Cancer Chemother Pharmacol (1992) 31: 61–70

Cancer Chemotherapy and Pharmacology

Mechanistic implications of alterations in HL-60 cell nascent DNA after exposure to 1-β-D-arabinofuranosylcytosine*

Douglas D. Ross, Dennis P. Cuddy, Noam Cohen, and David R. Hensley

Division of Developmental Therapeutics, University of Maryland Cancer Center, and Division of Hematology (DDR), Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Received 14 September 1991/Accepted 29 May 1992

Summary. To improve our understanding of the mechanism of 1-β-D-arabinofuranosylcytosine (ara-C) incorporation into DNA, we investigated the physical properties (size, position of nucleoside incorporation) of small fragments of nascent DNA (nDNA) obtained by pH-step alkaline elution of intact HL-60 cells following their exposure to ara-C. In the pH-step alkaline elution procedure, the smallest fragments of nDNA elute at pH 11. Anion-exchange high-performance liquid chromatography (HPLC) of nDNA obtained by 1 h elution at pH 11.0 of lysed HL-60 cells revealed a preponderance of nDNA fragments ranging from 0.5 to 40 kb in control ([^{3}H]-dThd-labeled) cells. Exposure of cells to ara-C (0.8–1 μM) resulted in a loss of the preponderance of radiolabel in fragments of 0.5–40 kb along with redistribution of the radiolabel (from [^{3}H]-dThd or [^{3}H]-ara-C) into smaller nDNA fragments (predominantly <100 bases in length) as determined by HPLC. We used the ability of pH-step alkaline elution to provide these small nDNA fragments produced by ara-C to investigate the paradoxical behavior of ara-C as a chain terminator in cell-free DNA synthetic systems while being incorporated into an internucleotide position in intact cells. Following the digestion of purified nDNA with micrococcal nuclease and spleen phosphodiesterase II, the proportion of radiolabel in 3′-dNMP (indicating an internucleotide position) or free nucleoside (indicating a chain terminus position) was determined by reverse-phase HPLC. In digests of prelabeled genomic DNA, as expected, >90% of the radiolabel from [^{14}C]-dThd or [^{3}H]-ara-C was found to exist in an internucleotide position (as determined by co-chromatography with authentic 3′-dTMP or 3′-ara-CMP). In contrast, digests of nDNA that eluted at pH 11.0 revealed a significantly higher proportion of radiolabel in the chain terminus position (29%–35%) when the nDNA was obtained from cells exposed to 1 μM [^{3}H]-ara-C as compared with cells exposed to [^{3}H]-dThd or [^{3}H]-dCyd alone (<10%). These data obtained from pH-step alkaline elution of intact cells suggest that by causing the inhibition of chain elongation while failing to inhibit the formation of new nDNA replication intermediates, ara-C exposure leads to the production of very small nDNA fragments. This relative chain-terminating effect of ara-C is most apparent in the small nDNA replication fragments that elute at pH 11.0. Since ara-C is accumulated predominantly in an internal position, even in the small nDNA fragments that elute at pH 11.0, ligation and gap-filling mechanisms in the intact cell must rapidly transform ara-C from a chain terminus to an internal position.

* Supported in part by grant RO-1-CA40188 from the NIH, National Cancer Institute. Presented in part at the 81st and 82nd Annual Meetings of the American Association for Cancer Research in Washington, D. C. (May 1990), and Houston, Texas (May 1991), respectively

Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine ara-CMP, 1-β-D-arabinofuranosylcytosine monophosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; dNMP, deoxynucleoside monophosphate; nDNA, nascent DNA; dThd, dCyd, 2′-deoxycytosine; PBS, phosphate-buffered saline (0.85% NaCl, 6.7 mM potassium phosphate, pH 7.4); nuclease buffer, 0.5 mM TRIS, 2 mM $CaCl_2$, pH 8; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid

Correspondence to: D. D. Ross, University of Maryland Cancer Center, Bressler Research Building, Room 9-015, 655 West Baltimore St., Baltimore, MD 21201, USA

Introduction

There is consensus at present that the incorporation of ara-C into mammalian DNA results in its cytotoxic effects [12, 13]. Despite this agreement, the precise mechanism(s) by which incorporated ara-C interferes with the DNA replicative mechanism and effects cytotoxicity remain(s) unknown. To improve our understanding of the molecular interaction of ara-C with DNA replication, we have applied pH-step alkaline elution [5, 21], since this is a useful method to study the kinetics of transit of a radiolabeled precursor both through nDNA and into genomic DNA in intact cells. Using pH-step alkaline elution, we recently observed

that radiolabeled ara-C accumulated predominantly (>70%) in nDNA during the exposure of intact HL-60 cells to [^{3}H]-ara-C [21]. Furthermore, we noted that concentrations of ara-C as high as 10 μM caused relatively little inhibition of the incorporation of [^{3}H]-dThd into the smallest nDNA replication intermediates (those eluting at pH 11.0 and 11.3) as compared with the rather profound inhibition of incorporation observed among large nDNA replication intermediates (eluting at pH 11.5 or 12.1 or retained on the filter) [21]. We interpreted these findings as being consistent with ara-C's inhibiting chain elongation while failing to inhibit the formation of new nDNA fragments.

In cell-free systems, evidence has accumulated suggesting that DNA polymerase α cannot elongate an nDNA strand following the addition of an ara-CMP to the 3′ terminus [17, 18]. For example, in early in vitro cell-free studies using purified DNA polymerase α, Momparler [18] has demonstrated that following the addition of an ara-CMP residue, chain termination occurs. More recent studies by Mikita and Beardsley [17], who used synthetic oligonucleotides containing ara-C, serve to confirm this. Paradoxically, when DNA is isolated from cells that have been exposed to radiolabeled ara-C, the majority of the ara-C is found incorporated in an internucleotide position [7, 11], with only 5%–15% being found at the chain terminus, although this percentage increases to approximately 30% at high (10–100 μM) extracellular concentrations of ara-C [11]. An internucleotide position for incorporated ara-C in intact cells is also suggested by our previous pH-step alkaline-elution studies [21]; following the exposure of HL-60 cells to [^{3}H]-ara-C, we observed an orderly transit of radiolabel through progressively larger nDNA intermediates and, finally, into full-length nDNA (retained on the filter).

In the present work, we sought to expand our previous pH-step alkaline-elution studies of ara-C by investigating the effects of ara-C on the size of small nDNA replication intermediates. In addition, because of the ability of pH-step alkaline elution to obtain a variety of nDNA replication intermediates, we also sought to determine whether a species of nDNA obtained from intact cells exposed to ara-C could be identified wherein ara-C incorporation occurred significantly in a chain terminus position. The most likely candidates for this may be small, newly formed fragments of nDNA. Therefore, we undertook the present studies to augment our understanding of the nature of the small nDNA replication intermediates that are formed in the presence of ara-C, i.e., those that elute at pH 11.0 in the pH-step alkaline-elution procedure.

Materials and methods

Cell culture

HL-60 human leukemic cells (passages 30–80) were grown in RPMI 1640 medium precisely as described elsewhere [20]. Cells in the logarithmic growth phase were used in all studies. The HL-60 cells used in these studies were tested to ensure the absence of contamination by *Mycoplasma* (Gen Probe, San Diego, Calif.).

Radiolabeled compounds

[5-^{3}H]-Ara-C (specific activity, 30 Ci/mmol, [5-methyl-^{3}H]-dThd (specific activity, 45 or 86 Ci/mmol), [5-^{3}H]-dCyd (specific activity, 25.6 Ci/mmol), and [2-^{14}C]-dThd (specific activity, 51 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). Radiolabeled compounds were added to cultures of HL-60 cells as described in Results or in the figure legends.

pH-Step alkaline elution

This procedure was performed exactly as previously described [21]. Briefly, HL-60 cells labeled with the appropriate radioactive DNA precursor were deposited on Nucleopore filters (25 mm, 3.0-μm pore size; Costar Corp., Cambridge, Mass.) held in a "smokestack"-type filter-funnel apparatus, washed with ice-cold saline, and then lysed with 5 ml of a buffer consisting of 2.0 M NaCl, 0.02 M Na_2-EDTA, and 0.3% (v/v) Sarkosyl detergent (pH 7.0). Thereafter, the lysed cells were washed with 3 ml of a solution containing 0.02 M Na_2-EDTA to remove the NaCl. Then, the exit tubing from the filter funnel was connected to a peristaltic pump (Minipuls III; Gilson Instrument Corp., Middleton, Wis.) and a solution containing 20 mM EDTA (free acid form) and tetrapropyl ammonium hydroxide (RSA Corp.; Ardsley, N. Y.) was added to the filter funnel and was pumped through the filter at a rate of 0.08 ml/min. After 1 h, the solution in the filter funnel was changed to one at pH 11.3 and the elution was continued for another hour; this procedure was repeated for successive elutions at pH 11.5 and 12.1. Following each application and prior to its reuse, each alkaline-elution filter funnel was washed extensively with detergent, after which a "mock" lysis, wash, and pH-step procedure was carried out (using the appropriate buffers but no cells). The radioactivity of the eluate of each step of this "mock" procedure was counted to ensure that the filters were absolutely free of radiolabel from previous experiments.

