Biochemical Pharmacology, Vol. 60, pp. 403–411, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00336-1

Differential Inhibition of the Human Cell DNA Replication Complex-Associated DNA Polymerases by the Antimetabolite 1-β-D-Arabinofuranosylcytosine Triphosphate (ara-CTP)

Suhua Han, Robert J. Hickey,*† Timothy D. Tom,‡ Philip W. Wills,* Juhani E. Syväoja§ and Linda H. Malkas*‖*

*Department of Pharmacology and Experimental Therapeutics, and †Greenebaum Cancer Center, School of Medicine, University of Maryland, Baltimore, MD, U.S.A.; ‡Anesthesia Services, Northwest Regional Hospital, Corpus Christi, TX, U.S.A.; and §Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN 90570, Oulu, Finland

ABSTRACT. The antimetabolite 1-β-D-arabinofuranosylcytosine (ara-C) has been used as a highly effective agent for the treatment of leukemia. The active metabolite 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP) is a potent inhibitor of DNA polymerases α, δ, and ε, and is responsible for inhibiting intact cell DNA synthesis. We have shown that a multiprotein complex, exhibiting many of the properties expected of the human cell DNA replication apparatus, can be readily isolated from human cells and tissues and is capable of supporting origin-dependent DNA synthesis *in vitro*. DNA polymerases α, δ, and ε are components of this multiprotein complex, termed the DNA synthesome, and we report here that the activities of these DNA synthesome-associated DNA polymerases are inhibited differentially by ara-CTP. Inhibition of the DNA synthesome-associated DNA polymerase α increased in a concentration-dependent manner, and was correlated closely with the inhibition of simian virus 40 (SV40) origin-dependent *in vitro* DNA replication, whereas DNA synthesome-associated DNA polymerase δ activity was not inhibited significantly by ara-CTP at 100 μM. Recent work has shown that the synthesome-associated DNA polymerase ε does not function in *in vitro* SV40 DNA replication, suggesting that only polymerases α and δ drive the DNA replication fork. Therefore, our results suggest that inhibition of the activity of the mammalian cell DNA synthesome by ara-CTP is due primarily to the inhibition of the DNA synthesome-associated DNA polymerase α. This observation implies that the drug may target specific phases of the DNA synthetic process in human cells. BIOCHEM PHARMACOL **60**;3: 403–411, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. DNA synthesome; DNA polymerase; ara-C; *in vitro* model; anticancer drugs

Ara-C¶ is one of the most effective agents used in the treatment of acute leukemia in humans [1], yet the precise mechanism(s) responsible for ara-C-induced cytotoxicity remains unresolved. Earlier studies suggested that the active metabolite of ara-C (ara-CTP) competes with dCTP for binding to DNA polymerase, which results in the inhibition of cellular DNA synthesis [2–4]. Kinetic studies, however, indicated that ara-CTP was only a weak competitive inhibitor of DNA polymerase, indicating that simple competitive inhibition of the enzyme could not explain the effects of this agent on cellular DNA replication and cytotoxicity [5]. Additional studies suggested that the incorporation of ara-CMP served to terminate replication at the 3′ ara-CMP of the daughter DNA strands [6–11].

However, the concept of ara-C acting solely as a growing daughter DNA chain terminator has been challenged by studies utilizing intact cells [3, 12–14]. Graham and Whitmore [3] analyzed high-molecular-weight DNA from mouse L cells labeled with [^{3}H]ara-C. They found only a small percentage of the incorporated [^{3}H]ara-CMP at the 3′ termini of the DNA; most was formed at internucleotide positions. In another study, a pH-step alkaline elution was used to demonstrate that although ara-C slows DNA replication, ara-CMP eventually becomes incorporated into genomic length DNA, and ara-CMP incorporation does not cause absolute chain termination [12]. The studies of Dijkwel and Wanka [13] also demonstrated that short, newly synthesized DNA strands containing ara-CMP eventually are converted to full-size DNA molecules [13]. Thus,

‖ Corresponding author: Linda H. Malkas, Ph.D., Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore St., BRB 4–002, Baltimore, MD 21201. Tel. (410) 706-2313 or 1798; FAX (410) 706-0032; E-mail: lmalkas@umaryland.edu

¶ *Abbreviations*: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CMP, 1-β-D-arabinofuranosylcytosine monophosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate; dNTP, deoxyribonucleotide triphosphate; and SV40, simian virus 40.

Received 16 August 1999; accepted 14 December 1999.

for intact cells, the presence of an ara-CMP residue at the 3′ terminus of a newly synthesized DNA strand initially inhibits further elongation of the strand, but eventually the addition of dNTPs to the 3′-ara-CMP occurs, resulting in ara-CMP being found in internucleotide linkages. In contrast, cell-free studies utilizing purified DNA polymerase enzyme have suggested that incorporated ara-CMP results in a profound inhibition of further DNA strand elongation [6–11].

We have been exploring the utility of a mammalian multiprotein DNA replication complex to serve as a novel and valid *in vitro* model system for the evaluation of anticancer drug actions on DNA synthesis. This multiprotein complex has been designated the DNA synthesome, and has been isolated from a variety of human cell lines and tissues [15–18]. The human cell DNA synthesome was shown to support semi-conservative DNA replication *in vitro* [15–18], using the SV40 based *in vitro* replication model (reviewed in Ref. 19). The DNA synthesome also exhibits a uniform sedimentation coefficient throughout its purification [15–17] and has been visualized as a discrete multiprotein species in native polyacrylamide gels [20].

