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ORIGINAL ARTICLE

Philip W. Wills · Robert Hickey · Linda Malkas

Ara-C differentially affects multiprotein forms of human cell DNA polymerase

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Abstract *Purpose*: The antimetabolite 1-β-D-arabinofuranosylcytosine (ara-C) has proven to be one of the most effective agents available for the treatment of acute leukemia although the precise mechanism by which ara-C induces cytotoxicity remains unclear. Our laboratory has previously isolated from human cells a DNA replication complex, termed the DNA synthesome, which is fully competent to orchestrate, in vitro, all of the reactions required to efficiently and faithfully replicate DNA. Using this system and the active metabolite of ara-C, ara-CTP, we demonstrated that the human DNA synthesome can efficiently incorporate ara-CTP into internucleotide positions of newly replicated DNA in vitro mimicking results obtained using intact cells and isolated nuclei. We then hypothesized that DNA polymerase auxiliary proteins, present within the DNA synthesome, may aid in incorporating this nucleotide analog into DNA. *Methods*: To test this hypothesis, we utilized three distinct multiprotein complexes each of which contained human DNA polymerase α and examined with standard in vitro polymerase assays the effectiveness of ara-C in inhibiting various aspects of their polymerase function. *Results and conclusion*: These polymerase-mediated elongation assays, which included ara-CTP- or ara-C-containing primers in the reaction mixture, showed that the rate of DNA elongation in the presence of ara-CTP was significantly enhanced when the DNA polymerase was associated with its auxiliary proteins, and that the elongation resulted in the formation of internucleotide ara-CMP. Nevertheless, the enhanced activities resulting from the association of these auxiliary proteins with polymerase α did not fully account for the remarkable efficiency with which the DNA synthesome incorporated ara-C into internucleotide positions during DNA replication.

Key words Cytosine arabinoside · Anticancer drugs · Mammalian cells · Multiprotein DNA replication complex · DNA synthesis

Abbreviations *Ara-C* 1-β-D-arabinofuranosylcytosine · *ara-CMP* 1-β-D-arabinofuranosyl-cytosine monophosphate · *ara-CTP* 1-β-D-arabinofuranosylcytosine triphosphate · *BSA* bovine serum albumin · *DTT* dithiothreitol · *SDS* sodium dodecyl sulfate · *SV40* simian virus 40

L. Malkas (✉)
University of Maryland School of Medicine,
Department of Pharmacology and Experimental Therapeutics,
the Program in Molecular and Cellular Biology,
the Program in Oncology, and the Program in Toxicology,
685 West Baltimore Street, Baltimore, MD 21201, USA
Tel.: +1-410-7062313; Fax: +1-410-7060032

P. W. Wills
University of Maryland School of Medicine,
Department of Pharmacology and Experimental Therapeutics,
655 West Baltimore Street, Baltimore, MD 21201, USA

R. Hickey
University of Maryland School of Medicine,
Department of Pharmacology and Experimental Therapeutics,
the Program in Molecular and Cellular Biology,
the Program in Toxicology, and the Marlene
and Stewart Greenebaum Cancer Center,
655 West Baltimore Street, Baltimore, MD 21201, USA

Introduction

Since its introduction as a chemotherapeutic agent, ara-C has been the subject of numerous scientific investigations. The intense interest in ara-C results from the drug's large clinical success in treating acute leukemia, and the general uncertainty about its precise mechanism of cytotoxicity [4, 8, 15]. Biochemically, it has been determined that ara-CTP, the active metabolite of ara-C, competes only weakly with dCTP for binding to DNA polymerase. This observation suggests that the mechanism responsible for ara-C-mediated cytotoxicity is more complicated than simple competitive inhibition of DNA polymerase [23]. Subsequent molecular studies have demonstrated that inhibition of DNA synthesis follows the incorporation of ara-CTP into growing

daughter DNA strands whereupon the incorporated ara-CMP acts as a block to further chain elongation [17, 22, 23, 24, 25, 26, 30]. In fact, some studies have concluded that, once ara-CTP is incorporated into the 3′ end of a DNA strand, elongation of the deoxyribonucleotide chain by human DNA polymerase is terminated [23, 24]. However, studies using intact cells and isolated nuclei have shown that ara-C appears to be incorporated primarily into internucleotide positions, despite being a profound inhibitor of DNA polymerase [6, 16, 18, 20, 27]. It has been found that at concentrations which severely inhibit DNA synthesis and cause cell death, over 95% of the drug is situated in nonterminal positions [9, 19]. In fact, even at extremely high concentrations of the drug (i.e. several hundredfold greater than the concentrations used in other studies [17, 22–26, 30]), less than 30% of the incorporated analog is found at the 3′ terminus of a DNA strand [20]. Thus, the data from intact cell investigations indicate that ara-C is an effective inhibitor of DNA replication, a potent cytotoxic agent, and primarily incorporated into internucleotide positions.