Purification of DNA

Genomic DNA was purified by the phenol/chloroform extraction-ethanol precipitation method as described elsewhere [3]. DNA collected by pH-step alkaline elution was purified by Centricon filtration as follows. An aliquot of 2.5 ml pH 11.0 buffer containing nDNA was neutralized with 1 *N* HCl and added to a Centricon 30 filter funnel (Amicon, W. R. Grace, Danvers, Mass.), which was centrifuged (5,000 *g*, 30 min). The filter was then washed twice with 2 ml 0.5 mM TRIS (pH 8) and inverted. Next, 200 μl 0.5 mM TRIS (pH 8) was added to the top, the funnel was centrifuged (2,000 *g*, 10 min), and the filtrate was collected. Finally, a 1/10 vol. of 20 mM $CaCl_2$ was added, and the filtrate was immediately used for enzyme digestion (see below). The yield of this procedure was 80%–90% for nDNA obtained from cells exposed to [^{3}H]-dThd or [^{3}H]-dCyd and 60%–80% for nDNA obtained from cells exposed to [^{3}H]-ara-C. Prior to their use, the Centricon 30 funnels were filled with a solution of salmon-sperm DNA (1 mg/ml, Sigma) for 1 h to prevent the binding of HL-60 DNA to the filters or plastic.

Enzymatic digestion of DNA

All nuclease digestions were performed immediately after the placement of DNA in calcium-containing nuclease buffer (0.5 mM TRIS, 2 mM $CaCl_2$, pH 8). To the purified DNA in nuclease buffer (usually 0.5–1 ml), 50 units micrococcal nuclease (EC 3.1.31.1, Sigma) was added and allowed to digest (37°C) for 45 min. The pH was then adjusted to 6 with 2 *N* HCl, and 5 units spleen phosphodiesterase II (EC 3.1.16.1, Sigma) was added. After a 30-min period of incubation at 37°C, an additional 5 units phosphodiesterase II was added and the mixture was incubated for an additional 30 min. The digest was then placed in a Centrifree (Amicon) filter funnel and centrifuged (2,000 *g*, 40 min), and the filtrate was used for HPLC analysis.

HPLC studies

Nucleoside/nucleotide chromatography. A reverse-phase C18 column (Radial Pak; Waters Associates, Milford, Mass.) installed in a Z-Module Radial Compression System (Waters) was used for nucleoside/nucleotide chromatography. Samples included the DNA digests (see above) and the appropriate authentic standards dissolved in distilled H_2O. The authentic standards included dThd, dCyd, 3′dTMP, and 3′CMP (all obtained from Sigma) as well as ara-C and 3′ara-CMP (generously donated as a gift from the Upjohn Company, Kalamazoo, Mich.). Linear-gradient elution conditions were applied (curve 6, Waters Model 660 Solvent Programmer) over a period of 30 min using 100% buffer A (0.01 M KH_2PO_4, pH 5.5) for the initial phase and 15% buffer B (80% methanol in water) in buffer A for the final phase at a flow rate of 1.5 ml/min. For each run of unknown DNA digest, the appropriate authentic standards were simultaneously injected. The radiolabel eluted from the DNA digests was detected by collecting fractions every 30 s during the run and then counting the radioactivity with a liquid scintillation counter (LS 5801; Beckman Instruments, Fullerton, Calif.). Detection of the authentic standards was accomplished by measuring the absorption at 254 nm (LC-85 Spectrophotometric Detector; Perkin Elmer, Norwalk, Conn.) and was recorded and integrated using a Model 740 Data Module (Waters). By the use of authentic radioactive and corresponding nonradioactive standards, a time delay of 30 s was noted between the radioactivity from the standard collected in a fraction and the optical detection of that same standard. This time lag was taken into account in the identification of unknown radioactive peaks through the use of authentic standards.

DNA chromatography. A Gen-Pak FAX column (Waters) coupled to a precolumn filter (Waters) was used for DNA chromatography. For these studies, a 5-ml sample-injection loop was added to the HPLC system described above. To each unknown sample (pH-step alkaline-elution fraction), 25 μg of a 1-kb DNA ladder (BRL, Gaithersberg, Md.) was added to serve as an internal standard. For certain samples, 10 μg each of $pd(A)_{12-18}$ and $pd(A)_{25-30}$ (Pharmacia LKB Biotechnology Inc., Piscataway, N. J.) were also added. Prior to injection, samples were filtered through a 0.45 μm filter (Millex-HV_4, Millipore Corp., Bedford, Mass.). Elution buffers consisted of buffer A (25 mM TRIS, pH 11.0) and buffer B (25 mM TRIS, pH 11.0, 1 M NaCl). The flow rate was 0.8 ml/min. Following the injection of sample and standards, the initial phase (see below) was continued for 10 min, then a linear gradient (increasing the percentage of buffer B in buffer A) was performed, after which the final phase was continued for another 5 min. For the best resolution of low-molecular-weight DNA fragments (<100 bases), the initial phase comprised 100% buffer A, and the gradient was increased from 0 to 50% buffer B in buffer A over a 100-min period. For optimal resolution of higher-molecular-weight DNA fragments (100–12,000 bases), the initial phase consisted of 37% buffer B in buffer A, with the gradient increasing from 37% to 49% buffer B in buffer A over a 50-min period. Detection of authentic standards was accomplished by UV absorption at 254 nm; radiolabeled DNA was detected by collecting fractions at 1-min intervals, after which the radioactivity in each fraction was determined by liquid scintillation counting.

Results

pH-Step alkaline elution of nDNA from HL-60 cells

Our previous kinetics studies of HL-60 cells using pH-step alkaline elution have demonstrated that the radiolabel eluting from cells exposed to [^{3}H]-dThd or [^{3}H]-ara-C represents incorporation into nDNA replication intermediates [21]. We find that 1 h exposure of HL-60 cells to [^{3}H]-dThd is sufficient to produce steady-state labeling of the discrete sizes of replication intermediates that elute under the conditions used in the pH-step alkaline-elution procedure (data not shown). When HL-60 cells in the log