Studies published by us examining the effect of ara-C (i.e. ara-CTP) on synthesome-mediated DNA replication indicate that the DNA synthesome can utilize ara-C as a substrate for *in vitro* DNA synthetic reaction [21]. We determined that ara-CTP slows synthesome-mediated DNA replication [21], but synthesis of full-length daughter form I plasmid DNA occurs in the presence of ara-CTP. We also observed that newly replicated DNA, isolated from synthesome-mediated DNA replication reactions, contains ara-CMP in internucleotide linkages [21]. This observation is consistent with the observations of other investigators who found that isolated nascent DNA, replicated in the presence of ara-C by intact cells, contains at least 70% of the incorporated ara-CMP in internucleotide positions [12, 22, 23]. Our results agreed with those demonstrations that intact cells incorporate [^{3}H]ara-C into short DNA fragments initially, but with time the radiolabeled ara-C moves into longer DNA fragments [2, 3, 12, 13]. Our results also correlated with those of Magnusson *et al.* [22], who demonstrated the incorporation of radiolabeled ara-CMP into internucleotide linkages of progeny polyomavirus form I DNA produced by infected cells. Their results imply that ara-C (i.e. ara-CTP) is a substrate for DNA polymerase in intact cells, and that the drug only slows the growth of newly replicating daughter DNA strands in intact cells.

In this report, studies are described that examined in greater detail the effect ara-CTP has on the synthesome-associated target proteins, DNA polymerases α and δ, while they are components of an assembled replication complex. The results indicate that ara-CTP differentially affects the activities of the synthesome-associated DNA polymerases. This observation implies that the drug may target specific phases of the DNA synthetic process in human cells.

MATERIALS AND METHODS

Materials

Poly[dG-dC][dG-dC] and ara-CTP were purchased from the Sigma Chemical Co. Poly[dG-dC][dG-dC] was dissolved in 50 mM HEPES (pH 5.9), ara-CTP was dissolved in 10 mM Tris–HCl (pH 8.0), and both reagents were stored in aliquots at -80°. [α-^{32}P]dGTP (3000 Ci/mmol) was purchased from New England Nuclear. M13mp18 single-stranded DNA and its universal sequencing primer (23 mer) were purchased from New England Biolabs.

Cell Culture and Harvest

Suspension cultures of HeLa cells were grown in Joklik's Modified Eagle's Medium supplemented with 5% each of calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/mL medium) were harvested and washed three times with PBS (8.4 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4). The cells then were pelleted by low-speed centrifugation (200 *g*, 5 min, 4°), and the cell pellets were stored at −80° until used to isolate the DNA synthesome.

Monolayer cultures of MCF-7 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine in T-150 flasks and split every 4–5 days. The cells were harvested as described for the HeLa cells. The cell pellets were stored at −80° prior to isolation of the DNA synthesome.

Purification of the DNA Synthesome from HeLa and MCF-7 Cells

The DNA synthesome was purified as described previously [15–18].

Purification of SV40 Large T-antigen

The SV40 large T-antigen, used in the *in vitro* SV40 DNA replication studies, was purified as described previously [15].

DNA Polymerase α Enzymatic Assay

The DNA polymerase α enzymatic assay was performed essentially as described [15] with the modifications by Wills *et al.* [21].

Enzymatic Assay for DNA Polymerases δ

The assay for DNA polymerase δ enzymatic activity was performed as described previously, with some modification [24]. Poly[dG-dC][dG-dC] was used as the template, at a concentration of 0.2 OD_{260} U/mL, and the assay contained 1–2 μg of purified DNA synthesome protein, 10 mM $MgCl_2$, 10 μM dCTP, 25 mM HEPES (pH 5.9), 200 μg/mL

of bovine serum albumin, 100 μCi/mL of [^{32}P]dGTP, 5% glycerol (v/v). The poly[dG-dC][dG-dC] template was boiled for 5 min and then chilled on ice prior to use. The reaction was carried out by incubating the reaction mixture at 37° for 15 min, and then the reaction mixtures were spotted onto Whatman DE81 filters. The amount of radiolabeled nucleotide bound to the filters was quantified as described [25].

In Vitro *SV40 DNA Replication Assay*

Assay reaction mixtures (25 μL) contained 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2–4 μg of purified DNA synthesome protein, 1.0 to 2.0 μg of purified SV40 large T-antigen, 50 ng of plasmid pSVO$^+$ containing an insert of the SV40 replication-origin DNA sequences [26], 100 μM each of dTTP and dATP, 200 μM each of rCTP, rGTP, and UTP, 1 mM ATP, 25 μM [α-^{32}P]dGTP, 40 mM creatine phosphate, and 1 μg of creatine kinase. The concentrations of dCTP and ara-CTP used in the reactions are described in the Results. The reactions were incubated at 37° for 4 hr, and the amount of radiolabeled nucleotide incorporated into newly synthesized replication products was measured by spotting the reaction mixtures onto Whatman DE81 filters, washing the filters, and quantifying the radiolabeled material retained by the filters by liquid scintillation, as previously described [25].

DNA Polymerase α Processivity Assay

The DNA polymerase α processivity assay was conducted using an excess of single-stranded DNA M13mp18 as the template. M13mp18 universal sequencing primer (23 mer) was labeled by [γ-^{32}P]ATP using T4 polynucleotide kinase. M13mp18 single-stranded DNA and a 4-fold molar excess of end-labeled M13mp18 universal sequencing primer were annealed in 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT by heating the mixture to 90° for 3 min, cooling the solution to 65° for 2 min, and then gradually cooling the solution to room temperature over 2 hr. Then the DNA synthesome (1 μg) was incubated with 20 μg of primed M13mp18 single-stranded DNA and the DNA polymerase α reaction buffer [14] at 37° for 30 min. The reaction was stopped by adding SDS to a final concentration of 1%, and the DNA products were extracted with phenol/chloroform. The size of the products was determined by resolving the products on an 8% sequencing gel. A 25-bp and a 10-bp DNA ladder were used to determine the size of the DNA products formed in the reaction. After electrophoresis, gels were dried, and the DNA products were visualized using a PhosphorImager. The processivity of polymerase α was determined by subtracting the base value of the primer from that of the longest band appearing on the gel.