To seek to determine whether ara-C's toxicity is mediated through its incorporation into internucleotide linkages or its placement at the 3′ terminus of growing DNA strands, we initially examined the effect of ara-C on the DNA synthesome during in vitro DNA replication [34]. The DNA synthesome is a multiprotein form of DNA polymerase that has been shown to be fully competent to replicate in vitro any supercoiled plasmid DNA containing a SV40 origin of DNA replication. The SV40 viral large T-antigen protein is the only viral protein required for the reaction to be carried out [2, 11, 21, 35]. The DNA synthesome possesses all of the known enzymatic activities required for DNA replication, including DNA polymerase α, DNA polymerase δ, DNA polymerase ε, DNA ligase, DNA helicase, DNA primase, and topoisomerases I and II [2, 11, 35]. Due to its complex nature, the DNA synthesome itself has proved to be an attractive model for examining the mechanism of action of ara-C [34]. Results obtained from the in vitro SV40 replication assays using the DNA synthesome and ara-C more closely correlate with the results of intact cell studies than those utilizing purified DNA polymerase α. The DNA synthesome is able to both incorporate ara-C primarily into internucleotide positions and produce full-length daughter DNA molecules. The presence of ara-CTP in the reaction mixture significantly decreases the rate of synthesis, but does not terminate production of full-length DNA. Following our initial observations with the DNA synthesome, several other studies have also utilized in vitro SV40 origin-dependent DNA replication assays to examine the mechanism of action of ara-C, camptothecin and doxorubicin on the DNA synthetic process [1, 3, 5, 13, 20].

These studies have indicated that the in vitro DNA replication assays utilizing the purified DNA synthesome can serve as a powerful in vitro model for studying the mechanism of action of both established and new anticancer drugs that directly affect cellular DNA synthesis. One possible reason for the ability of the DNA synthesome to produce replication products which closely resemble those produced by intact cells could be the association of the appropriate accessory factors with the DNA polymerases within the structure of the DNA synthesome. In the study reported here, we examined the contribution these accessory factors make toward enhancing the ability of the DNA synthesome-associated DNA polymerases to the replication of DNA in the presence of ara-CTP. We examined in simple enzymatic assays containing ara-CTP three different forms of human DNA polymerase: the replication competent 18S DNA synthesome [2, 21], the 10S DNA polymerase α_2 complex [33], and the DNA polymerase α-primase complex.

The DNA synthesome is a multiprotein complex consisting of approximately 35 polypeptides and possesses the intrinsic ability to carry out all of the reactions required to efficiently replicate DNA in vitro [2, 21, 35]. In contrast, the polymerase α_2 complex contains ten polypeptides that cannot support large T-antigen and SV40 origin-dependent DNA replication in vitro [33]. The polymerase α-primase complex, which comprises only four proteins, cannot support in vitro DNA synthesis either [18, 29]. We report here the results of our analyses of these three distinct forms of DNA polymerase demonstrating that the association of the DNA polymerases with their appropriate accessory factors facilitates the incorporation of ara-CTP into elongating DNA chains.

Materials and methods

Cell culture

All enzymes were isolated from HeLa S3 cells grown in suspension cultures in Joklik's modified Eagle's medium with 5% each of irradiated calf and fetal bovine serum.

Protein purification procedures

The human DNA synthesome was isolated from HeLa S3 cells using our previously published procedures [20]. The readily sedimentable P-4 fraction was further purified by Q-Sepharose chromatography employing a continuous salt gradient (50–500 m*M* KCl) to elute the bound proteins.

The polymerase α_2 complex was isolated using the procedure of Vishwanatha et al. [33] with modification. We modified the previous centrifugation scheme by preparing the P4 as described [2, 20, 35] and then applying the P4 to the chromatography columns described by Vishwanatha et al. [33]. The only modification to the chromatography steps was the replacement of the final Bio-Rad DEAE-BIOGEL column with a Whatman DEAE-cellulose column.

The polymerase α-primase complex was isolated as described by Lamothe et al. [18].

DNA polymerase assay

The assay reaction mixtures (10 µl) contained: 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 200 µ*M* dATP, dGTP and

TTP, 1 μg activated DNA, 50 μ*M* of either [^{3}H]dCTP or [^{3}H]ara-CTP, 2 Ci/mmol, and 1–10 μg of enzyme. The reaction mixture was incubated at 37 °C for the times indicated. The reaction was then stopped by spotting the sample onto Whatman DE81 filters, drying the filters, and then washing them with 10 ml per filter of 0.1 *M* NaPPi, followed by three consecutive washes with 10 ml per filter of 0.3 *M* ammonium formate. The filters were dried, and the amount of nucleotide incorporated into the DNA template by each of the three forms of DNA polymerase was quantified using liquid scintillation counting.

Nuclease digestion and HPLC analysis of the DNA products formed during the polymerase assay

The assay reaction mixtures (50 μl) contained: 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 200 μ*M* dATP, dGTP and TTP, 10 μg activated DNA, 150 μ*M* [^{3}H]ara-CTP, 2 Ci/mmol, and 5–50 μg of enzyme. Reactions were terminated by the addition of SDS to a final concentration of 0.4%. Unincorporated nucleotide was removed from the assay mixtures by centrifugal concentration with repeated washes using a Centricon-30 filter (Amicon, W.R. Grace, Mass.). After final resuspension in 10 m*M* Tris-HCl, pH 8.0, $CaCl_2$ was added to a final concentration of 2 m*M*. The purified DNA products were then digested with 50 U of micrococcal nuclease at 37 °C for 45 min. This was followed by two rounds of digestion at 37 °C with 3 U of spleen phosphodiesterase II for 30 min. Prior to HPLC analysis, the samples were filtered through a Centrifree (Amicon) filter membrane to remove proteins.