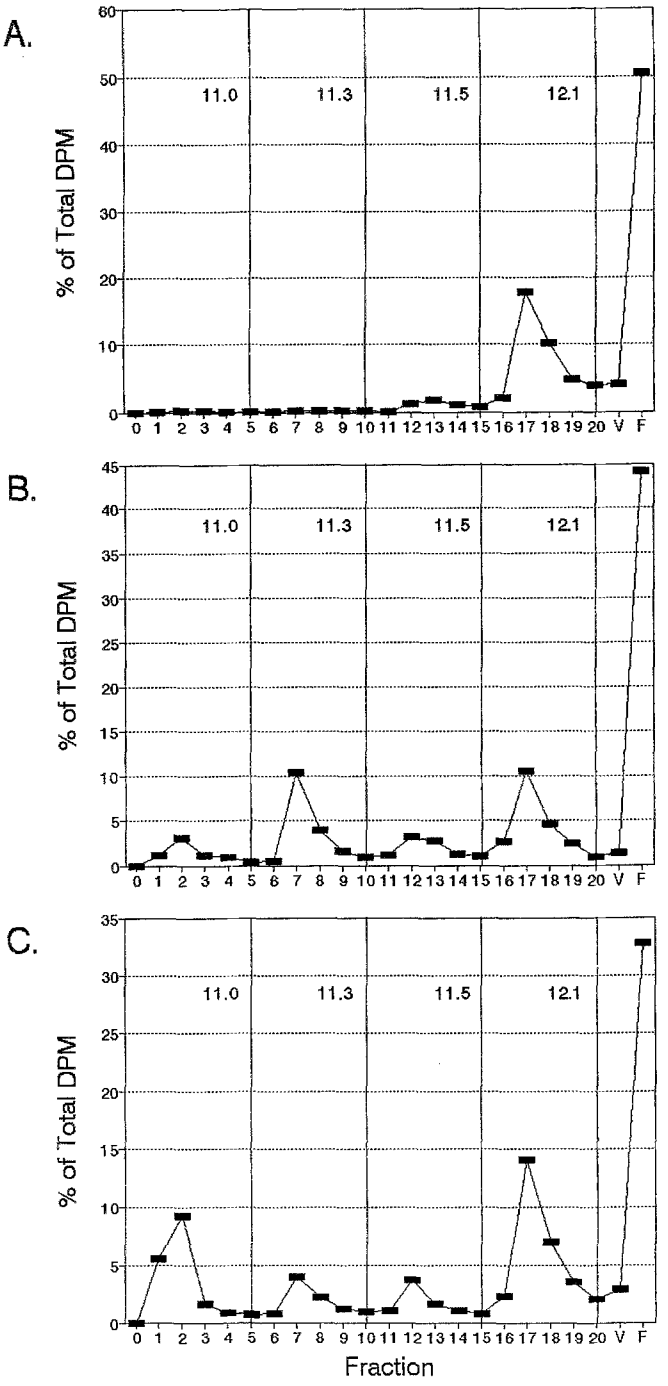

Fig. 1 A–C. Typical pH-step alkaline-elution profiles of HL-60 cells in the log growth phase following their exposure to radiolabeled compounds. **A** Cells were exposed to [^{3}H]-dThd (100 μCi ml^{-1} 0.75×10^{-6} cells; specific activity, 45 Ci/mmol) for 1 h. Total incorporation into DNA was 1,438,412 dpm 10^{-6} cells h^{-1}. **B** Cells were exposed to 1 μM ara-C for 1 h after which [^{3}H]-dThd (100 μCi ml^{-1} 0.75×10^{-6} cells; specific activity, 45 Ci/mmol) was added for an additional hour. Total incorporation into DNA was 39,541 dpm 10^{-6} cells h^{-1}. **C** [^{3}H]-ara-C (25 μCi ml^{-1} 1.1×10^{-6} cells; specific activity, 30 Ci/mmol) for 2 h. The final ara-C concentration was 0.8 μM. Total incorporation into DNA equaled 6,343 dpm 10^{-6} cells h^{-1}

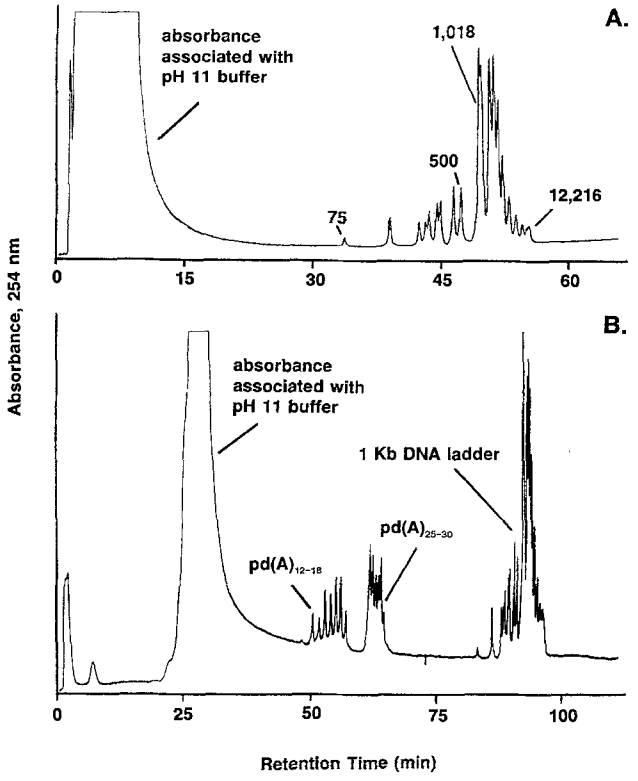

Fig. 2. A Typical Gen-Pak FAX HPLC chromatogram of 25 μg of a 1-kb DNA ladder, obtained using HPLC conditions favoring resolution of higher-molecular-weight DNA as described in Materials and methods. **B** Typical Gen-Pak FAX HPLC chromatogram of 10 μg each of $pd(A)_{12-18}$ and $pd(A)_{25-30}$ and of 25 μg of a 1-kb DNA ladder, obtained using HPLC conditions favoring resolution of low-molecular-weight DNA as described in Materials and methods

growth phase are exposed to [^{3}H]-dThd for 1 h and then subjected to pH-step alkaline elution, an elution pattern typical of that shown in Fig. 1 A is obtained. Note that most of the radiolabel distributes into what should be very large nDNA replication intermediates (those eluting at pH 12.1 or retained on the filter – see [4, 18] for a description of the nDNA size range obtained using pH-step alkaline elution). When [^{3}H]-dThd is added to ara-C-pretreated cultures (Fig. 1 B) or when cells are exposed to [^{3}H]-ara-C alone (Fig. 1 C), in contrast to the data displayed in Fig. 1 A, a greater proportion of radiolabel distributes into what would be expected to be smaller nDNA replication intermediates (i.e., those eluting at pH 11.0, 11.3, and 11.5). These findings are consistent with the inhibition of DNA elongation caused by ara-C and are in agreement with the results of our previous pH-step alkaline-elution studies of ara-C [21]. The data in Fig. 1 are shown because they represent pH-step alkaline-elution profiles that are typical for the studies discussed in this paper.

Gen-Pak FAX HPLC analysis of the size of nDNA eluted from HL-60 cells at pH 11

We used a high-performance anion-exchange column (Gen-Pak FAX) [16, 22, 24, 25] and a 5-ml sample-injection loop for this investigation. Thus, the method enabled us to inject virtually all of a single 60-min pH-step fraction (4.8 ml) immediately after its collection. We selected this method rather than denaturing agarose or polyacrylamide gels or alkaline sucrose gradients because the HPLC method not only provides good resolution of low-molecular-weight DNA species but also enables the direct application of the sample (without prior concentration) and the ability to detect directly the chromatographic distribution of radiolabel in the collected fractions. One drawback of the HPLC procedure is its inability to resolve very large DNA fragments (>50,000 bases), which makes it unsuitable for the sizing of nDNA fragments that elute at pH 11.3 or higher under control conditions. To maintain the nDNA in a single-stranded state and to prevent aggregation by random annealing, we kept the mobile phase at a denaturing pH (11.0), yet managed to accomplish satisfactory chromatographic separation of authentic DNA standards.

Figure 2 A shows a typical chromatogram of 25 μg of a 1-kb DNA ladder (DNA marker standard, obtained from Bethesda Research Labs, Gaithersberg, Md.) injected in a volume of 4.8 ml of pure pH 11.0 alkaline-elution buffer and run under conditions favoring resolution of higher-molecular-weight DNA (see Materials and methods). The material showing high absorption at 254 nm and eluting at between 0 and 15 min represents the solute in the pH 11.0 buffer injected, since no absorption occurs at this point if the 1-kb ladder is injected in the absence of this buffer. No significant difference is seen in the chromatogram if the 1-kb ladder is first heated to 100°C for 15 min and then rapidly cooled. The chromatogram obtained for the 1-kb DNA-ladder standard is in excellent agreement with those obtained for this reference standard by the manufacturer of the Gen-Pak FAX column [16, 25]. From the DNA fragment sizes shown in Fig. 2 A it can be seen that under the chromatographic conditions used, we managed to obtain particularly good resolution of small DNA fragments (from 75 to 500 bases). Figure 2 B shows a typical chromatogram of $pd(A)_{12-18}$, $pd(A)_{25-30}$, and the 1-kb DNA ladder, obtained under elution conditions favoring resolution of low-molecular-weight DNA fragments (see Materials and methods). Note that excellent chromatographic separation of the components of the $pd(A)_{12-18}$ and $pd(A)_{25-30}$ standards was obtained. When run under the conditions used for the resolution of larger DNA fragment sizes (Fig. 2 A), both $pd(A)_{12-18}$ and $pd(A)_{25-30}$ eluted during the initial 10 min (data not shown).