DNA Polymerase δ Processivity Assay

The DNA polymerase δ processivity assay was conducted using an excess of single-stranded M13mp18 DNA as the template. M13mp18 single-stranded DNA and a 4-fold molar excess of M13mp18 universal sequencing primer were annealed as described for the polymerase α processivity assay. To inhibit the synthesome-associated DNA polymerase α activity during the assay, the DNA synthesome (1.9 μg) was preincubated for 1 hr with the anti-polymerase α antibody SJK 132–20 [27], and then the reaction was incubated with 20 mM MOPS (pH 5.9), 5% glycerol (v/v), 5 mM β-mercaptoethanol, 250 μg/mL of bovine serum albumin, 200 μM dATP, 200 μM dTTP, 10 μM dCTP, 10 μM dGTP, 400 μCi/mL of [α-^{32}P]dGTP, and 5 μg of primed M13mp18 single-stranded DNA at 37° for 30 min. Ten percent of the reaction mixture was spotted onto Whatman DE81 filters, and the amount of radiolabeled nucleotide incorporated into newly polymerized DNA products was quantified [25]. The polymerase activity in the remaining 90% of the reaction was stopped by adding SDS to a final concentration of 1%, and the DNA products were extracted with phenol/chloroform and precipitated with ethanol as previously described [25]. The length of the reaction products was determined by resolving the products on an 8% denaturing polyacrylamide gel containing 100 mM Tris-borate (pH 8.3), 8 M urea, and 2 mM EDTA [25]. A 25-bp and a 100-bp DNA ladder were resolved in a separate lane and used to determine the size of the DNA products formed in the reaction. After electrophoresis, the gels were soaked for 30–45 min in a solution containing 5% methanol and 5% acetic acid (v/v), and the gel was dried and stored with Kodak XAR-5 film in order to visualize the DNA products formed during the reaction [25]. The processivity of polymerase δ is determined by subtracting the base value of the primer from that of the longest band appearing on the gel.

RESULTS

Effects of ara-CTP on the Synthesome-Associated DNA Polymerase Activities

SYNTHESOME-ASSOCIATED DNA POLYMERASE α. Experiments were initiated to examine the effect of ara-CTP on the activity of the synthesome-associated DNA polymerase α. To carry out this analysis, it was first necessary to determine if the replication complex-associated DNA polymerases δ and ε contribute to the activity measured under the conditions used for the polymerase α assay. This was accomplished by measuring polymerase activity in the presence of increasing amounts of an antibody that specifically inhibits DNA polymerase α enzyme activity (SJK 132–20 [27]). Should polymerases δ and ε contribute to the total activity observed under the conditions used to assay polymerase α, then it could be anticipated that the antibody directed against polymerase α would only partially inhibit the measured polymerase activity. As shown in Fig.

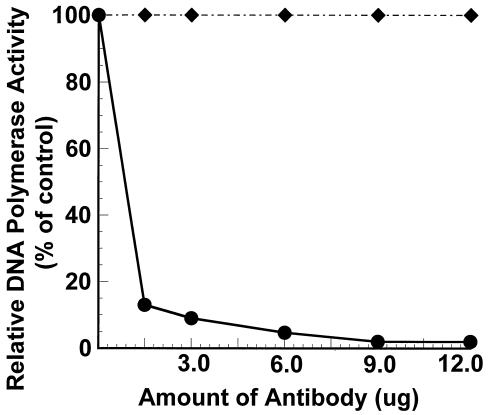

FIG. 1. Inhibition of the DNA synthesome-associated DNA polymerase α activity by monoclonal antibody SJK 132–20. A 1.9-μg portion of the synthesome protein fraction (Q-Sepharose pooled peak fraction derived from MCF-7 cells) was preincubated for 1 hr on ice with increasing concentrations of antibody (1.5 μg/μL). Control reactions contained equivalent volumes of Tris-buffered saline in place of the antibody. Polymerase α activity assays then were performed by incubating the reaction mixture for 1 hr at 37° in the polymerase α reaction buffer [15]. The reactions were stopped by spotting the reaction mixture onto Whatman DE81 filters, and relative DNA synthesis was determined by measuring the amount of radiolabeled nucleotide incorporated into Whatman DE81 filter-bound material. The experiments were repeated five times, and the average values for enzymatic activity at each antibody concentration were reported in the figure. Key: (♦) buffer only, and (●) antibody. (One hundred percent control = 130 fmol.)

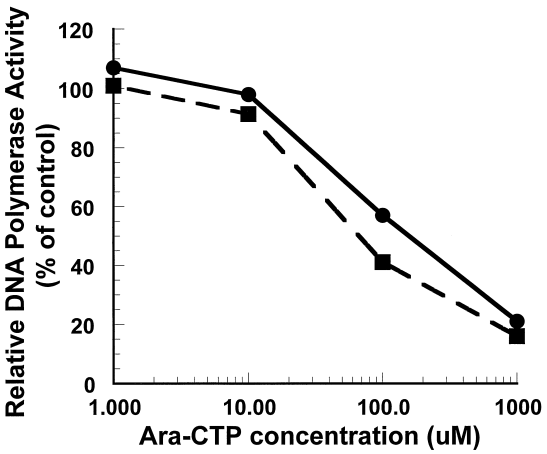

FIG. 2. Effect of ara-CTP on the activity of the DNA synthesome-associated DNA polymerase α. Nucleotide-polymerizing activity assays of synthesome-associated DNA polymerases were performed in the presence of 10 μM dCTP and increasing concentrations of ara-CTP. Spotting and drying the reaction mixture onto Whatman DE81 filters stopped the reactions, and the relative amount of DNA synthesis was determined as described [25]. Assays were conducted using the DNA synthesome (1–2 μg) isolated from HeLa (■) and MCF-7 (●) cells. The experiments were repeated three times with each cell line, and the average values are reported. (HeLa 100% control = 350 fmol; MCF-7 100% control = 70 fmol.)

