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Imbalanced DNA synthesis induced by cytosine arabinoside and fludarabine in human leukemia cells

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Abstract

Previous studies have demonstrated that cytosine arabinoside (araC) induces an accumulation of Okazaki fragments, while fludarabine (FaraA) inhibits Okazaki fragment synthesis. We extended these observations in the present study to provide insights into various mechanisms by which these anticancer drugs affect DNA replication and induce genomic instability in human CEM leukemia cells. Neither araC nor FaraA induced a detectable amount of re-replicated DNA in S-phase cells, which indicated that drug-induced alterations in Okazaki fragment synthesis were not accompanied by DNA re-replication. Synthesis on both leading and lagging DNA strands within the c-*myc* locus was measured in cells incubated with equitoxic concentrations of araC or FaraA. In araC-treated cells, nascent DNA from the lagging strand was enriched about 5-fold compared with the leading strand. In contrast, FaraA did not induce any replication imbalance. AraC- and FaraA induced changes in the frequency of *N*-(phosphonacetyl)-l-aspartate (PALA) resistance and the extent of CAD gene amplification were monitored as markers of drug-induced genomic instability. At concentrations that reduced cloning efficiency by 50% (IC_{50}), araC increased the frequency of PALA resistance about 4-fold, while FaraA did not have a significant effect on the frequency of PALA resistance. Pretreatment with araC also increased the extent of CAD gene amplification. We propose that the imbalanced DNA synthesis induced by araC leads to the accumulation of Okazaki fragments on the lagging arms and single-stranded DNA regions on the leading arms of replication forks. The formation of these abnormal replication structures was associated with the generation of genomic instability.

Keywords: Imbalanced DNA synthesis; Cytosine arabinoside; Fludarabine; Genomic instability; DNA replication

1. Introduction

The anticancer drug araC is a deoxycytidine analog, and its active triphosphate metabolite is a competitive inhibitor of dCTP [1]. When incorporated into DNA, araC is a potent inhibitor of DNA chain elongation by polymerases α, δ, and ϵ [2–6]. Previous studies from our laboratory using a whole cell lysate system have shown that araC does not inhibit primer RNA formation but rather induces an accumulation of Okazaki fragments at concentrations that inhibit DNA synthesis [7]. The accumulation of primer RNA is consistent with the results of Hu *et al.* [8], who showed that primer RNA likewise accumulated when DNA polymerase α activity was slowed following incubation of the purified DNA polymerase α/primase complex with subphysiological concentrations of the deoxyribonucleoside triphosphate substrates. Also, Ross and co-workers [9] have reported that araC induces marked inhibition of DNA chain elongation in whole cells, but does not affect replicon initiation or the synthesis of Okazaki fragments. An explanation of these effects is provided, in part, by the observation that araC is a weak inhibitor of DNA primase [5,7]. Furthermore, arabinonucleosides, such as araC and araA, are efficiently incorporated by DNA polymerase α into RNA primers but do not prevent elongation of RNA primers by this enzyme, thus allowing for Okazaki fragment formation [10,11]. Taken together, these data suggest that the inhibitory effects of araC on DNA replication do not involve inhibition of RNA

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Abbreviations: araC, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside); araA, 1-β-D-arabinofuranosyladenine; BrdUrd, 5-bromo-2′-deoxyuridine; FaraA, 1-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine); IC_{50}, concentration that reduces cloning efficiency by 50%; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PALA, *N*-(phosphonacetyl)-L-aspartate; and SSC, standard saline citrate.

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primer synthesis and origin activation, but most likely result from inhibition of DNA chain elongation.

In contrast to araC, other studies have demonstrated that the triphosphate metabolite of the purine nucleoside analogue, FaraA, is a potent inhibitor of DNA primase [5,7,12]. FaraATP is actually a more efficient substrate for DNA primase than ATP [12]. As a result of its incorporation into primer RNA, FaraATP induces primer RNA chain termination. This has been shown to reduce the synthesis of RNA primers and Okazaki fragments in cell-free systems and in cell lysates [5,7].

The opposing effects of araC and FaraA on Okazaki fragment accumulation could provide insights into the mechanisms by which these agents inhibit DNA replication and induce genomic instability in cells. One possible mechanism is that the araC-induced accumulation of Okazaki fragments may lead to repriming at the origins of replication as the cells attempt to overcome inhibition of DNA synthesis. After drug removal, one or more rounds of DNA re-replication may occur within a single cell cycle [13]. Another possibility relates to the asymmetric nature of DNA replication and the different effects of araC and FaraA on the enzymes involved in leading and lagging strand synthesis [14]. By affecting DNA synthesis on the two strands of the replication fork to a different extent, araC may induce an imbalance between leading and lagging strand synthesis and generate asymmetric replication forks. FaraA seemed less likely to induce asymmetric replication forks, since primer RNA synthesis is required on both the lagging strands and on the leading strands at replication origins. To directly test these ideas, we monitored DNA replication on the leading and lagging strands at a specific genetic locus (*c-myc*) in cells treated with either araC or FaraA. To the best of our knowledge a direct analysis of the effects of araC and FaraA on the generation of asymmetric replication forks in cells has not been reported previously.

Alterations in DNA synthesis, such as re-replication or imbalanced synthesis, may lead to a loss of genomic stability. AraC and other anticancer agents are known to induce certain forms of genomic instability, such as gene amplification [15–18]. Drug-induced amplification of proto-oncogenes and drug resistance genes can promote tumor progression [19] and the development of resistance to cancer chemotherapy [20]. Thus, studies into the mechanisms by which the anticancer drugs araC or FaraA inhibit DNA synthesis and induce genomic instability may have clinical relevance.