First, we used HPLC conditions favoring the resolution of relatively high-molecular-weight DNA to study the nDNA that eluted at pH 11.0 in the pH-step alkaline-elution procedure. Although the percentage of label eluting at pH 11.0 in Fig. 1 A *appears* to be low (approximately 0.5% of the total radioactivity incorporated), over 5,000 dpm of incorporated label was actually recovered from this fraction, which was sufficient for subsequent Gen-Pak FAX HPLC studies. Figure 3 A shows a typical chromatogram of nDNA eluted at pH 11.0 from HL-60 cells exposed to [^{3}H]-dThd for 1 h, along with the retention times of some of the fragments in the 1-kb DNA-ladder standard. The relationship between DNA-standard fragment size and retention time typically displays a complex nature, as reported previously [16], with the highest degree of resolu-

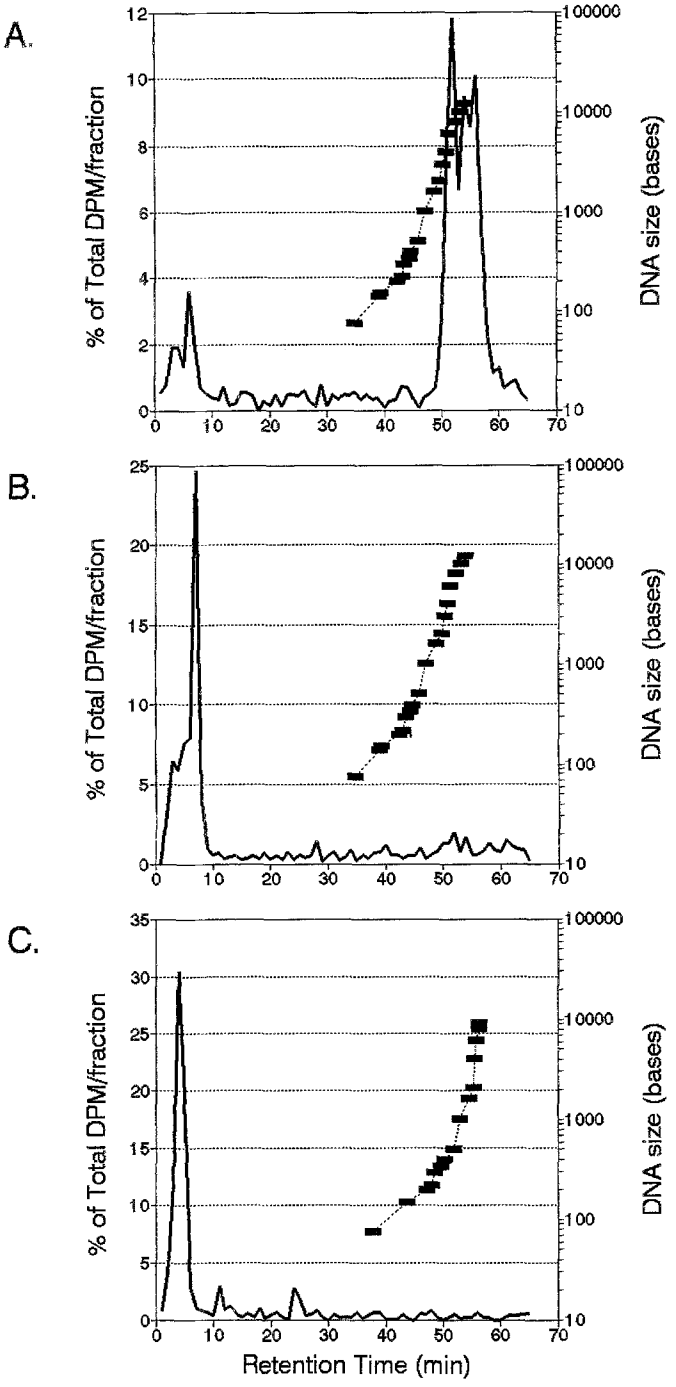

Fig. 3 A–C. Typcial Gen-Pak FAX HPLC chromatograms of nDNA collected by 1 h alkaline elution at pH 11.0 of HL-60 cells in the log growth phase following their exposure to radiolabeled compounds. **A** Cells were exposed to [^{3}H]-dThd (100 μCi ml^{-1} 0.75×10^{-6} cells; specific activity, 45 Ci/mmol) for 1 h. The total radioactivity applied to the column was 3,260 dpm; the recovery of radioactivity from the column was 100%. **B** Cells were exposed to 1 μM ara-C for 1 h, after which [^{3}H]-dThd (100 μCi ml^{-1} 0.75×10^{-6} cells; specific activity, 45 Ci/mmol) was added for an additional hour. The total radioactivity applied was 2,300 dpm; the recovery of radioactivity was 100%. **C** Cells were exposed to [^{3}H]-ara-C (25 μCi ml^{-1} 1.1×10^{-6} cells; specific activity, 30 Ci/mmol) for 2 h. The final ara-C concentration was 0.8 μM. The total radioactivity applied to the column was 1,350 dpm; the recovery of radioactivity from the column was 100%. The elution conditions used were those favoring resolution of nDNA fragments consisting of <30 bases (see Materials and methods). *Solid lines*, Radiolabeled nDNA eluted; ■ - - - - ■, DNA standards (1-kb DNA ladder)

tion being obtained for smaller (<500 bases) DNA fragments.

In Fig. 3A it can be seen that the majority (approximately 70%) of the radiolabel is associated with a heterogenous peak that elutes between 50 and 60 min, corresponding to DNA fragments of between 1,000 and 40,000 bases in length. Smaller peaks elute at between 0 and 10 min, which may correspond to very small oligonucleotides considerably smaller than 75 bases in length. A low-level continuum of radiolabel is also visible eluting at between 10 and 50 min, which corresponds to the incorporation of [^{3}H]-dThd into nDNA fragments varying in size up to 1 kb. In contrast, preexposure of cells to ara-C (1 μM) causes 65% of the [^{3}H]-dThd label to accumulate in material eluting in less than 10 min (Fig. 3B), with the remainder eluting as a broad continuum of size ranges throughout the rest of the chromatogram. It is noteworthy that the preponderance of label eluting in the heterogeneous 1- to 40-kb species as seen in the control is lost and an increase in the proportion of label eluting in the first 10 min is evident. This indicates that nDNA fragments formed in the presence of ara-C are indeed quite small, certainly smaller than 75 bases, and probably <30 bases [since pd(A)$_{25-30}$ elutes in the first 10 min under the elution conditions described in Fig. 3]. Similarly, the Gen-Pak FAX chromatogram of nDNA obtained by pH 11 elution of cells exposed to [^{3}H]-ara-C (0.8 μM, Fig. 3C) is very similar to that of cells pretreated with ara-C and then exposed to [^{3}H]-dThd (Fig. 3B). In each experiment shown in Fig. 3, we recovered virtually 100% of the radioactivity applied to the column. The alkaline-elution profiles that correspond to Figs. 3A, 3B, and 3C are shown in Figs. 1A, 1B, and 1C, respectively. These studies were repeated numerous times with similar results.