1, approximately 90% of the DNA synthesome-associated DNA polymerase α activity was inhibited using 1.5 μg of the purified SJK132–20 antibody, whereas 99% of the activity of the enzyme was inhibited by 9 μg of the antibody. These results indicate that DNA polymerase α is the major polymerase responsible for elongating the DNA template under the assay conditions. Control reactions lacking the SJK132–20 antibody did not inhibit the activity of the synthesome-associated DNA polymerase α (Fig. 1).

Once the specificity of the DNA polymerase α assay was established, an evaluation of the effect of ara-CTP on the synthesome-associated DNA polymerase α activity derived from both HeLa and MCF-7 cells was made. The assay was conducted in the presence of 10 μM dCTP and increasing concentrations of ara-CTP. The results of these experiments are shown in Fig. 2. It was observed that ara-CTP significantly inhibited the activity of the synthesome-associated DNA polymerase α derived from both HeLa and MCF-7 cells. The most significant decrease in polymerase α activity occurred between 10 and 100 μM ara-CTP.

SYNTHESOME-ASSOCIATED DNA POLYMERASE δ. To conduct these studies, it was first necessary to establish that the assay conditions used to evaluate DNA polymerase δ were specific to only this enzyme. To determine if polymerase α contributed to the activity measured in the polymerase δ assay, the DNA synthesome was preincubated for 1 hr with increasing amounts of the anti-DNA polymerase α antibody SJK132–20 prior to initiating the assay for polymerase δ activity. If polymerase α contributes to the activity measured in the polymerase δ assay, then the anti-polymerase α antibody should inhibit the observed total activity significantly. The results of these experiments are shown in Fig. 3. Four micrograms of monoclonal antibody SJK132–20 barely inhibited the observed total DNA polymerase activity measured under the polymerase δ assay conditions. Control reactions containing buffered saline in place of the purified antibody also did not exhibit any inhibition of the total measured polymerase activity. These results indicate that DNA polymerase α is essentially silent under the conditions of the polymerase δ assay.

Once it was determined that DNA polymerase α did not contribute to the measured activity in the synthesome-associated DNA polymerase δ reaction, it then was necessary to establish whether the activity of the complex-associated DNA polymerase ϵ contributed to that of polymerase δ in the DNA polymerase δ assay. To accomplish this, the DNA synthesome was preincubated with increasing amounts of the DNA polymerase ϵ inhibitory antibody K18 [28]. After preincubation with the K18 antibody, the activity of the synthesome-associated DNA polymerase δ enzyme was assessed (see Materials and Methods). The results of these experiments showed no inhibition of polymerase δ activity by the DNA polymerase ϵ specific antibody (Fig. 4).

It has been demonstrated previously that synthesome-associated DNA polymerases α and δ both participate in synthesome-mediated *in vitro* SV40 DNA replication [15–18]. Recently, it was shown that inhibition of DNA

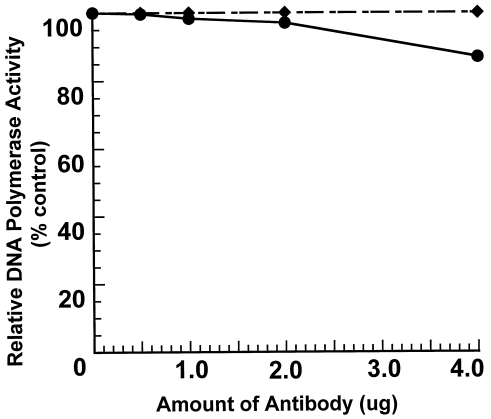

FIG. 3. Inhibition of the activity of the DNA synthesome-associated DNA polymerase δ by anti-DNA polymerase α monoclonal antibody SJK 132–20. A 1.9-μg portion of the enzyme was preincubated for 1 hr on ice with increasing concentrations of the anti-DNA polymerase α antibody SJK 132–20 (as indicated in Fig. 1). Control reactions contained equivalent volumes of Tris-buffered saline lacking the purified antibody. The activity of DNA synthesome-associated DNA polymerase δ was determined by using poly[dG-dC][dG-dC] as the template, and incubating the treated synthesome and template in the presence of 10 mM $MgCl_2$, 10 μM dCTP, 25 mM HEPES (pH 5.9), 200 μg/mL of bovine serum albumin, 100 μCi/mL of [α-^{32}P]dGTP, and 5% glycerol. The reaction mixtures were spotted and dried onto Whatman DE81 filters, and the amount of radiolabeled nucleotide incorporated into DNA during the reaction was quantified by measuring the amount of radiolabeled material retained on the washed DE81 filters (as described in Materials and Methods). Key: (◆) buffer only, and (●) antibody. (One hundred percent control = 2 fmol.) The experiments were repeated three times.

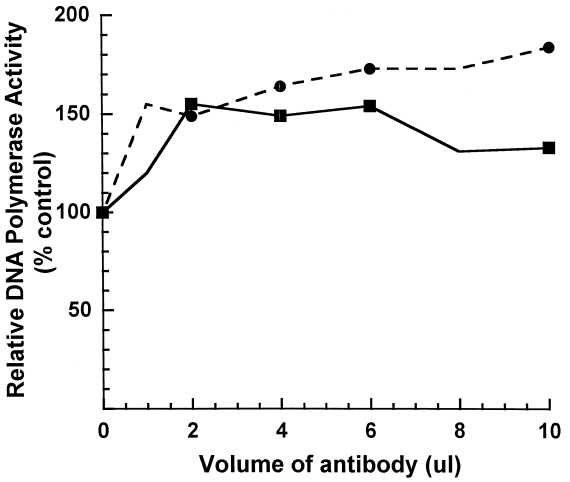

FIG. 4. Effect of anti-DNA polymerase ε antibody on synthesome-associated DNA polymerase δ activity. A 1.9-μg portion of the enzyme was preincubated for 1 hr on ice with increasing concentrations of the anti-DNA polymerase ε antibody (■) [K18, 0.71 μg/μL, in 30 mM HEPES (pH 7.66)]. Control reactions (●) contained equivalent volumes of 30 mM HEPES (pH 7.66) lacking the purified antibody. The activity of DNA synthesome-associated DNA polymerase δ was determined as described in Materials and Methods. The reaction mixtures were spotted and dried onto Whatman DE81 filters, and the amount of radiolabeled nucleotide incorporated into DNA during the reaction was quantified by measuring the amount of radiolabeled material retained on the washed DE81 filters (as described in Materials and Methods) (100% control = 1.65 fmol). The experiments were done six times.