2. Materials and methods

2.1. Reagents

Tissue culture medium, serum, antibiotics, and the DNA labeling kit for random primers were obtained from GIBCO/BRL. The pHSR-1 plasmid, which contained a 9.0-kb genomic insert of the c-*myc* gene, and the human cDNA probes for GAPDH (1.7 kb) and fibronectin-1 (1.3 kb) were purchased from the American Type Culture Collection. Thea Tlsty of the University of California at San Francisco provided a plasmid containing an insert of the human CAD gene that was described by Davidson *et al.* [21]. The plasmid was digested with *Eco*RI and *Pst*I to yield a 1.8-kb fragment that comprises a portion of the exon encoding the ATCase domain of the human CAD gene. FaraA was a gift of Berlex Laboratories, and araC was obtained from the Sigma Chemical Co. PALA was a gift of U.S. Bioscience. [α-^{32}P]CTP (specific radioactivity of 3000 Ci/mmol) was purchased from Dupont/NEN Radiochemicals. [*Methyl*-^{3}H]thymidine, [6-^{3}H]BrdUrd, and [2-^{14}C]thymidine with specific radioactivities of 50 mCi/mmol, 25 Ci/mmol, and 65 Ci/mmol, respectively, were purchased from Moravek Biochemicals, Inc. Restriction endonucleases, Whatman GF-C glass fiber filters, and the pGEM-3Z vector were purchased from the Fisher Scientific Co. Bio-spin columns were obtained from Bio-Rad Laboratories. RNase-free pancreatic DNase I and RNase A were obtained from the Worthington Biochemical Co. SSC buffer (20X) consisted of 3 M NaCl and 0.3 M sodium citrate (pH 7).

2.2. Cell culture and isolation of the CEM-2B clone

Human leukemia CEM cells were grown at 37^{o} under 95% air–5% CO_2 in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum. To carry out the studies with a homogeneous cell population, CEM cells were cloned in soft agar, and single colonies were picked and expanded. Karyotypic analysis was performed on a number of clones to determine their ploidy. Clone CEM-2B, which was near tetraploid (93 chromosomes), was used in all the studies.

2.3. Centrifugal elutriation

Exponentially growing cells (approximately 5×10^8 in 10 mL) were loaded in a standard elutriation chamber of a JE-6B Beckman rotor at a flow rate of 10 mL/min. Elutriation was performed at a rotor speed of 1500 rpm in a J2-MI centrifuge. The cells were collected in 6 fractions of 100 mL each by increasing the flow rate from 10 to 22 mL/min in increments of 2 mL/min. The mean cell volume of the elutriated cells was measured with a Coulter cell counter equipped with a multichannel analyzer. Aliquots of elutriated cells were stained with propidium iodide and analyzed by flow cytometry to determine DNA content and cell cycle distribution as previously described [22].

2.4. DNA re-replication assay

To determine whether araC or FaraA induced DNA re-replication, we modified the procedure used by Schimke *et al.* [13] for detecting re-replication within a single cell

cycle. Exponentially growing CEM cells were uniformly prelabeled with [^{14}C]thymidine for 72 hr. Early S-phase cells were isolated by centrifugal elutriation and incubated for 2 hr with [^{3}H]BrdUrd at 37°. Either 100 nM araC or 5 μM FaraA was then added to the culture medium, and the cells were incubated for an additional 6 hr. After 6 hr, the drugs were removed, and the cells were resuspended in fresh medium containing BrdUrd alone for an additional 12 hr. Untreated control cells were incubated with BrdUrd for 10 hr. Control and drug-treated cells were centrifuged, lysed, and incubated with proteinase K for 4 hr at 37°. After the addition of NaCl to a final concentration of 200 mM, the DNA was sheared by repeated passages through a 25-gauge needle and mixed with a neutral solution of CsCl (final density 1.742 g/mL). The samples were centrifuged at 35,000 rpm in a 70 Ti Beckman rotor for 60 hr at 20°. Gradient fractions were collected from the bottom of the tubes, the DNA was precipitated with trichloroacetic acid, and the radioactivity in the DNA precipitates was then measured by liquid scintillation counting.

2.5. Preparation of strand-specific probes

Single-stranded RNA probes were generated to quantify leading and lagging strand DNA synthesis on the c-*myc* gene. We isolated a 367-bp fragment downstream to the origin of replication of the c-*myc* gene by cutting the pHSR-1 plasmid with *Xba*I and *Sac*I. The fragment was subcloned in pGEM-3Z vector containing Sp6 and T7 promoter sites in opposite orientation. Transcription with the specific RNA polymerases in the presence of [α-^{32}P]CTP yielded radiolabeled strand-specific RNA probes complementary to either the leading or lagging strand of the replication fork. Radiolabeled probes were purified with Biospin columns.

2.6. Analysis of replication fork progression

This was done as previously described with minor modification [22–24]. Briefly, CEM cells were labeled with 0.01 μCi/mL of [^{14}C]thymidine for 72 hr and chased for 24 hr in radioactive-free medium. The cells were then incubated at 37° with 10 μM [^{3}H]BrdUrd (0.025 μCi/mL) in either the presence or the absence of drugs. All of the following steps were carried out under subdued light. After 4 hr, DNA was isolated from control and drug-treated cells. Samples were incubated with RNase A (100 μg/mL) for 1 hr at 37° followed by proteinase K (0.2 mg/mL) at 37° overnight. DNA was extracted with phenol and chloroform, denatured by the addition of 0.15 M NaOH, and then mixed with a solution consisting of 50 mM NaOH, 3 mM EDTA, and CsCl at a final density of 1.806 g/mL. Samples were centrifuged at 35,000 rpm in a 70.Ti Beckman rotor for 60 hr at 25° to separate single-stranded BrdUrd–DNA from unreplicated DNA. Fractions of 0.45 mL were collected from the bottom of the tubes, and the amount of radioactivity was determined by liquid scintillation counting. Equal amounts of BrdUrd–DNA (2–4 μg) were denatured by the addition of 0.1 vol. of 3 M NaOH and incubation at 100° for 5 min. The samples were then neutralized with 1 vol. of 6X SSC and applied to nylon membranes using a slot-blot apparatus. The blots were baked for 1 hr at 80° and hybridized to ^{32}P-labeled strand-specific RNA probes. Hybridization conditions were as previously described [22]. Each experiment was repeated twice with two different DNA preparations. Following the first round of hybridization, each blot was stripped and re-hybridized to the probe for the opposite strand. The hybridization bias (i.e. the ratio between the hybridization signals of the two complementary probes) was determined by densitometry using Gel-Pro software and calculated as:

$$\frac{\text{lagging strand synthesis / leading strand synthesis in drug-treated cells}}{\text{lagging strand synthesis / leading strand synthesis in control cells}}$$

2.7. DNA synthesis inhibition

Cells (5×10^4/mL) were preincubated for 4 hr with or without the drug and then incubated for 30 min at 37° in the presence of [^{3}H]thymidine (0.25 μCi/mL). Aliquots (100 μL) of the cell suspension were transferred to glass fiber filters. Filters were dried and placed in scintillation vials, and the amount of radioactivity on each filter was determined by liquid scintillation counting.