Because the HPLC gradient conditions used in the experiments depicted in Fig. 3 could not resolve DNA oligomers of <30 bases in length [the pd(A)$_{12-18}$ and pd(A)$_{25-30}$ eluted in the first 10 min of initial conditions – see above], we next adjusted the HPLC elution conditions to favor resolution of low-molecular-weight DNA species (Fig. 4 displays the data obtained in a typical experiment). Controls (Fig. 4A) that were exposed to [^{3}H]dThd but not to ara-C displayed an HPLC elution profile similar to that shown in Fig. 3A, with a predominance of fragments ranging from approximately 0.5 to 40 kb being eluted, accompanied by a low-level continuum of radiolabeled fragments eluting at between 10 and 90 min. In contrast (Fig. 4B), the HPLC elution profile of nDNA obtained from cells exposed to [^{3}H]-ara-C (1 μM) displayed only the broad continuum of radiolabeled DNA sizes ranging from molecules that may be as small as dimers or trimers (if one extrapolates the DNA-standard curve) to those over 40 kb in length, with distinct loss of the preponderance of labeling in the size range of 0.5–40 kb occurring as seen in the control. In Fig. 4B it can be seen that only 25% of the radioactivity eluted during the first 10 min (initial conditions), in contrast to the 65% that was observed to elute during the first 10 min under gradient conditions favoring resolution of high-molecular-weight DNA species (Figs. 3B, C), indicating that the material that eluted in the first 10 min during the experiments depicted in Figs. 3B

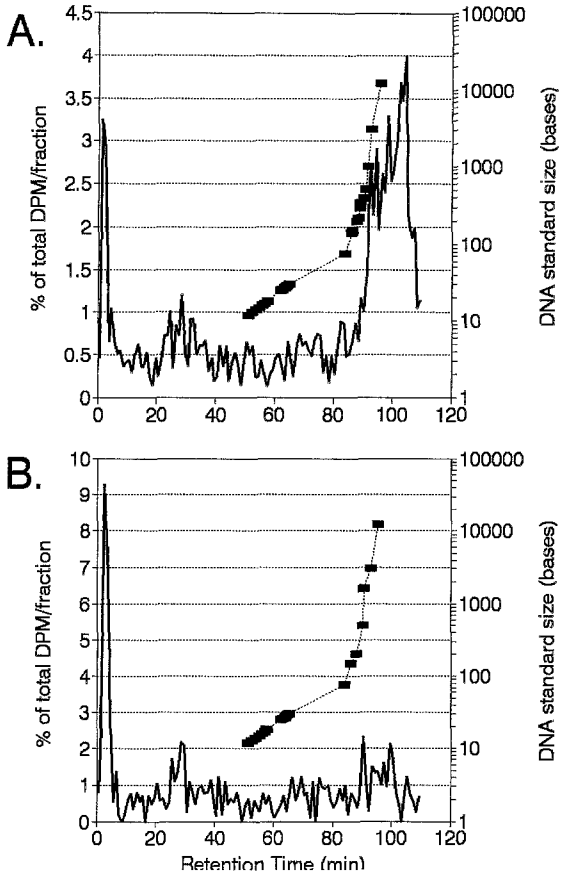

Fig. 4 A, B. Gen-Pak FAX HPLC chromatograms of nDNA collected by 1 h alkaline elution at pH 11.0 of HL-60 cells in the log growth phase following their exposure to radiolabeled compounds. **A** Cells were exposed to [^{3}H]-dThd (25 μCi ml^{-1} 0.9×10^{-6} cells; specific activity, 45 Ci/mmol) for 30 min. The total radioactivity applied to the column was 4,750 dpm; the recovery of radioactivity from the column was 93%. **B** Cells were exposed to [^{3}H]-ara-C (30 μCi ml^{-1} 0.9×10^{-6} cells; specific activity. 30 Ci/mmol) for 13 h. The final ara-C concentration was 1.0 μM. The total radioactivity applied to the column was 2,120 dpm; the recovery of radioactivity from the column was 99.5%. The elution conditions used were those favoring resolution of small nDNA fragments (see Materials and methods). *Solid lines*, radiolabeled nDNA eluted; ■ - - - - ■, DNA standards (1-kb DNA ladder), pd(A)$_{12-18}$ and pd(A)$_{25-30}$

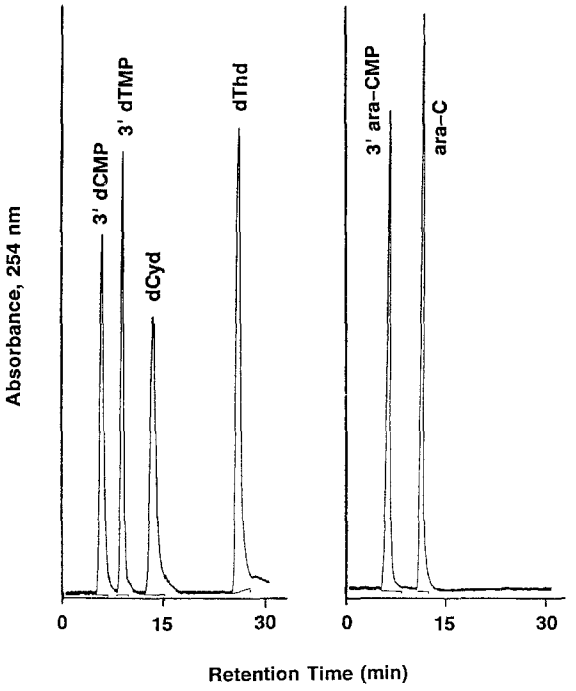

Fig. 5. Typical HPLC chromatograms of authentic nucleoside and nucleotide standards. In all, 25 nmol each of either 3′dCMP, 3′dTMP, dCyd, and dThd or 3′ara-CMP and ara-C were injected, after which HPLC analysis was performed as described in Materials and methods. Detection was accomplished by monitoring absorbance at 254 nm

and 3C consisted mostly of short oligonucleotides rather than unincorporated, radiolabeled monomeric nucleotides or nucleosides. The radioactivity seen eluting with the solvent front (i.e., within <10 min) in Figs. 4A and 4B may indeed represent unincorporated nucleoside or nucleotide (nucleoside and dNTPs eluted in this area under the conditions used for the experiments shown in Fig. 4 – data not shown) or very small oligonucleotides (e.g., ranging from dimers to pentamers). Hence, considering the data shown in Figs. 3 and 4, we estimate that the length of the nDNA fragments that accumulate in the presence of ara-C is predominantly <30 bases and may actually be as small as a few bases. The experiments depicted in Fig. 4 were repeated numerous times with similar results.

Position of radiolabeled nucleoside in DNA

To determine the position of radiolabeled precursor in DNA, we used a procedure involving digestion of the DNA with micrococcal nuclease and spleen phosphodiesterase II [9]. This procedure results in a digest consisting of 3′-deoxynucleoside monophosphates. However, a nucleotide occupying a chain terminus position would be converted to the corresponding free nucleoside. We used HPLC to quantify the proportion of radiolabel from a given labeled precursor in 3′-deoxynucleoside monophosphate versus the free nucleoside. Figure 5 shows typical chromatograms obtained for our authentic nucleoside and 3′-deoxynucleoside monophosphate standards.

To confirm that the enzyme-digestion system was functioning properly, we labeled HL-60 genomic DNA by pulse-chase exposure to [^{14}C]-dThd (24-h pulse, 24-h chase) or [^{3}H]-ara-C (3-h pulse, 12-h chase) and then isolated it by phenol/chloroform extraction as described in Materials and methods. The pulse-chase method of exposure to ara-C selected was one whereby the majority of radiolabeled ara-C would be expected to appear in genomic DNA as based on our previous pH-step alkaline-elution studies [21]. As expected, the [^{14}C]-dThd label was found predominantly (90%) in 3′dTMP following DNAse digestion, indicating an internucleotide position (Fig. 6A). Similarly, and consistent with previous reports [7, 11], ara-C was distributed predominantly in an internal position in genomic DNA, as evidenced by recovery of 95% of the radioactivity in 3′ara-CMP, following the exposure of HL-60 cells to 1 μM [^{3}H]-ara-C (Fig. 6B).