polymerase ε by the K18 antibody in intact cells inhibits DNA synthesis as well, whereas synthesome-driven SV40 DNA replication *in vitro* was not inhibited by this antibody [28]. This observation suggested that while polymerase ε is required by intact cells for DNA replication, its role may be associated with replication-coupled DNA repair rather than with the DNA replication reaction itself. Therefore, for our continuing studies examining the action of ara-CTP on synthesome-associated DNA polymerase activity and the DNA replication function of the synthesome, we concentrated on the effect of ara-CTP on synthesome-associated DNA polymerase α and δ activities.

Using assay conditions that selectively measure the activities of DNA synthesome-associated DNA polymerases, we evaluated whether increasing concentrations of ara-CTP inhibit the activity of synthesome-associated DNA polymerase δ. The activities of HeLa and MCF-7 cell-derived synthesome-associated DNA polymerase δ were measured in reactions containing 10 μM dCTP and increasing concentrations of ara-CTP. Figure 5 shows the results of these studies. Unlike the observed significant inhibition of synthesome-associated DNA polymerase α activity by ara-CTP (Fig. 2), it was found that the drug did not inhibit DNA polymerase δ activity readily at concentrations shown to greatly suppress polymerase α function (Fig. 5). At 100 μM ara-CTP, the synthesome-associated DNA polymerase δ of MCF-7 and HeLa cells was inhibited by only 5 and 12%, respectively. In contrast, at the same concentration of ara-CTP, synthesome-associated DNA polymerase α activity was inhibited by 45% for the synthesome isolated from MCF-7 cells and by 65% for the HeLa cell-derived synthesome (Fig. 2). Altogether, the results suggest that the activity of DNA synthesome-associated DNA polymerase α was more sensitive to the inhibitory effects of ara-CTP than the activity of the replication complex-associated DNA polymerase δ. In addition, this effect of ara-CTP on polymerase α function was observed regardless of the cell line used to prepare the DNA synthesome.

Suppression of Synthesome-Driven In Vitro *SV40 DNA Replication*

Ara-CTP has been shown to inhibit synthesome-driven SV40 DNA replication [21]. However, despite this inhibition, full-length daughter DNA molecules containing incorporated ara-CMP are produced in the synthesome-driven replication reactions [21], suggesting that the synthesome-associated DNA polymerases could readily incorporate ara-CMP into newly synthesized daughter DNA molecules. We wanted to determine whether the observed preferential inhibition of DNA polymerase α by

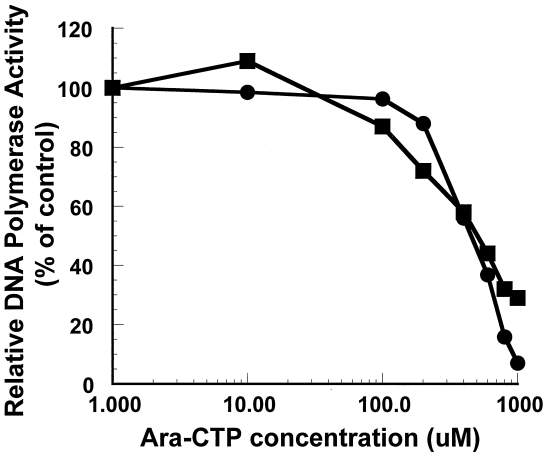

FIG. 5. Effect of ara-CTP on the synthesome-associated DNA polymerase δ. Nucleotide-polymerizing activity assays of synthesome-associated DNA polymerase δ were performed in the presence of 10 μM dCTP and increasing concentrations of ara-CTP. The reactions were stopped by spotting the reaction mixture onto Whatman DE81 filters, and the relative amount of DNA synthesis was determined as described in the legend of Fig. 1. Assays were conducted using the DNA synthesome from HeLa (■) and MCF-7 (●) cells. (HeLa 100% = 6.2 fmol; MCF-7 100% control = 8.3 fmol.) The experiments were repeated five times with each cell line.

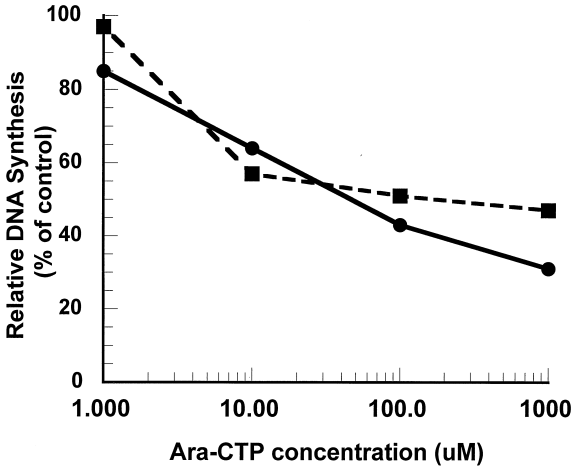

FIG. 6. Effect of ara-CTP on DNA synthesome-mediated *in vitro* DNA replication. DNA synthesome-mediated SV40 origin-dependent *in vitro* DNA replication reactions were performed in the presence of 10 μM dCTP and increasing concentrations of ara-CTP. The DNA replication reaction mixtures were incubated for 4 hr at 37°. The reactions then were stopped by spotting the reaction mixture onto Whatman DE81 filters, and the amount of newly synthesized DNA was quantified by measuring the amount of [α-^{32}P]dGTP incorporated into DE81 filter-bound material during the reaction. Assays were conducted using the DNA synthesome from HeLa (■) and MCF7 (●) cells. (HeLa 100% control = 11.6 fmol; MCF-7 100% control = 4.76 fmol.) The experiments were repeated three times with each cell line.