2.8. Clonogenic assay

Exponentially growing CEM-2B cells were allowed to grow for 24 hr. Then cells were incubated for 16 hr with either no drugs or various concentrations of araC (10–100 nM) or FaraA (1–20 μM). To select for PALA-resistant clones, cells were washed in drug-free medium, counted, and then plated (10^3 to 10^4 cells/plate) in 0.3% soft agar containing 15% dialyzed fetal bovine serum and either 0, 120, or 240 μM PALA. These concentrations were 3- and 6-fold higher than the concentration of PALA IC_{50} as determined in preliminary experiments. Twelve days later, colonies of at least 50 cells were counted in each plate to determine the cloning efficiency. The cloning efficiency of araC- and FaraA-pretreated cells was corrected for the cell kill caused by these drugs alone in the absence of PALA as previously described [17]. The results are expressed as fold increase in PALA resistance in the drug-treated samples relative to cells treated with PALA alone.

2.9. Assessment of gene amplification

Colonies of 50–100 cells were isolated at random from plates of untreated control and each drug treatment group. Colonies were mechanically disrupted and propagated to

1×10^6 cells in the presence of various concentrations of the selective agent, PALA. DNA was extracted with phenol/chloroform, and the DNA concentration in each sample was determined by measuring the absorbance at 260 nm. Equal amounts of DNA were applied to nylon membranes by the slot-blot technique. The blots were hybridized to a radiolabeled CAD probe as previously described [22]. Blots were stripped and re-hybridized to the GAPDH probe to control for the amount of DNA applied on the nylon filter. Duplicate blots were also hybridized to a probe for the fibronectin-1 gene, which is located on the q arm of chromosome 2 to determine whether or not changes in the CAD gene copy number were due to acquisition of extra copies of this chromosome.

3. Results

3.1. Effects of araC and FaraA on DNA re-replication

It is possible that cells initiate multiple rounds of replication within a single cell cycle (re-replication) in an attempt to overcome replication fork arrest induced by either araC or FaraA. The araC-induced accumulation of RNA-primed DNA (Okazaki fragments) [7] may facilitate DNA re-replication. To determine whether araC or FaraA induced re-replication, the DNA synthesized after drug removal was analyzed by CsCl density gradient centrifugation. In a neutral CsCl gradient, DNA synthesized in the presence of BrdUrd is expected to band at a density higher than that of unsubstituted DNA. DNA re-replication within the same S phase and in the presence of BrdUrd would lead to the formation of DNA in which both strands contain BrdUrd, termed heavy-heavy (HH) DNA. To monitor any synthesis of HH DNA, exponentially growing CEM cells were first uniformly prelabeled with [^{14}C]thymidine for 72 hr. Early S-phase cells were isolated by centrifugal elutriation and then incubated for 2 hr with [^{3}H]BrdUrd. Either 100 nM araC or 5 μM FaraA was added to the culture medium, and the cells were incubated for an additional 6 hr. After 6 hr, the drugs were removed, and the cells were resuspended in fresh medium containing [^{3}H]BrdUrd alone for an additional 12 hr to allow the cells to replicate and possibly re-replicate the DNA. Data from flow cytometry and [^{3}H]thymidine incorporation assays showed that within this 12-hr period most of the drug-treated cells had completed S phase and entered G_2/M, but had not entered a second cell cycle (data not shown). Untreated control cells were incubated in the presence of [^{3}H]BrdUrd for 10 hr. By this time the control cells had moved to the G_1 phase but had not entered a second S phase (data not shown).

As shown in Fig. 1A, the ^{14}C-prelabeled DNA from control cells incubated with [^{3}H]BrdUrd for 10 hr banded in two distinct peaks. One peak corresponded to DNA not substituted with [^{3}H]BrdUrd [light-light (LL) DNA]. The second peak represented the parental strand of [^{14}C]DNA