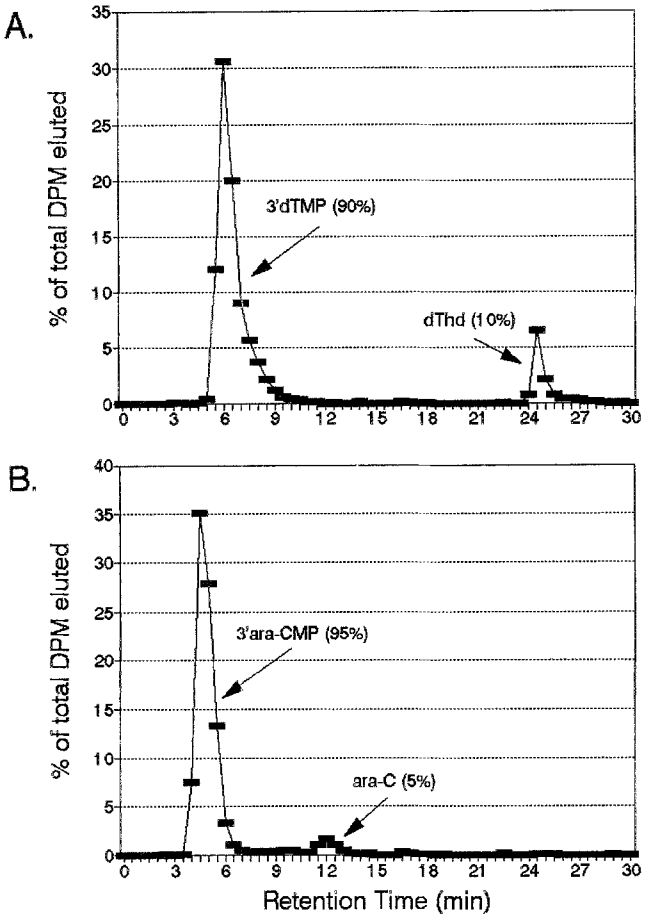

Fig. 6 A, B. HPLC analysis of DNA from HL-60 cells prelabeled with [^{14}C]-dThd or [^{3}H]-ara-C, obtained after digestion with micrococcal nuclease and spleen phosphodiesterase II. **A** Cells were exposed to 1 μM [^{14}C]-dThd (0.05 μCi ml^{-1} 10^{-6} cells; specific activity, 51 mCi/mmol) for 24 h, then washed free of radiolabel and placed in culture for another 48 h. Genomic DNA was isolated by phenol/chloroform extraction, digested, and analyzed as described in Materials and methods. The yield of the purification procedure was 25%. The total radioactivity applied to the column was 9,184 dpm. The identity of the radioactive peaks was established by co-chromatography with authentic 3′dTMP and dThd, detected by absorption at 254 nm. **B** Cells were exposed to 1 μM [^{3}H]-ara-C (30 μCi ml^{-1} 10^{-6} cells; specific activity, 30 Ci/mmol) for 3 h, then washed free of radiolabel and placed in culture for another 12 h. Genomic DNA was isolated by phenol/chloroform extraction, digested, and analyzed as described in Materials and methods. The yield of the purification procedure was 23%. The total radioactivity applied to the column was 22,302 dpm. The identity of the radioactive peaks was established by co-chromatography with authentic 3′ara-CMP and ara-C, detected by absorption at 254 nm

We focused next on nDNA eluting at pH 11.0 in the pH-step alkaline-elution procedure. Our previous pH-step alkaline-elution studies [21] served to indicate that the radiolabeled DNA thus obtained consists predominantly of nDNA and not of mitochondrial DNA or parental DNA fragments that have been radiolabeled via DNA repair synthesis. For these investigations, nDNA was purified directly from the neutralized pH 11.0 alkaline-elution buffer by the use of Centricon 30 filters [1]. As controls, we performed studies on the position occupied by [^{3}H]-dThd or -dCyd incorporated into nDNA. The results are summarized in Table 1, and Fig. 7 shows typical chromatograms

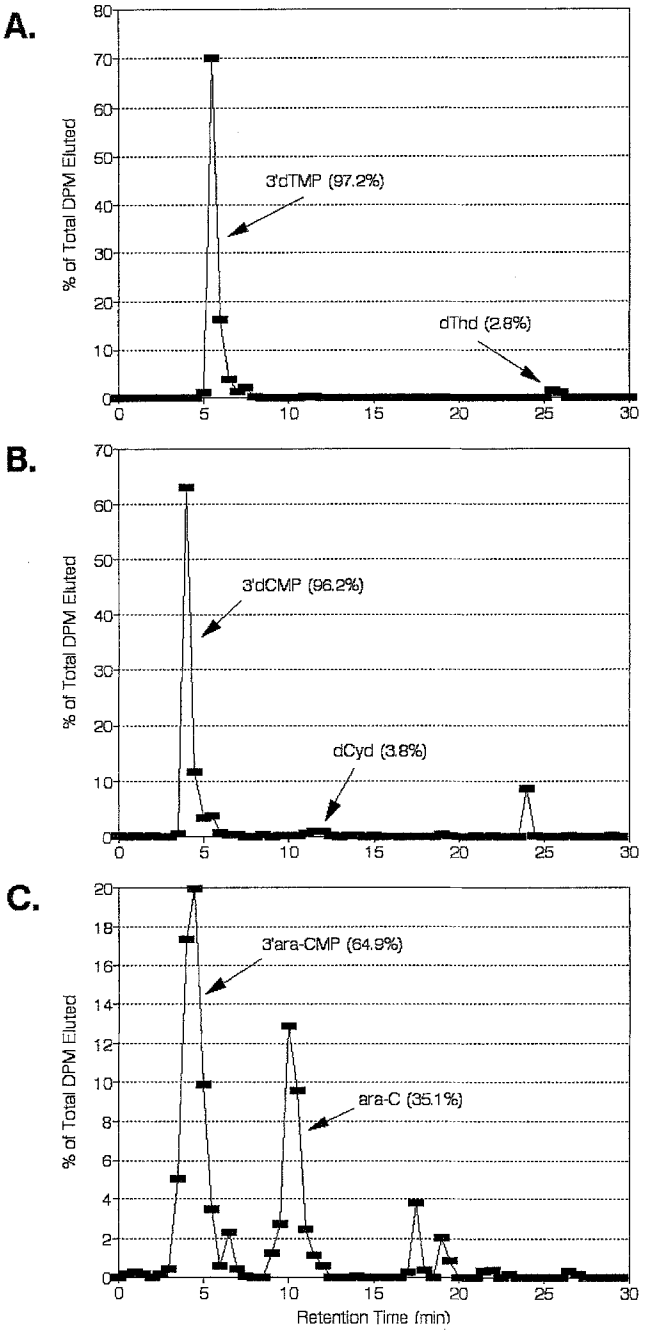

Fig. 7 A–C. HPLC analysis of nDNA obtained by a single pH-step elution (pH 11.0, 1 h) or HL-60 cells in the log growth phase after their exposure to [^{3}H]-dThd, [^{3}H]-dCyd, or [^{3}H]-ara-C and subsequent digestion with micrococcal nuclease and spleen phosphodiesterase II. The nDNA was purified by Centricon 30 filtration as described in Materials and methods. The data shown are typical of those obtained in repeated experiments (see Table 1). **A** Cells were exposed to 0.3 μM [^{3}H]-dThd (25 μCi ml^{-1} 10^{-6} cells; specific activity, 86 Ci/mmol) for 1 h (corresponding to dThd experiment 2, Table 1). The identity of the radioactive peaks was established by co-chromatography with authentic 3′dTMP and dThd, detected by absorption at 254 nm. **B** Cells were exposed to 1.0 μM [^{3}H]-dCyd (25 μCi ml^{-1} 0.7 × 10^{-6} cells; specific activity, 25.6 Ci/mmol) for 1 h (corresponding to dCyd experiment 1, Table 1). The identity of the radioactive peaks was established by co-chromatography with authentic 3′dCMP and dCyd, detected by absorption at 254 nm. **C** HL-60 cells were exposed to 1.0 μM [^{3}H]-ara-C (30 μCi ml^{-1} 10^{-6} cells; specific activity, 30 Ci/mmol) for 17 h (corresponding to ara-C experiment 1, Table 1). The identity of the radioactive peaks was established by co-chromatography with authentic 3′ara-CMP and ara-C, detected by absorption at 254 nm

Table 1. Distribution of [^{3}H]-dThd, [^{3}H]-dCyd, or [^{3}H]-ara-C in nDNA as isolated by elution at pH 11.0 for 1 h

Labeled compound	Experiment	Radioactivity recovered by HPLC (dpm)	% 3′-dNMP (internucleotide)	% Nucleoside (chain terminus)
[^{3}H]-dThd	1	32,980	94.8	5.2
	2	71,088	97.2	2.8
[^{3}H]-dCyd	1	5,137	96.2	3.8
	2	5,718	90.1	9.9
[^{3}H]-ara-C	1	3,507	64.9	35.1
	2	2,396	70.5	29.5