ara-CTP (Fig. 2) would correlate with an inhibition in synthesome-driven DNA replication. To accomplish this, synthesome-driven *in vitro* SV40 DNA replication reactions were performed (see Materials and Methods) in the presence of 10 μM dCTP and increasing concentrations of ara-CTP. The results of these experiments are shown in Fig. 6. Synthesome-driven *in vitro* SV40 DNA replication was inhibited approximately 50% at an ara-CTP concentration of 100 μM. This inhibitory concentration of ara-CTP was similar to that needed to inhibit approximately 50% of the activity of the synthesome-associated DNA polymerase α enzyme (Fig. 2).

Effect of ara-CTP on Synthesome-Associated DNA Polymerase α and δ Processivity

The effect of ara-CTP on the processivity function of the synthesome-associated DNA polymerases α and δ also was examined. Polymerase processivity is a measure of the ability of the enzyme to remain clamped to a DNA template following each successive addition of a nucleotide to a growing DNA chain. The processivity value is measured readily by the chain length of the DNA products synthesized under conditions of DNA template excess. Template excess minimizes the likelihood of reassociation of the enzyme with the DNA template it has abandoned.

Processivity assays (see Materials and Methods) were performed on the MCF-7 synthesome-associated DNA polymerases with increasing concentrations of ara-CTP. The gel electrophoretic analyses of the resulting DNA products are shown in Fig. 7. We observed that, in the absence of drug, DNA polymerase α (Fig. 7, A) was a much less processive enzyme than DNA polymerase δ (Fig. 7, B). The processivity values for synthesome polymerases α and δ were determined to be 13 and > 500 bases, respectively, in the absence of ara-CTP. These processivity values for these enzymes were consistent with those reported by others [29]. At first glance, the figures suggest that there is stronger inhibition of polymerase δ than of polymerase α. However, there are great differences between the processivity values of polymerase α and polymerase δ. Hence, we felt that it would be more reasonable to compare the degree to which increasing concentrations of ara-CTP inhibited both polymerase reactions than to compare the numerical values for processivity. In Fig. 7 (A and B), we can see that the processivity of both DNA polymerases α and δ was inhibited moderately at 10 μM ara-CTP and that processivity was strongly inhibited at a concentration of 100 μM ara-CTP, whereas no obvious inhibitions were found at 1 μM ara-CTP. This was true for both polymerase α and polymerase δ. Thus, we concluded that ara-CTP similarly inhibited the processivity of both DNA polymerases α and δ. Therefore, the primary effect of ara-CTP does not appear to influence the processivity of the synthesome-associated DNA polymerases α and δ differentially, but rather their overall polymerizing activity.

DISCUSSION

In this report, we present the first evidence describing the effect of ara-CTP on human cell DNA polymerases that