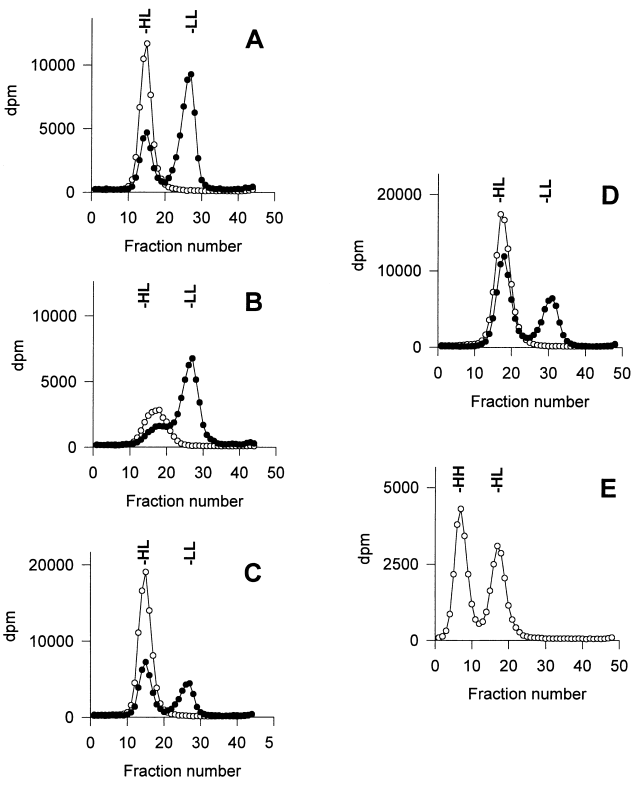

Fig. 1. Analysis of DNA re-replication. CEM cells were preincubated with [^{14}C]thymidine for 72 hr. Early S-phase cells were isolated by centrifugal elutriation and then incubated for 2 hr with [^{3}H]BrdUrd. The cells were incubated with either no drug (A) or with 100 nM araC (B) or 5 μM FaraA (C). After 6 hr, the cells were incubated with BrdUrd for an additional 12 hr. Untreated control cells were incubated with BrdUrd for 10 hr. The DNA was centrifuged to equilibrium in a CsCl gradient. Radioactivity in the gradient fractions was measured by liquid scintillation counting. (D) CEM cells were preincubated with [^{14}C]thymidine for 36 hr. Then [^{3}H]BrdUrd was added for 12 hr to label only one DNA strand. (E) CEM cells were incubated with [^{3}H]BrdUrd for 48 hr (about two cell cycles) to label both DNA strands. Key: HH, heavy-heavy DNA; HL, heavy-light DNA; LL, light-light DNA; (μ) [^{3}H]BrdUrd; and (λ) [^{14}C]thymidine.

that had replicated during the 10-hr incubation with [^{3}H]BrdUrd and, thus, had a density of heavy-light (HL) DNA. Panels B and C of Fig. 1 show a similar distribution of [^{3}H]BrdUrd and ^{14}C-labeled DNA between HL and LL DNA in araC- and FaraA-treated cells. As observed in control cells, neither araC nor FaraA induced the formation of HH DNA. To verify that these conditions were able to separate any HH DNA present in the sample, we incubated CEM cells for either 12 or 48 hr in the presence of [^{3}H]BrdUrd. After 12 hr, only one DNA strand would have incorporated [^{3}H]BrdUrd and, thus, only HL DNA is expected in this sample (Fig. 1D). During the 48-hr incubation, a fraction of the cells would have entered a second S phase and incorporated [^{3}H]BrdUrd into both DNA strands. As a result, the 48-hr sample would contain both HL and HH DNA. Figure 1E shows that the gradient clearly separated LL, HL, and HH DNA. Therefore, the absence of HH DNA in cells incubated with either araC or FaraA indicated

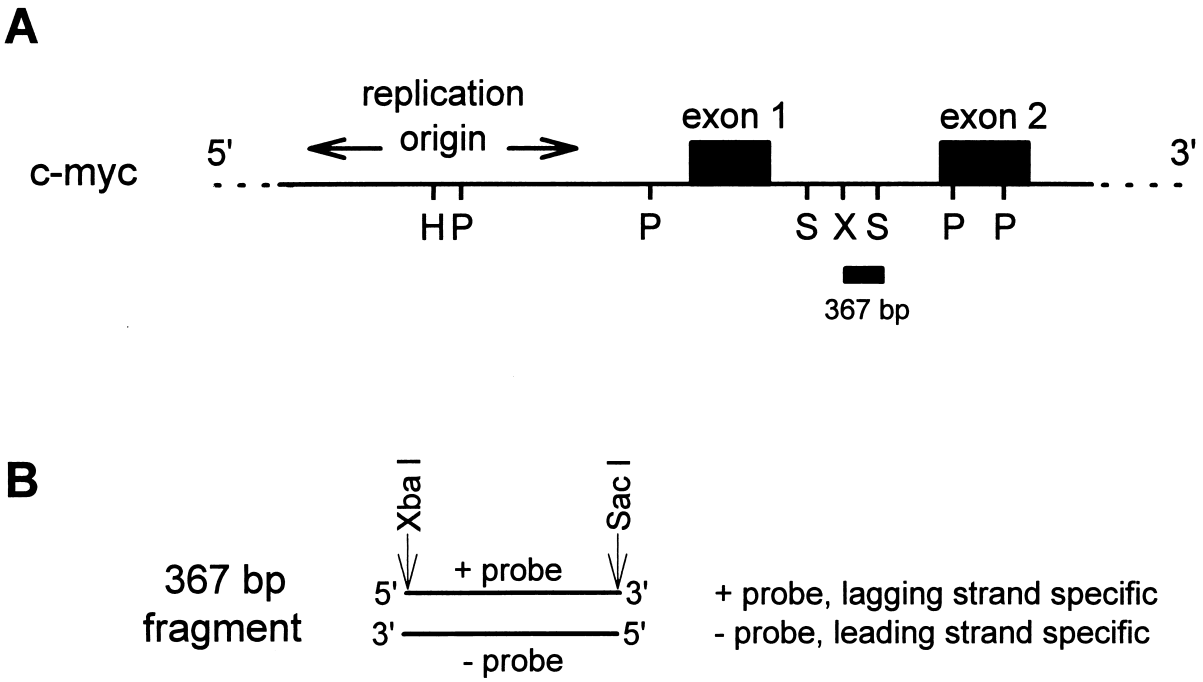

Fig. 2. Analysis of leading and lagging strand DNA synthesis in the c-*myc* gene. (A) Map of the *c-myc* gene. Restriction endonuclease sites: H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xba*I. A replication origin has been mapped in the 5′ flanking region of the gene. Arrows indicate the direction of replication forks originating from this site. A small bar indicates the position of the 367-bp fragment that was used to monitor lagging and leading strand synthesis. (B) Strand-specific RNA probes. The *Xba*I/*Sac*I fragment was subcloned into a pGEM transcription vector. Transcription of the 367-bp fragment by either T7 or SP6 RNA polymerases yielded strand-specific RNA probes. The 5′ → 3′ strand of the *Xba*I/*Sac*I fragment is the *plus* (+) probe complementary to newly replicated DNA from the lagging strand. The 3′ → 5′ strand is the *minus* (−) probe complementary to newly replicated DNA from the leading strand.

that neither of these agents induced a detectable amount of DNA re-replication.

