg/mL (30% inhibition of incorporation of [^{14}C]thymidine) to 0.1 μg/mL (50% inhibition), no change in the modal position of nascent DNA is observed. This result has been invoked previously as evidence that ara-C inhibits replicon initiation as a primary mode of action (Fridland, 1977).

The results obtained from the alkaline sucrose sedimentation studies suggest that ara-C may interfere with chain elongation and may also inhibit replicon initiation to some degree. In order to assess the distribution of [^{14}C]thymidine in CHO DNA, the samples from the experiment described in Figure 2A were digested with *Eco*RI endonuclease, electrophoresed on agarose gels, transferred to DBM paper, and autoradiographed. The autoradiographs of such digests show that, in the absence of ara-C, radioactivity is distributed throughout the entire spectrum of genomic restriction fragments (Figure 2B, lane 4). As the dose of ara-C increases, labeling in all size classes is reduced and becomes progressively limited to three discrete *Eco*RI fragments approximately 8.4, 4.4, and 3.0 kb in length (Figure 2B, lanes 1–3). The labeled bands constitute a smaller proportion of the total signal at lower drug doses, but the bands themselves appear to be labeled to the same degree at both 1.0 and 0.1 μg of ara-C/mL. This observation suggests that the synthesis of these three fragments represents the refractile component of DNA synthesis observed in the ara-C dose–response experiment shown in Figure 1B.

ara-C-Insensitive Component of CHO DNA Is Denser Than Main-Band DNA. DNA synthesized in the beginning of the S period in the presence of ara-C was also analyzed by cesium chloride gradient sedimentation in order to assess the density distribution of incorporated [^{3}H]thymidine after our labeling protocol. Figure 3 shows the effect of ara-C on the labeling of the light main-band DNA species (average density 1.708 g/cm^3) vs. a heavier species (average density 1.722 g/cm^3). In the absence of inhibitor, the heavier species accounts for 30–40% of the total label incorporated into DNA by 12 h

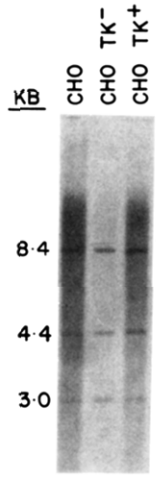

FIGURE 4: Pattern of labeling of DNA in CHO TK⁻ cells in the presence of ara-C. Cells were labeled for 12 h in the presence of 2.5 μg of ara-C/mL with 1 μCi/mL [^{14}C]thymidine after release from the G1 block. Following trypsinization, DNA samples were prepared and digested with *Eco*RI endonuclease; 2.5 μg of each sample was electrophoresed on a 0.8% agarose gel and was then transferred to DBM paper for autoradiography. Parental CHO cell DNA is compared to DNA from CHO cells lacking a functional nuclear thymidine kinase gene (CHO TK⁻) and to CHO TK⁻ cells transformed with the herpes simplex thymidine kinase gene (CHO TK⁺). Estimated sizes in kilobases of the labeled DNA fragments are indicated.

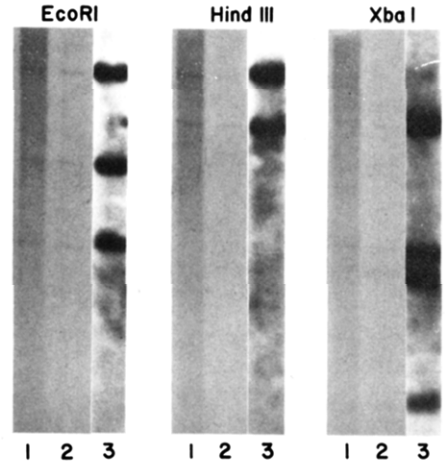

FIGURE 5: Specific hybridization of Chinese hamster mtDNA to ara-C-insensitive restriction fragments. CHO cells were released from a G1 block in the presence of 1.0 μCi/mL [^{14}C]thymidine and 2.5 (lanes 1) or 5.0 (lanes 2) μg/mL ara-C. After 12 h, DNA was isolated and digested with the indicated restriction endonuclease; 2.5 μg of each digest was electrophoresed on a 0.8% agarose gel prior to transfer to DBM paper. The fragments labeled with [^{14}C]thymidine were visualized by exposure to X-ray film for 21 days (lanes 1 and 2). The digests were then probed with ^{32}P-labeled Chinese hamster mtDNA prepared by nick translation, and the fragments hybridizing to mtDNA were visualized by exposure to X-ray film for 2 days (lanes 3).