HL-60 cells in culture in the log growth phase were exposed to [^{3}H]-dThd (0.3 μM, 86 Ci/mmol; 25 μCi ml^{-1} 10^{-6} cells, 1 h), to [^{3}H]-dCyd [1 μM, 25.6 Ci/mmol; 25 μCi ml^{-1} 10^{-6} cells, for 1 h (experiment 1) or 2 h (experiment 2)], or to [^{3}H]-ara-C (1 μM, 30 Ci/mmol; 30 μCi ml^{-1} 10^{-6} cells, 17 h) and were then subjected to pH-step alkaline elution as described in Materials and methods. Material in the pH 11.0 eluate was purified by Centricon 30 filtration and subjected to enzyme digestion, and the digest was then analyzed by HPLC as described in Materials and methods. Radioactivity in the HPLC eluate was identified using the appropriate nonradioactive 3′-dNMP or deoxynucleoside as internal standards, which were detected by absorption at 254 nm as described in Materials and methods. Recovery of radioactivity following HPLC was >95% of the amount injected

of the radioactivity eluted in these studies. The percentages given in Table 1 are based on the sum of radioactivity that co-chromatographed with the appropriate deoxynucleoside and 3′-deoxynucleoside monophosphate standards. This sum was routinely >80% of the total radioactivity applied to the HPLC system. For cells exposed to [^{3}H]-dThd (0.3 μM, 1 h) in replicate experiments (Table 1, Fig. 7A), >90% of the radiolabel was found in an internucleotide position (3′dTMP) following digestion of the nDNA that eluted at pH 11.0, exactly as was observed for [^{14}C]-dThd incorporated into genomic DNA. Similarly, when [^{3}H]-dCyd was used (cells were exposed to 1.0 μM for 1 or 2 h), >90% of the radiolabel was found in 3′dCMP (Table 1, Fig. 7B). The total amounts of radioactivity incorporated in the [^{3}H]-dCyd studies shown in Table 1 were lower than those incorporated in the [^{3}H]-dThd experiments because the specific activity of the [^{3}H]-dCyd used (25.6 Ci/mmol) was lower than that of the [^{3}H]-dThd applied (86 Ci/mmol) and because any deamination of the [5-^{3}H]-dCyd would cause a loss of the tritium radiolabel from the 5-carbon of dCyd. Finally, when [^{3}H]-ara-C was used (cells were exposed to 1.0 μM for 17 h; Table 1 and Fig. 7C) in replicate experiments, only 64.9% and 70.5% of the ara-C incorporated into nDNA was found in an internucleotide position, with 35.1% and 29.5% of the radiolabeled ara-C, respectively, being detected in a chain terminus position (significantly different from the incorporated position of labeled dThd or dCyd; $P < 0.05$, Student's t-test).

Discussion

The present studies demonstrate that ara-C causes a marked overall reduction in the size of nDNA replication intermediates that elute at pH 11.0 in the pH-step alkaline-elution procedure. The position of ara-C incorporated into the nDNA is predominantly internucleotide; however, ara-C occupies a significantly higher proportion of chain termini (30%–35%) in nDNA eluting at pH 11.0 than do the naturally occurring analogues dCyd or dThd (<10%).

In ara-C-treated cells, the most striking alteration is loss of the preponderance of radiolabeled nDNA in the 0.5- to 40-kb size range that predominates in control ([^{3}H]-dThd-treated) cultures. The overall effect is a net decrease in nDNA fragment size in response to ara-C. These data obtained in pH-step alkaline elution and Gen-Pak FAX chromatography are in agreement with those of other investigators (e.g., [4, 7, 26]), who observed a reduction in nDNA fragment size in response to ara-C using alkaline sucrose gradients. What is apparent from our Gen-Pak FAX HPLC studies is that the size of these nDNA fragments that accumulate in the presence of ara-C is very small and may frequently amount to only a few bases in length (see Figs. 3, 4). Since we did not use synchronized cells, it is likely that a population of replication intermediates of 0.5–40 kb in length previously existed in the cells and that these intermediates were actively undergoing chain elongation at the time of the addition of ara-C. Thus, one would expect at least a portion of the radiolabel in ara-C-treated cells to be associated with these preformed intermediates. Instead, we observed very little radiolabel that was specifically associated with these 0.5–40 kb fragments following exposure to [^{3}H]-ara-C (Figs. 3C, 4B) or to [^{3}H]-dThd in ara-C-pretreated cells (Fig. 3B). Thus, the number of sites of chain elongation available for incorporation of radiolabeled dThd or ara-C in the preformed 0.5- to 40-kb fragments during exposure to ara-C is much smaller than the number available for incorporation of radiolabeled precursor among the small nDNA fragments. This suggests that the majority of the radioisotope incorporated in the presence of ara-C occurs in newly formed nDNA strands. This is also consistent with ara-C's causing a profound inhibition of chain elongation while failing to inhibit (or perhaps even stimulating) the formation of nDNA replication intermediates either within an active replicon (see below) or during the initiation process itself, in agreement with our previous pH-step alkaline-elution studies [20] and with the work of other investigators [4, 27–29].

In view of the above findings, it is possible that the observed accumulation of small nDNA fragments in the presence of ara-C might be attributable to a reduction in the size of the Okazaki fragments as a result of ara-C inhibition of chain elongation. Alternatively, one could invoke a hy-

pothesis wherein the inhibition of DNA elongation caused by ara-C treatment might lead to RNA priming and to the formation of nDNA replication intermediates within the replicon at sites other than the replication origins associated with uninhibited replicative DNA synthesis [4, 27–29]. This process, termed "aberrant re-replication" by some investigators [27], can occur in response to inhibition of DNA synthesis by a variety of agents and is thought to arise as a result of a cellular defense response to avoid an unreplicated DNA segment. In the case of ara-C, elongation of these newly formed nDNA fragments would continue until the next C site, whereby incorporation of ara-C at that site would lead to inhibition of elongation. This may serve to promote further formation of nDNA replication intermediates adjacent to the site of the block, with the continuation of this process resulting in the net accumulation of very small nDNA fragments as observed in the present study.

Given the known profound inhibition of chain elongation caused by ara-C, one would expect to find a high proportion of nDNA chains terminating in ara-C *during* drug exposure. Moreover, given the above discussion concerning the formation of small nDNA replication intermediates in response to ara-C, one would expect the highest proportion of ara-C incorporated into a chain terminus position to be among these small fragments as compared with larger nDNA intermediates. Indeed, among the nDNA fragments that elute at pH 11.0, ara-C incorporates into a chain terminus position in a significantly greater proportion (30%–35%) than does dCyd or dThd (<10%; $P < 0.05$). However, it is noteworthy that even among the small nDNA fragments thus isolated, ara-C exists *predominantly* in an internucleotide position (65%–70%, Table 1). This relatively efficient internalization of ara-C residues could occur by a number of mechanisms.

First, it should be noted that replicative DNA synthesis in the intact cell is probably accomplished by a multiprotein complex that contains the essential enzymatic elements necessary for the efficient production of DNA [15]. In contrast to the behavior of purified DNA polymerases in cell-free systems that incorporate ara-C in a chain terminus position [17, 18], the multienzyme-DNA replication complex may be more efficient in the internalization of incorporated ara-C. Furthermore, Mikita and Beardsley [17] have shown that DNA ligase is capable of joining a DNA strand with ara-CMP at the 3′ terminus, with an adjacent strand ending in a 5′ nucleoside monophosphate. Hence, DNA ligation in the intact cell offers another mechanism for internalization of incorporated ara-C. Finally, a number of studies [19, 20, 23] using a cell-free primer-elongation assay have shown that the inhibition of DNA polymerase α- or β-catalyzed DNA-strand elongation by aranucleosides displays sequence specificity; in certain single cytosine sites, ara-C is incorporated internally in the nDNA strand with relative ease (with little inhibitory effect on elongation), whereas at other sites (e.g., double cytosine), elongation is greatly inhibited by the insertion of an ara-CMP residue. This may at least partly account for the predominantly internal position of incorporated ara-C observed in intact cells.

An alternative explanation for the observed accumulation of very short nDNA fragments in ara-C-treated cells would be that they result from the breakdown of larger DNA fragments as a consequence of alkali-labile sites introduced by incorporated ara-C [6, 14]. We feel that this is unlikely. This issue was addressed in our previous pH-step alkaline-elution studies of ara-C [21], in which concentrations of ara-C as high as 1 mM did not enhance the elution at pH 11.0 of parental DNA prelabeled with [^{14}C]-dThd. Furthermore, using a conventional alkaline-elution single-strand-break assay, we found no difference in the rate of elution of DNA prelabeled with [^{14}C]-dThd versus [^{3}H]-ara-C (data not shown).