3.2. Effects of AraC and FaraA on replication fork progression

Previous studies from our laboratory have shown that araC and FaraA differ with regard to their effects on primer RNA and Okazaki fragment synthesis. AraC does not inhibit primer RNA synthesis and induces an accumulation of Okazaki fragments at the replication fork. In contrast, FaraA inhibits primer RNA and Okazaki fragment synthesis [7,12]. In light of the effects of araC on Okazaki fragment synthesis, we hypothesized that araC allowed activation of the origin of replication but altered the normal progression of the replication forks by producing an imbalance between the synthesis of the leading and lagging strands. To test this hypothesis, we analyzed the effects of araC on the replication of the leading and lagging strand within the c-*myc* gene. Figure 2 shows a map of the human c-*myc* locus. This is one of the few human loci for which the origin of replication has been consistently mapped in the 5′ flanking region of the gene by various methods [25,26]. Furthermore, we previously confirmed the 5′ to 3′ direction of replication fork progression within the c-*myc* locus in CEM cells [22]. Figure 2 also demonstrates that the strand-specific RNA probes, which were used to monitor leading and lagging strand synthesis, corresponded to a 367-bp fragment of the first intron located downstream of the replication origin.

The relative amounts of leading and lagging strand DNA synthesized in control and araC-treated cells were assessed using a modification of the replication fork polarity assay described previously [23,24]. Cells were incubated for 4 hr with either 0, 10, or 25 nM araC. These concentrations of araC inhibited DNA synthesis 84 and 92% compared with control cells, respectively. BrdUrd was present in the medium during the 4-hr incubation to label newly replicated DNA. After isolation of newly replicated DNA by CsCl density gradient centrifugation, equal amounts of newly replicated DNA from untreated and araC-treated cells were applied to nylon membranes by the slot-blot technique. Then the blots were hybridized to c-*myc* strand-specific probes. The intensity of hybridization of newly replicated DNA to each probe was determined by densitometry, and the hybridization bias in drug-treated samples relative to control samples was calculated as described in section 2. Newly replicated DNA from araC-treated cells hybridized preferentially to the probe complementary to the lagging strand compared with the leading strand probe (Fig. 3). This result indicated that in the presence of araC newly replicated DNA originated preferentially from the lagging strand of the forks. The magnitude of the hybridization bias was 1.6

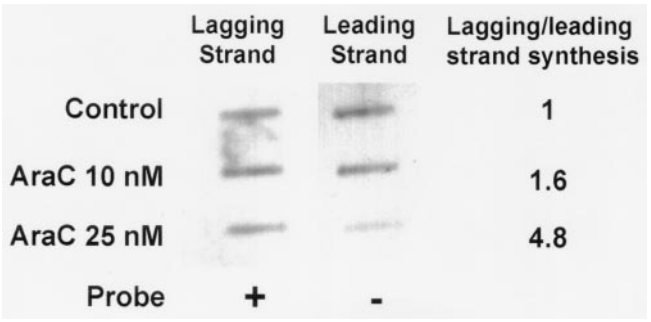

Fig. 3. Effects of araC on leading and lagging strand DNA synthesis. Duplicate slot blots, which contained equal amounts of BrdUrd–DNA from untreated cells and araC-treated cells, were hybridized to the ^{32}P-labeled strand-specific RNA probes complementary to the lagging (+) and the leading (−) strand of the c-*myc* fragment. Hybridization intensities were determined by densitometry. The hybridization bias (lagging/leading strand DNA synthesis) was calculated in araC-treated samples after normalization to the bias that existed in untreated control cells. A value > 1 indicated that in the experimental sample lagging strand DNA synthesis was greater than leading strand DNA synthesis.

and 4.8 at 10 and 25 nM araC, respectively. Similarly, hybridization biases of 1.5 and 3.5 at 10 and 25 nM araC, respectively, were obtained when the experiment was repeated. These results demonstrated that araC generated an imbalance between the synthesis of the two strands of the replication fork with greater inhibition of the leading than of the lagging strand. Therefore, treatment of cells with araC generates asymmetric replication forks, which may lead to genomic instability.

In contrast to araC, FaraA is a strong inhibitor of primer RNA formation [5,7,12], which is required for both the initiation of DNA replication on the leading and lagging strands and for Okazaki fragment synthesis on the lagging strand. Therefore, FaraA could block both leading and lagging strand synthesis at the origins of replication and, thus, might not induce imbalanced DNA synthesis. To test this hypothesis, we analyzed replication fork progression in the c-*myc* locus in control and FaraA-treated cells. Figure 4 shows the results of an experiment in which newly replicated DNA from control and FaraA-treated cells was hybridized to the leading and lagging strand RNA probes. In this experiment we treated cells with 5 μM FaraA, a concentration that induced a 90% inhibition of DNA synthesis. This concentration of FaraA was, therefore, equivalent to 25 nM araC. Newly replicated DNA from FaraA-treated cells hybridized to both probes to a degree similar to newly replicated DNA from untreated cells. The hybridization bias was 0.8 in FaraA-treated cells compared with the control. A hybridization ratio of 0.7 was obtained when this experiment was repeated. Therefore, residual DNA synthesis in cells treated with 5 μM FaraA was still coupled and coordinated between the two arms of the replication fork, similar to that in untreated control cells.

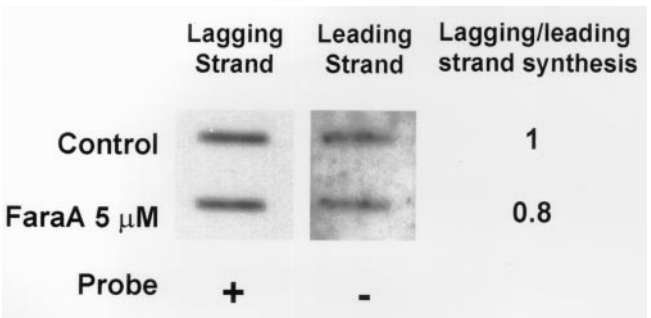

Fig. 4. Effects of FaraA on leading and lagging strand DNA synthesis. Duplicate blots, which contained equal amounts of BrdUrd–DNA from control cells and cells treated with FaraA (5 μM), were hybridized to the ^{32}P-labeled strand-specific RNA probes. The hybridization bias in the FaraA-treated sample was determined by densitometry and was normalized to the bias in untreated control cells as described in the legend to Fig. 3.