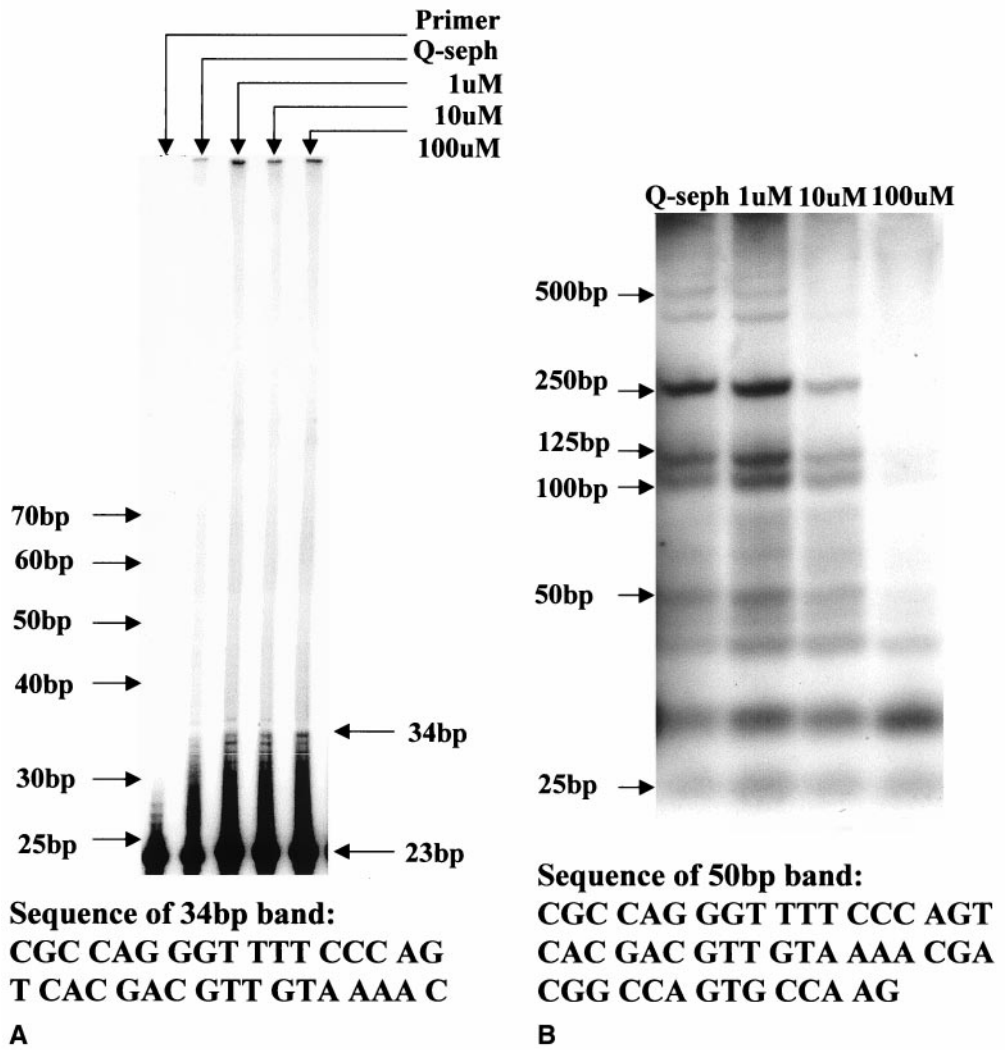

FIG. 7. Effect of ara-CTP on the processivity of MCF-7 synthesome-associated DNA polymerases α and δ. Processivity assays were performed (as described in Materials and Methods) in the presence of 10 μM dCTP and increasing concentrations of ara-CTP using assay conditions that fully supported the activity of the synthesome-associated DNA polymerase α or δ. The DNA template was present in excess. The reactions were stopped by the addition of SDS to a final concentration of 1%, and the DNA reaction products were phenol-extracted and analyzed by resolving the reaction products on an 8% denaturing polyacrylamide gel. The left panel shows the results with DNA polymerase α, and the right panel shows the results using DNA polymerase δ. The processivity assays were performed as independent assays on 3 consecutive days.

remain assembled to a functional DNA replication complex. In this context, the data indicate that synthesome-associated DNA polymerase α was inhibited preferentially by ara-CTP when compared with that of polymerase δ. Also, there was a correlation observed between the inhibition of synthesome-associated DNA polymerase α by ara-CTP and synthesome-mediated DNA replication. This would suggest that the action of ara-CTP at the mammalian DNA replication fork may be driven primarily by its preferential inhibition of DNA polymerase α. Data were also presented that indicate that ara-CTP affects, to a similar extent, the processivity function of DNA polymerases α and δ.

The rate-limiting process for SV40 replication is considered to be assembly of the T-antigen/replication proteins at the origin. Thus, the measured inhibition of steady-state replication would be expected to understate the actual inhibition of DNA synthesis in this system. However, this is true only if a reconstituted replication system employing individually purified proteins is used. In fact, assembly of these replication proteins to form a complex with T-antigen at the origin does not occur. The DNA synthesome exists as a fully assembled replication complex whose activity must be modulated throughout the cell cycle. Using atomic force microscopy data,* we demonstrated that the complex is already assembled and simply engages the origin in the presence of T-antigen. The synthesome has been purified to essential homogeneity,† and no evidence for

* Sekowski JW, personal communication. Cited with permission.
†Jiang HY, Hickey RJ, Tom TD, Wills PW, Abdel-Aziz WF and Malkas LH, Manuscript submitted for publication.

assembly has been formed in the atomic force microscopy studies using the highly purified synthesome. Thus, the estimates of ara-CTP inhibition are anticipated to be relatively accurate.

The mammalian DNA polymerases involved in replication have been shown to have very specialized functions at the replication fork. DNA polymerase α currently is believed to function primarily in the initiation of Okazaki fragment synthesis on the lagging DNA strand during mammalian DNA replication as well as to initiate the first DNA polymerization on both the leading and lagging DNA strands at the onset of DNA synthesis (reviewed in Refs. 19 and 30). The replication protein Replication Factor-C then is proposed to mediate a process termed polymerase switching [31, 32], allowing DNA polymerase δ access to the replication fork to facilitate the elongation phase of DNA synthesis. The specialized function of the DNA polymerases in DNA replication, together with our observations regarding the preferential inhibition of synthesome-associated polymerase α by ara-CTP, suggests that this drug may target a specific phase(s) of the DNA synthetic process. Since polymerase α plays a critical role in replication initiation as well as Okazaki fragment formation, our results regarding the action of ara-CTP suggest that the drug preferentially inhibits DNA replication initiation and the synthesis of Okazaki fragments. Support for this premise has been reported by other investigators. Heintz and Hamlin [33] reported that ara-C inhibits the initiation of DNA synthesis at a defined mammalian replicon initiation site. Ross and colleagues [23], using an alkaline elution technique, demonstrated that the exposure of human leukemia cells to ara-C results in the inhibition of nascent DNA chain elongation and the accumulation of newly synthesized DNA replication intermediates, including Okazaki fragments as well as long and short subgenomic-length DNA.

The finding that ara-CTP differentially inhibits the DNA polymerases of an intact human cell DNA replication apparatus is novel and points to a mechanism of action other than simple induced chain termination. We plan studies to examine this intriguing finding more closely.

This work was supported, in part, by research award CA57350 from the National Institutes of Health to L. H. M. and research award CA74904 from the National Institutes of Health/National Cancer Institute to R. J. H.