An intriguing effect of ara-C (as well as a variety of other cytotoxic agents) reported recently involves its ability to activate a form of programmed cell death called apoptosis [8, 10]. This process is characterized by the formation of an internucleosomal pattern of DNA fragmentation [30], apparently as the result of the activation of a cellular endonuclease [2], which results in the production of DNA fragments in multiples of approximately 200 base pairs. The small DNA fragments we observed in response to ara-C were much smaller than even the smallest (200 bp) fragment of a nucleosomal ladder. Hence, we feel strongly that the ara-C-induced diminution in the size of fragments eluting at pH 11.0 is not the result of apoptosis. Moreover, the duration of exposure of cells to ara-C (0.8–1 μM, 1–13 h) used in our studies was shorter than that previously reported to produce a discernable apoptotic effect in HL-60 cells [10], whereby exposure to 3 μM ara-C for 24–48 h was required. Furthermore, the nucleosomal fragmentation pattern arises from genomic DNA; our studies using radiolabeled precursor or ara-C were designed to measure incorporation into nDNA. If intranucleosomal fragmentation of radiolabeled DNA had occurred in our studies, we are confident that the Gen-Pak FAX HPLC system used would have readily detected the expected DNA fragments of multiples of 200 bp.

In their classic paper, Graham and Whitmore [7] have reported that the frequency with which ara-C occupies a chain terminus position is <10% for both nDNA and genomic DNA. This was accomplished by the exposure of L-cells to 1 μM [^{3}H]-ara-C for 4 h followed by "chasing" for 6 h with 0.1 mM dCyd. DNA was obtained first from a portion of the cells after the 4-h ara-C "pulse" and again after the dCyd "chase" by precipitation with perchloric acid followed by alkaline hydrolysis (0.5 *N* NaOH) of RNA. The nuclease-digestion technique applied was identical to that used in the present studies. The DNA that the above-mentioned authors digested immediately after the 4-h exposure to [^{3}H]-ara-C should represent ara-C incorporated into nDNA. Since they found ara-C to reside almost exclusively in an internal position in their nDNA fraction, their results differ substantially from those presented herein for nDNA-containing ara-C obtained by pH 11.0 alkaline elution. When corrected for differences in cell number, the total amount of acid-insoluble radioactivity detected by Graham and Whitmore following the 6-h chase was 2.3-fold that obtained following the 4-h ara-C pulse. Although this may have been due to the utilization of preexisting intracellular ara-CTP pools during the 6-h peri-

od of dCyd exposure, it is possible that the crucial small fragments of nDNA that were present immediately after the 4-h ara-C exposure might have been lost during the DNA purification process, and this may account for the discrepancy between our findings and those of Graham and Whitmore.

By demonstrating that an enhanced proportion of ara-C residues was incorporated into the chain terminus of short nDNA fragments, we reconciled the previously reported chain-terminating effects of ara-C in cell-free systems with the observed internal incorporation of ara-C in DNA obtained from intact cells. Thus, the present studies serve to elucidate further the processes by which ara-C interacts with the mammalian DNA replicative mechanism. We are continuing our efforts to define precisely the molecular effects of ara-C on DNA synthesis and function with the goal translating such knowledge into schemes to enhance its clinical effectiveness.

Acknowledgements. We thank Drs. L. Malkas and R. Hickey of the Department of Pharmacology and Program of Oncology, University of Maryland School of Medicine, for their helpful suggestions and critical reading of this manuscript.

References

1. Amicon technical manual (1990) Publication 950. W. R. Grace & Co., Danvers, Mass.
2. Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am J Pathol 136: 593–608
3. Davis LG, Dibner MD, Battey JF (1986) Preparation of DNA from eukaryotic cells: general method. In: Davis/Dibner/Battey (eds) Basic methods in molecular biology, vol 368. Elsevier, New York, pp 44–46
4. Dijkwel PA, Wanka F (1978) Enhanced release of nascent single strands from DNA synthesized in the presence of arabinosylcytosine. Biochim Biophys Acta 520: 461–471
5. Erickson LC, Ross WE, Kohn KW (1979) Isolation and purification of large quantities of DNA replication intermediates by pH-step alkaline elution. Chromosoma 74: 125–139
6. Fram RJ, Kufe DW (1982) DNA strand breaks caused by inhibitors of DNA synthesis: 1-β-D-arabinofuranosylcytosine and aphidicolin. Cancer Res 42: 4050–4053
7. Graham FL, Whitmore GF (1970) Studies in mouse L-cells on the incorporation of 1-β-D-arabinofuranosylcytosine into DNA and on inhibition of DNA polymerase by 1-β-D-arabinofuranosylcytosine 5′-triphosphate. Cancer Res 30: 2636–2644
8. Gunji H, Kharbanda S, Kufe D (1991) Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. Cancer Res 51: 741–743
9. Josse J, Kaiser AD, Kornberg A (1961) Enzymatic synthesis of deoxyribonucleic acid: VIII. Frequencies of nearest neighbor base sequences in deoxyribonucleic acid. J Biol Chem 236: 864–875
10. Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 49: 5870–5878
11. Kufe DW, Major PP (1982) Studies on the mechanism of action of cytosine arabinoside. Med Pediatr Oncol [Suppl] 1: 49–67
12. Kufe DW, Major PP, Egan EM, Beardsley GP (1980) Correlation of cytotoxicity with incorporation of ara-C into DNA. J Biol Chem 255: 8997–9000
13. Major PP, Egan EM, Beardsley GP, Minden ME, Kufe DW (1981) Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. Proc Natl Acad Sci USA 78: 3235–3239
14. Major PP, Eagan EM, Herrick D, Kufe DW (1982) Instability of (ara-C)DNA under alkaline conditions. Biochem Pharmacol 31: 861–866
15. Malkas L, Hickey R, Li C, Pedersen N, Baril E (1990) A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication in vitro. Biochemistry 29: 6362–6374
16. Merion M, Warren W, Stacey C, Dwyer ME (1988) High resolution purification of DNA restriction fragments using HPLC. BioTechniques 6: 246–251
17. Mikita T, Beardsley GP (1988) Functional consequences of the arabinosylcytosine structural lesion in DNA. Biochemistry 27: 4698–4705
18. Momparler RL (1969) Effect of cytosine arabinoside 5′-triphosphate on mammalian DNA polymerase. Biochem Biophys Res Commun 34: 465–471
19. Ohno Y, Spriggs D, Matsukage A, Ohno T, Kufe D (1988) Effects of 1-β-D-arabinofuranosylcytosine incorporation on elongation of specific DNA sequences by DNA polymerase β. Cancer Res 48: 1494–1498
20. Ohno Y, Spriggs D, Matsukage A, Ohno T, Kufe D (1989) Sequence-specific inhibition of DNA strand elongation by incorporation of 9-β-D-arabinofuranosyladenine. Cancer Res 49: 2077–2081
21. Ross DD, Chen S-RS, Cuddy DP (1990) Effects of 1-β-D-arabinofuranosylcytosine on DNA replication intermediates monitored by pH-step alkaline elution. Cancer Res 50: 2658–2666
22. Stowers DJ, Keim JM, Paul PS, Lyoo YS, Merion M, Benbow RM (1988) High-resolution chromatography of nucleic acids on the GenPak FAX column. J Chromatogr 444: 47–65
23. Townsend AJ, Cheng Y-C (1987) Sequence-specific effects of ara-5-aza-CTP and ara-CTP on DNA synthesis by purified human DNA polymerases in vitro: visualization of chain elongation on a defined template. Mol Pharmacol 32: 330–339
24. Warren W, Merion M (1988) High resolution purification of synthetic oligonucleotides using HPLC. BioChromatography 3: 118–126
25. Waters Life Science Notes (1988) High resolution DNA separations with HPLC that are faster, easier and with higher loads than electrophoresis
26. Wist E, Krokan H, Prydz H (1976) Effect of 1-β-D-arabinofuranosylcytosine triphosphate on DNA synthesis in isolated HeLa cell nuclei. Biochemistry 15: 3647–3652
27. Woodcock DM (1987) Cytosine arabinoside toxicity: molecular events, biological consequences, and their implications. Semin Oncol 14: 251–256
28. Woodcock DM, Cooper IA (1979) Aberrant double replication of segments of chromosomal DNA following DNA synthesis inhibition by cytosine arabinoside. Exp Cell Res 123: 157–166
29. Woodcock DM, Fox RM, Cooper IA (1979) Evidence for a new mechanism of cytotoxicity of 1-β-D-arabinofuranosylcytosine. Cancer Res 39: 1418–1424
30. Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555–556