3.3. Drug-induced genomic instability

The above results demonstrated that araC inhibited leading strand synthesis to a greater extent than lagging strand synthesis, while in FaraA-treated cells DNA synthesis was inhibited to a similar extent on both arms of the replication fork. Additional studies were done to determine whether the imbalanced DNA synthesis induced by araC was accompanied by genomic instability.

Previous studies have shown that antiproliferative agents increase the frequency of resistance to the anticancer drug PALA in some cell lines by inducing CAD gene amplification [17,18,27,28]. PALA specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme, which is involved in *de novo* pyrimidine synthesis. Cells that acquire multiple copies of the CAD gene through gene amplification can be selected in PALA-containing medium [17,18,27]. Therefore, we monitored araC- and FaraA-induced changes in the frequency of PALA resistance as a marker of drug-induced genomic instability. Exponentially growing CEM-2B cells were pretreated with increasing concentrations of araC or FaraA for 16 hr. Control, araC- and FaraA-treated cells were then cloned in either the absence or the presence of 120 μM PALA. This concentration was three times higher than the concentration of PALA that reduced the cloning efficiency of these cells by 50% (IC_{50}). AraC at its IC_{50} (25 nM) (Fig. 5A) increased the frequency of PALA resistance about 4-fold (Fig. 5B). The frequency of PALA resistance increased up to 35-fold when cells were pretreated with 100 nM araC. A 65-fold increase in PALA resistance was observed in cells pretreated with 100 nM araC and cloned in 240 μM PALA (data not shown). FaraA was a less potent inducer of PALA resistance than araC when compared at equitoxic concentrations. At concentrations as high as its IC_{50} (5 μM) (Fig. 5C), FaraA did not affect significantly the frequency of PALA resistance (Fig. 5D). The frequency of PALA resistance was 4- and 7.5-fold greater in cells pretreated with 10 and 20 μM FaraA than in cells incubated with PALA alone. FaraA at high concentra-