References

1. Frei E III, Bickers JN, Hewlett JS, Lang M, Leary WV and Talley RW, Dose schedule and antitumor studies of arabinosyl cytosine (NSC 63878). *Cancer Res* **29:** 1325–1332, 1969.
2. Graham F and Whitmore G, The effect of 1-β-D-arabinofuranosylcytosine on growth, viability, and DNA synthesis of mouse L-cells. *Cancer Res* **30:** 2627–2635, 1970.
3. Graham F and Whitmore G, Studies in mouse L-cells on the incorporation of 1-β-D-arabinofuranosylcytosine. *Cancer Res* **30:** 2636–2644, 1970.
4. Furth JJ and Cohen SS, Inhibition of mammalian DNA polymerase by the 5′-triphosphate of 1-β-D-arabinofuranosylcytosine and the 5′-triphosphate of 9-β-D-arabinofuranosyladenine. *Cancer Res* **28:** 2061–2067, 1968.
5. Momparler R, Effect of cytosine arabinoside 5′-triphosphate on mammalian DNA polymerase. *Biochem Biophys Res Commun* **34:** 465–471, 1969.
6. Momparler R, Pharmacology of cytosine arabinoside. *Med Pediat Oncol Suppl* **1:** 45–48, 1982.
7. Momparler R, Kinetic and template studies with 1-β-D-arabinofuranosylcytosine 5′-triphosphate and mammalian deoxyribonucleic acid polymerase. *Mol Pharmacol* **8:** 362–370, 1972.
8. Townsend AJ and Cheng Y-C, 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, 1987.
9. Ohno Y, Spriggs A, Matsukage T, Ohno T and Kufe D, Effects of 1-β-D-arabinofuranosylcytosine incorporation on elongation of specific DNA sequences by DNA polymerase β. *Cancer Res* **48:** 1494–1498, 1988.
10. Mikita T and Beardsley GP, Functional consequences of arabinosylcytosine structural lesion in DNA. *Biochemistry* **27:** 4698–4705, 1988.
11. Perrino FW and Mekosh HL, Incorporation of cytosine arabinoside monophosphate into DNA at internucleoside linkages by human DNA polymerase α. *J Biol Chem* **267:** 23043–23051, 1992.
12. Ross DD, Chen S-RS and Cuddy DP, Effects of 1-β-D-arabinofuranosylcytosine on DNA replication intermediates monitored by pH-step alkaline elution. *Cancer Res* **50**: 2658–2666, 1990.
13. Dijkwel PA and Wanka F, Enhanced release of nascent single strands from DNA synthesized in the presence of arabinocytosine. *Biochim Biophys Acta* **520:** 461–471, 1978.
14. Zahn R, Muller W, Foster W, Mardnof A and Beyer R, Action of 1-β-D-arabinofuranosylcytosine on mammalian tumor cells. *Eur J Cancer* **8:** 391–396, 1972.
15. Malkas LH, Hickey RJ, Li C-J, Pedersen N and Baril EF, A 21S enzyme complex from HeLa cells functions in simian virus 40 DNA replication *in vitro*. *Biochemistry* **29:** 6362–6374, 1990.
16. Applegren N, Hickey RJ, Kleinschmidt AM, Zhou Q, Coll J, Wills P, Swaby R, Wei Y, Quan JY, Lee MYWT and Malkas LH, Further characterization of the human cell multiprotein DNA replication complex. *J Cell Biochem* **59:** 91–107, 1995.
17. Coll JM, Sekowski JW, Hickey RJ, Schnaper L, Yue W, Brodie AM, Uitto L, Syvaoja JE and Malkas LH, The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. *Oncol Res* **8:** 435–447, 1996.
18. Lin S, Hickey RJ and Malkas LH, The isolation of a DNA synthesome from human leukemia cells. *Leuk Res* **21:** 501–512, 1997.
19. Malkas L, DNA replication machinery of the mammalian cell. *J Cell Biochem Suppl* **30/31**(the 25th Anniversary Issue)**:** 18–29, 1998.
20. Jiang HY, Hickey RJ, Bechtel BE, Wills PW, Han S, Tom TD, Wei YT and Malkas LH, Whole gel eluter purification of a functional multiprotein DNA replication complex. *BioRadiations* **102:** 18–20, 1998.
21. Wills P, Hickey R, Ross D, Cuddy D and Malkas L, A novel *in vitro* model system for studying the action of ara-C. *Cancer Chemother Pharmacol* **38:** 366–372, 1996.
22. Magnusson G, Craig R, Närkhammar M, Reichard P, Staub M and Warner H, Replication of polyoma DNA: Effects of hydroxyurea and arabinosyl cytosine. *Cold Spring Harb Symp Quant Biol* **39** (Pt 1)**:** 227–233, 1975.

23. Ross DD, Cuddy DP, Cohen N and Hensley DR, Mechanistic implications of alterations in HL-60 cell nascent DNA after exposure to 1-β-D-arabinofuranosylcytosine. *Cancer Chemother Pharmacol* **31:** 61–70, 1992.
24. Lee MY, Jiang YQ, Zhang SJ and Toomey NL, Characterization of human DNA polymerase δ and its immunochemical relationships with DNA polymerase α and ϵ. *J Biol Chem* **266:** 2423–2429, 1991.
25. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning, A Laboratory Manual,* 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
26. Stillman BW and Gluzman Y, Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Mol Cell Biol* **5:** 2051–2060, 1985.
27. Tanaka S, Hu S-Z, Wang TS-F and Korn D, Preparation and preliminary characterization of monoclonal antibodies against human DNA polymerase α. *J Biol Chem* **257:** 8386–8390, 1982.
28. Pospiech H, Kursula I, Abdel-Aziz W, Malkas L, Uitto L, Kastelli M, Vihinen-Ranta M, Eskelinen S and Syväoja JE, A neutralizing antibody against human DNA polymerase ϵ inhibits cellular but not SV40 DNA replication. *Nucleic Acids Res* **27:** 3799–3804, 1999.
29. Kornberg A and Baker T, *DNA Replication*, 2nd Edn. W. H. Freeman, New York, 1992.
30. Hickey RJ and Malkas LH, Mammalian cell DNA replication. *Crit Rev Eukaryot Gene Expr* **7:** 125–157, 1997.
31. Waga S, Bauer G and Stillman B, Reconstitution of complete SV40 DNA replication with purified replication factors. *J Biol Chem* **269:** 10923–10934, 1994.
32. Tsurimoto T and Stillman B, Replication factors required for SV40 DNA replication *in vitro*. *J Biol Chem* **266:** 1961–1968, 1991.
33. Heintz NH and Hamlin JL, In *vivo* effects of cytosine arabinoside on deoxyribonucleic acid replication in Chinese hamster ovary cells. 2. Cytosine arabinoside affects the rate of synthesis but not the pattern of labeling of an amplified chromosomal sequence at the onset of the S period. *Biochemistry* **22:** 3557–3562, 1983.