(Figure 3A, peak I, open circles).

In the presence of 5.0 μg/mL ara-C, labeling of main-band DNA is greatly reduced (Figure 3B, peak II). Of the total radioactivity loaded on the gradient, approximately 30% of the label is recovered in the light density species. The remaining 70% of the radioactivity is recovered in the denser, ara-C-insensitive peak. Direct quantitative comparison of panels A and B is not possible since the gradients were loaded on a mass basis, and individual cells in the control sample had completed varying fractions of the S period by 12 h after release from the G1 block. It is qualitatively apparent, however, that the denser DNA species represents the major labeled DNA species in cells treated with high concentrations of ara-C after release from a G1 block. Although the mass of mtDNA in CHO cells is estimated to be approximately 1% of the total cellular DNA, on the basis of its density, we suspected that the bulk of the heavy DNA fraction represented mtDNA. The high proportion of mtDNA labeling both in the presence and absence of ara-C is probably due to the fact that mtDNA synthesis occurs throughout the labeling interval, which includes all of the G1 period and part of S; significant labeling of nuclear DNA does not begin until 5 h after release from the G1 block, when some cells begin to enter the S period.

ara-C-Insensitive DNA Fragments Are Mitochondrial in Origin. When an identical labeling protocol was performed in CHO cells lacking a nuclear thymidine kinase (TK) gene, the same three *Eco*RI fragments were labeled (Figure 4). However, in contrast to wild-type CHO cells (Figure 4, lane 1), synthesis of DNA in TK⁻ cells in the presence of 2.5 μg/mL ara-C is limited almost exclusively to these three *Eco*RI fragments (lane 2). Densitometric tracings of this autoradiograph show that the three fragments contribute greater than 75% of the total autoradiographic signal, and background labeling due to nuclear DNA synthesis is barely detectable. When CHO TK⁻ cells are transformed to the TK⁺ phenotype by transfection with a plasmid containing the herpes simplex TK gene, the background labeling is restored (Figure 4, lane 3), confirming that cells enter the S phase and begin to synthesize nuclear DNA in the presence of ara-C.

Since in TK⁻ cells incorporation of exogenous thymidine is limited almost exclusively to mtDNA (Attardi & Attardi, 1972; Clayton & Teplitz, 1972; Berk & Clayton, 1973), we tested whether the ara-C-insensitive restriction fragments were mitochondrial in origin by hybridizing ^{32}P-labeled Chinese hamster mtDNA to several different restriction digest of CHO DNA (Figure 5). It is apparent that all fragments labeled at a high dose of ara-C hybridize to legitimate mtDNA and, therefore, must be derived from the endogenous mtDNA present in DNA isolated from purified nuclei.

Discussion

Our laboratory is interested in the initiation of chromosomal DNA replication in mammalian cells. We are especially interested in determining if DNA replication begins at genetically fixed nucleotide sequences analogous to the origins of replication observed in bacteria, bacteriophages, and viruses. Since ara-C has been reported to act as a chain terminator in vitro (Atkinson et al., 1969) and has negligible effects on protein and RNA synthesis (Graham & Whitmore, 1970a; Jones & Moscona, 1974), we selected this drug in an effort to slow replication forks and, hence, to limit the labeling of DNA at the beginning of the S period to those sequences including and surrounding initiation sites.

When administered to cells entering the S phase, ara-C exhibited a biphasic dose–response curve of inhibition of radiolabeled thymidine incorporation into TCA-insoluble material, with a refractile component present at concentrations of ara-C greater than 1.0 μg/mL. Alkaline sucrose sedimentation studies suggested that the drug inhibited the synthesis of nascent DNA in all size classes, although a discrete ara-C-insensitive species was observed at high doses. This refractile DNA species has a density greater than nuclear DNA and similar to published values for mammalian mitochondrial DNA (Altman & Katz, 1976). When we subsequently examined the distribution of radiolabeled thymidine in DNA synthesized under these conditions by restriction endonuclease digestion, electrophoresis, and autoradiography, we found that high doses of ara-C limited DNA replication to several discrete restriction fragments common to all CHO