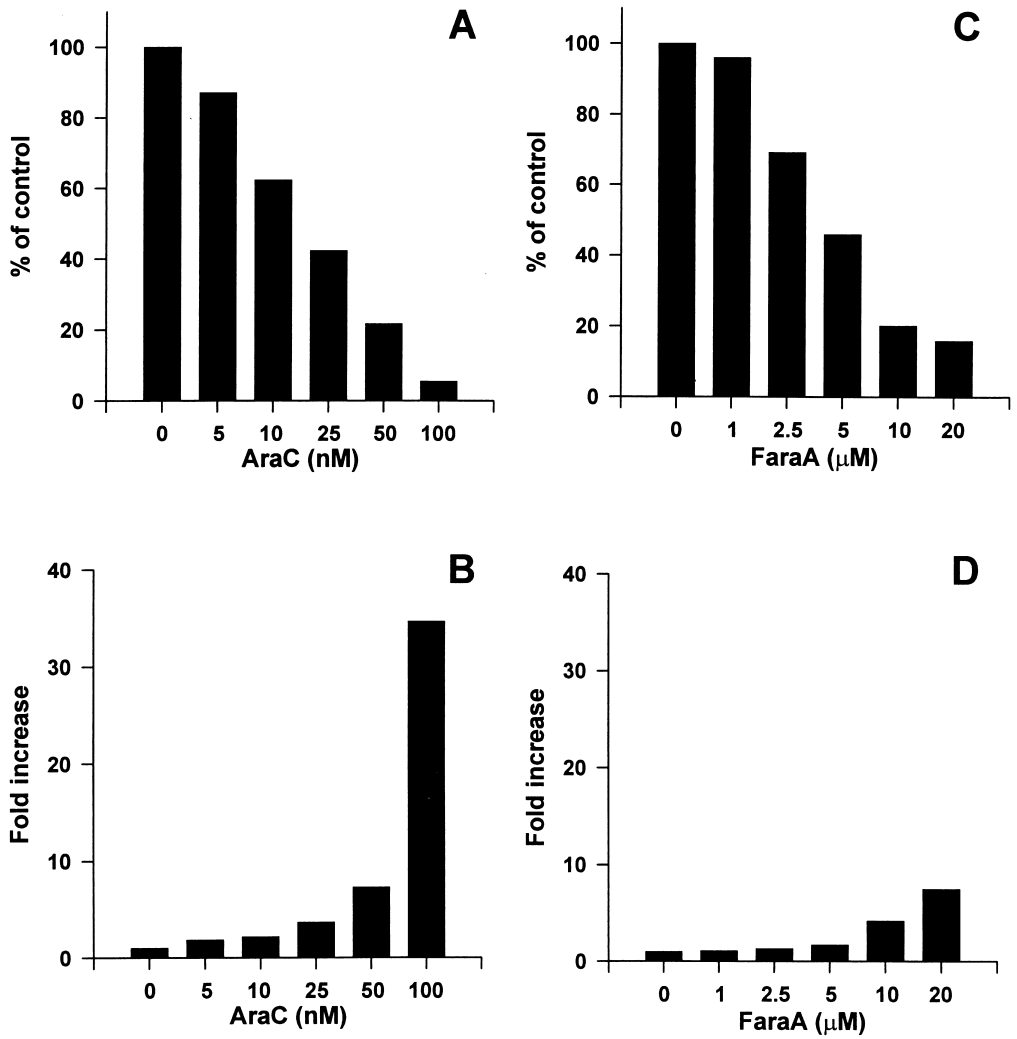

Fig. 5. Effects of araC and FaraA on the cloning efficiency and frequency of PALA resistance. CEM-2B cells were incubated with the indicated concentrations of araC or FaraA for 16 hr. The cells then were washed, counted, and cloned in 0.3% soft agar in either the presence or the absence of PALA (120 μM). After 12 days the colonies were counted. The experiment was done three times, and the results from a representative experiment are shown. (A) Cloning efficiency of araC-treated cells. Results are expressed as the percentage of the cloning efficiency of untreated control cells. The cloning efficiency of untreated cells was 31.4 $\pm$ 3.3% (mean $\pm$ SD). (B) Effect of araC on the frequency of PALA resistance. Results are expressed as fold increase in PALA resistance in araC-pretreated cells compared with control cells. The frequency of PALA resistance in control cells was $3 \pm 0.4 \times 10^{-3}$. To calculate the fold increase in PALA resistance, the cloning efficiency of araC-treated + PALA selected cells was corrected for the decrease in cell viability induced by the corresponding concentrations of araC alone. (C) Cloning efficiency of FaraA-treated cells. Results are expressed as the percentage of the cloning efficiency of untreated control cells. (D) Effect of FaraA on the frequency of PALA resistance. Results are expressed as fold increase in PALA resistance in FaraA-pretreated cells compared with control cells. In this experiment, the frequency of PALA resistance in control cells was $4 \pm 0.5 \times 10^{-3}$. The fold increase in PALA resistance was calculated as described in panel B.

tions may inhibit other polymerases in addition to DNA primase [29] and thereby induce genomic instability.

Gene amplification is one form of genomic instability that can be readily evaluated in drug-treated cells. PALA-resistant colonies were isolated from the agar plates that were used to assess cloning efficiency. The relative CAD gene copy number was then determined by slot-blot hybridization to ^{32}P-labeled cDNA probes for the CAD and GAPDH genes. Figure 6A shows the relative CAD gene copy number in control and various PALA-resistant clones. In cells not pretreated with araC but selected with the lowest concentration of PALA (120 μM), only one out of five colonies had a detectable increase (1.5-fold) in relative CAD gene copy number compared with control clones. In non-pretreated cells selected at the highest concentration of PALA (240 μM), CAD gene copy number was 1.5- to 2.5-fold greater than in control clones. AraC pretreatment increased both the frequency and the extent of CAD gene amplification in cells selected with either 120 or 240 μM PALA. At 50 nM araC + 240 μM PALA, the CAD gene copy number was increased 3- to 5-fold. Since CEM-2B cells have 4 copies of chromosome 2, a 3- to 5-fold increase is equivalent to 12–20 copies of the CAD gene per cell.

To verify that the increase in CAD gene copy number

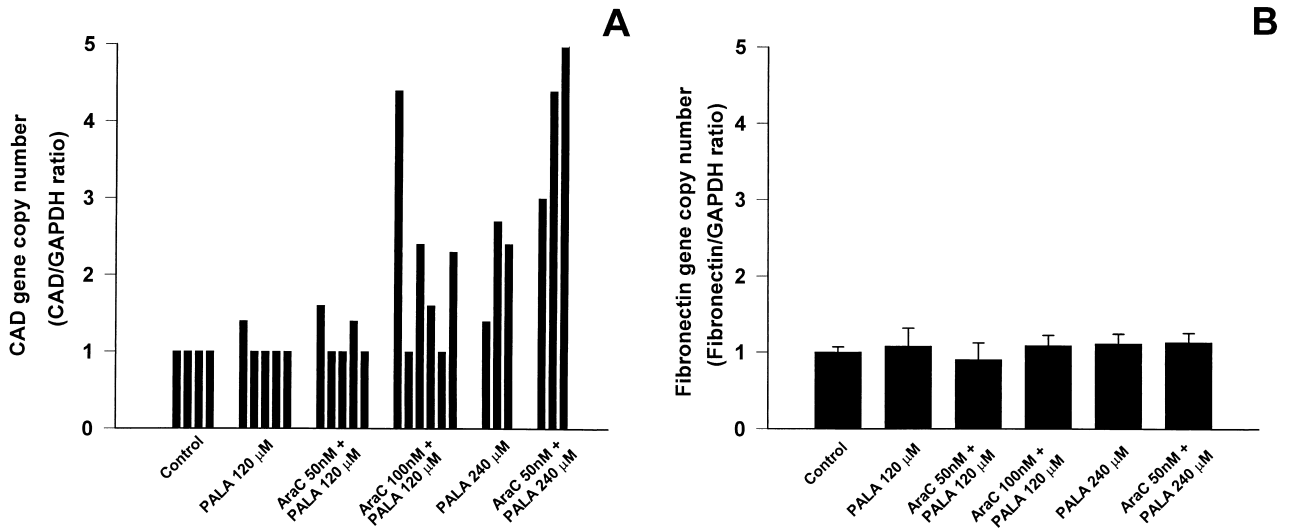

Fig. 6. Relative CAD gene copy number in control and PALA-resistant clones. Control and araC-treated cells were cloned in the presence of 0, 120, or 240 μM PALA. Individual colonies of $\sim$ 100 cells were isolated at random from plates after PALA selection and grown in the presence of the corresponding concentration of PALA. Cells were expanded to 1×10^6, and the DNA was extracted. Equal amounts of DNA were analyzed by slot-blotting. Triplicate blots were hybridized to radiolabeled probes for the CAD, GAPDH, or fibronectin-1 genes. Results are expressed either as the CAD/GAPDH ratio (A) or the fibronectin/GAPDH ratio (B) of the individual colonies that survived PALA selection.

was due to gene amplification rather than to changes in ploidy (e.g. extra copies of chromosome 2), we also measured the fibronectin-1 gene copy number relative to the GAPDH gene copy number in control and PALA-resistant colonies. The fibronectin-1 gene is located on the q arm of chromosome 2, whereas the CAD gene is on the p arm. Therefore, if the drug-resistant cells contained extra copies of chromosome 2, then the ratio of the fibronectin-1 gene relative to the GAPDH gene should also be increased in PALA-resistant cells compared with untreated control cells. In contrast, the CAD/GAPDH ratio should be higher than the fibronectin-1/GAPDH ratio in cells that have specifically amplified the CAD gene. There was no increase in the fibronectin-1/GAPDH ratio in any of the PALA-resistant colonies (Fig. 6B). This indicated that the increase in CAD gene copies observed in the PALA-resistant cells was not due to an increase in ploidy but to gene amplification.

It was not possible to determine whether FaraA induced genomic instability using gene amplification as an endpoint. None of the PALA-resistant colonies isolated from cells pretreated with 5–20 μM FaraA could be expanded to 1×10^6 cells in the presence of 240 μM PALA. This suggested that FaraA, which did not induce imbalanced DNA synthesis, likewise did not induce the formation of stable CAD amplicons. It was also possible that the accumulation of DNA damage in the FaraA-treated cells prevented their clonal expansion in the presence of PALA.

4. Discussion

The goal of the present study was to provide insights into the mechanisms by which araC and FaraA affect DNA replication and genomic stability. DNA re-replication is known to occur when mitosis is blocked (e.g. by microtubule inhibitors) in cells that have alterations in cell cycle checkpoints [30]. However, this phenomenon is not observed consistently in cells incubated with inhibitors of DNA synthesis [31]. Our results indicate that neither araC nor FaraA induced a detectable amount of DNA re-replication in CEM cells, although it was possible that DNA re-replication was induced at levels that were below the limit of sensitivity of the assay. In contrast, our data demonstrated that araC induces a specific alteration in replication fork progression. Analysis of newly replicated DNA synthesized in the presence of araC revealed a greater inhibition of leading strand synthesis compared with lagging strand synthesis. In cells incubated with 25 nM araC, a concentration that inhibited total DNA synthesis by about 90%, newly replicated DNA on the lagging strand was enriched about 5-fold compared with newly replicated DNA on the leading strand. In contrast, FaraA did not induce imbalanced DNA synthesis at a concentration that likewise decreased total DNA synthesis 90% compared with the untreated control. We analyzed nascent DNA at the level of the c-*myc* locus because both the origin of replication and the direction of replication fork movement had been determined previously in CEM cells [22,25,26]. However, one would expect to see similar effects of araC on lagging strand synthesis throughout the entire genome.

To the best of our knowledge, this is the first direct demonstration of imbalanced DNA synthesis induced by an anticancer drug in whole cells. The observed effects appear consistent with the known biochemical mechanisms of these arabinonucleoside analogues. AraCTP is an inhibitor of

DNA polymerases δ and ϵ [2–6,32], but does interfere with Okazaki fragment synthesis on the lagging strand [7,9]. Our analysis of replication fork progression indicates that DNA synthesis on the leading strand is reduced more than lagging strand synthesis, although araC reduces the overall rate of DNA synthesis to < 10% of control. This suggests that leading strand DNA synthesis, which is catalyzed by polymerase δ or possibly ϵ [33], is inhibited almost completely in the presence of araC. Lagging strand synthesis, which is mainly catalyzed by the DNA polymerase α–primase complex, is much less affected. This would lead to the accumulation of Okazaki fragments on the lagging strand of the fork in spite of inhibition of total DNA synthesis by araC [7]. In prokaryotic cells there is considerable evidence of physical and functional coupling of the leading and lagging strand polymerases [34–36]. A similar model has been proposed for eukaryotic replicative polymerases [33]. Our data also indicate that DNA synthesis on the two strands of the fork is uncoupled in the presence of araC. However, further studies will be needed to directly address the question of whether araC induces either physical uncoupling of the polymerases or uncoupling of their activities. Regardless of the exact mechanism of the uncoupling, this phenomenon may generate asymmetric forks with areas of single-stranded DNA corresponding to the site of blockage of DNA synthesis on the leading strand.

Our studies suggest that FaraA at equitoxic concentrations is less likely to induce imbalanced DNA synthesis than araC. The decreased ability of FaraA to induce imbalanced DNA synthesis may be related to the different mechanism of inhibition of DNA synthesis, is a primary target of FaraA [5,7,12]. FaraATP has been shown to inhibit primer RNA and Okazaki fragment synthesis in both a cell-free system and whole cell lysates [7,12]. By inhibiting the synthesis of RNA primer and Okazaki fragments at replication origins on both the lagging and leading strand, FaraA may prevent origin activation and progression of replication forks. Therefore, inhibition of DNA synthesis by FaraA would be less likely to result in an imbalance between lagging and leading strand synthesis and accumulation of asymmetric replication forks.

To date, only emetine, a protein synthesis inhibitor, has been shown to induce imbalanced DNA synthesis in cells, with greater inhibition of the lagging strand than the leading strand [24]. Electron microscopy revealed that replicating SV40 DNA molecules in emetine-treated cells contained single-stranded gaps on only one arm of the replication fork [24]. None of the SV40 replication forks in untreated cells contained single-stranded gaps. This suggests that single-stranded DNA regions can be formed when DNA synthesis is unequally inhibited on the two arms of the forks. Thus, it is likely that similar single-stranded gaps are formed during DNA replication in araC-treated cells. This phenomenon may have biologically relevant consequences. The aberrant gap-containing replication forks that accumulate in araC-treated cells are likely to be good substrates for DNA breakage and/or illegitimate recombination. The latter events have been closely associated with drug-induced genomic instability and gene amplification [31]. In normal cells, gapped DNA induces p53-dependent G_1 arrest [37]. However, CEM cells are permissive for gene amplification because both alleles of the *p53* gene in these cells contain missense mutations [38]. In support of this hypothesis, we observed a significant increase in the extent of CAD gene amplification in cells pretreated with araC but not with an equitoxic concentration of FaraA.

In this report we describe the effects of araC and FaraA on replication fork progression and propose a mechanism by which araC, and perhaps other anticancer and genotoxic compounds, may induce genomic instability and gene amplification. Further studies are necessary to assess more directly the role of imbalanced DNA synthesis in these processes. An understanding of the molecular pathways that lead to drug-induced gene amplification may shed light on the mechanisms by which amplification of cellular oncogenes and drug-resistance genes occurs. This may prove to be also valuable to the design of therapeutic strategies aimed at preventing this phenomenon.

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