cell types. We have presented several lines of evidence indicating that the ara-C-insensitive component is mtDNA. In CHO TK$^-$ cells, which lack the ability to utilize exogenous radiolabeled thymidine as a substrate for nuclear DNA synthesis, labeling of nascent DNA is limited to the same discrete *Eco*RI restriction fragments in the presence or absence of ara-C. These fragments total approximately 15.8 kb in length, the unit size of CHO mtDNA (M. Nass, personal communication), and we have shown that they hybridize to ^{32}P-labeled CHO mtDNA. The refractile component has an average density greater than main-band nuclear DNA, a characteristic of mammalian mtDNAs (Altman & Katz, 1976). Although the profiles presented in Figure 3 do not prove that all the labeled DNA migrating at the dense position is mitochondrial in origin, restriction endonuclease and hybridization analysis of these samples suggest that a major fraction of the dense species is mtDNA. In addition, mtDNA synthesis has been shown to be insensitive to ara-C in vitro (Pica Mattoccia & Sorrentino, 1979).

At concentrations of ara-C that inhibit greater than 90% of the total incorporation of labeled thymidine into cellular DNA, both densitometry of autoradiographs of restriction digests and cesium chloride gradient centrifugation show that mtDNA synthesis accounts for at least 30% of the total label incorporated into DNA when cells are labeled after release from a G1 block and DNA is prepared from CHO nuclei by our method. In our procedure, cells are hypotonically swollen prior to lysis with a nonionic detergent (NP-40), and cytoplasmic and nuclear fractions are separated by centrifugation. This treatment yields a cytoplasmic fraction that contains virtually all of the in vitro translatable mRNA and results in quantitative recovery of dihydrofolate reductase, a known cytoplasmic enzyme (unpublished observation). Other investigators have reported difficulty in obtaining complete separation of nuclear and mtDNAs in other isolation procedures (Bogenhagen & Clayton, 1974), and we suspect that this problem has complicated many studies on the mode of action of ara-C. In addition, a number of investigations on the action of the drug have utilized whole-cell DNA, ignoring the contribution of mtDNA (ca. 1% of cellular DNA).

The presence of this replicon considerably complicates the interpetation of the effects of ara-C on alkaline sucrose sedimentation profiles of nascent labeled DNA. Although at low concentrations of ara-C, mtDNA synthesis comprises a minor portion of total cellular DNA synthesis, in the presence of high concentrations of the drug, its synthesis becomes a significant concern (Figures 2 and 3). A large variety of labeled single-stranded species are generated from the synthesis of mtDNA (Clayton, 1982), and their contribution to sedimentation profiles is difficult to assess. Bell & Fridland (1980) analyzed the effect of ara-C on the synthesis of DNA by alkaline sucrose gradient sedimentation and observed a shift of the major sedimentation peak from 30S to 70S. They therefore concluded that initiation of new replicons is inhibited by the drug. This contrasts with the observations of Dijkwel & Wanka (1978), who observed little or no effect of ara-C on replicon initiation in sedimentation studies and, therefore, suggested that the primary mode of action of the drug is on the rate of polymerization. Neither study accounts for the contribution of mtDNA synthesis, even though whole-cell DNA samples were examined. Density sedimentation studies performed with bromodeoxyuridine revealed the presence of a minor heavy-heavy DNA species in cells in which replication had been interrupted by an hour-long incubation with ara-C (Woodcock & Cooper, 1979). It was concluded that the drug induces rereplication of selected chromosomal sequences since, upon removal of the drug, DNA was synthesized from template strands that had been replicated several hours previously. We consider it likely that this synthesis is mitochondrial in origin, since replication of mtDNA proceeds uninterrupted in the presence of ara-C and several rounds of replication of mtDNA could easily occur under their labeling conditions. We suggest that cells lacking a nuclear thymidine kinase gene would provide a useful control for separating the effects of ara-C (or any drug acting on α DNA polymerase) on nuclear DNA synthesis from effects on mtDNA replication.

In an accompanying paper, we examine the effects of ara-C on nuclear DNA synthesis under conditions in which mtDNA synthesis does not interfere. We have examined the effects of ara-C on the movement of DNA away from its site of synthesis on the nuclear matrix and on the synthesis of an amplified nuclear DNA sequence.

Acknowledgments

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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†

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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

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[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.

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