Samples were then loaded onto the HPLC column and eluted along with authentic deoxyribonucleotide and deoxyribonucleoside standards. Reverse-phase HPLC analysis was performed over a 15-min period with an Mbondapak C18 column (Waters) eluted with a mobile phase of 0.01 *M* KH_2PO_4, pH 5.5, containing a linearly increasing gradient of methanol (0–15%) at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, and samples of each fraction were counted on a Beckman LS 6000 beta-counter. The tritiated deoxyribonucleotides and deoxyribonucleosides produced during nuclease digestion were eluted with the authentic standards, and the elution was monitored by ultraviolet absorption at a wavelength of 245 nm.

DNA elongation assay

A 24-nucleotide oligonucleotide primer was labeled by incubation of 40 pmol of the primer with 200 μCi ([^{32}P]ATP, 3000 Ci/mmol; NEN) and 20 U T4 polynucleotide kinase (New England Biolabs) for 90 min at 37 °C in a 1× polynucleotide kinase buffer (New England Biolabs). The kinase was inactivated by heating the reaction mixture for 10 min at 75 °C, and the reaction mixture was spun through a TE-10 column (Clontech) to remove any free γ[^{32}P]ATP. The annealing of the primer to the template was accomplished by combining 0.04 μg labeled primer with 0.46 μg unlabeled primer and 54.0 μg M13mp18(+) DNA in 20 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 100 m*M* NaCl, and 1 m*M* DTT. This mixture was then incubated for 2 min at 90 °C, followed by 3 min at 65 °C, and then allowed to slowly cool to room temperature overnight.

The DNA elongation mixture (10 μl) contained 1.5 μg labeled 24mer/M13, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 1 μg BSA, 200 μ*M* dATP, dGTP and TTP, where appropriate, either 200 μ*M* dCTP or ara-CTP, and 1–10 μg of one of the three DNA polymerase complexes. The reaction mixture was incubated at 37 °C for the times indicated, and the reaction was then stopped by the addition of 150 μg carrier RNA containing SDS at a final concentration of 0.2%. The elongation assay reaction products were extracted with phenol/chloroform and precipitated with ethanol. Samples were then resuspended in 50% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol FF and loaded onto a 6% polyacrylamide sequencing gel. The reaction products were resolved by electrophoresis through the gel for 2 h using constant power at 30 W. The gel was then dried and subjected to autoradiography to visualize the elongated DNA products formed.

DNA elongation assay from ara-C primers

Assorted 20-bp primers (Fig. 4A) were first annealed to a 27-bp template (5′-AAAAAAAGGAGCAATTAAAGGTACTCT-3′) by mixing 4 μg primer with 4.6 μg template in 20 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 100 m*M* NaCl, and 1 m*M* DTT. This mixture was then incubated for 2 min at 90 °C, followed by 3 min at 65 °C, and finally it was allowed to slowly cool to room temperature overnight.

The ara-C primer elongation assay mixture (40 μl) contained 0.4 μg of assorted primer/template, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 1 μg BSA, 1 m*M* AMP, 30 μ*M* γ[^{32}P]TTP (specific activity 30 μCi/mmol). The reaction was initiated by the addition of 20–50 μg of either DNA synthesome or DNA polymerase α-primase complex and the reaction mixture allowed to incubate at 37 °C for specified times. The reaction was terminated by spotting samples of the reaction mixture onto DE81 filters which were then washed and analyzed as described for the DNA polymerase assay.

DNA repair assay

Primers B, C, and G (Fig. 4A) were annealed to a 28-bp template (5′-AAAAGGAGCAATTAAAGGTACTCTAAAA-3′) as previously described for the DNA elongation assay from ara-C primers. The reaction mixture contained 1.5 μg of primer B/template, primer C/template, or primer G/template, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 1 μg BSA, 5 μ*M* γ[^{32}P]-dCTP (specific activity 30 μCi/mmol). The reaction was initiated by the addition of 20–50 μg of either DNA synthesome or DNA polymerase α_2 complex. The reaction mixtures were incubated for 1 h at 37 °C after which the reactions were terminated by spotting samples of each reaction mixture onto DE81 filters. The filters were washed and analyzed as described for the DNA polymerase assay. Specific ara-C repair activity was calculated by subtracting nonspecific incorporation of γ[^{32}P]dCTP by both DNA polymerase α_2 and the DNA synthesome into the control reaction containing primer B/template from the incorporation values obtained from reactions containing primers having ara-C present at either internal or terminal positions.

Results

More highly complex forms of human DNA polymerase incorporate [^{3}H]ara-CTP into DNA more efficiently than purified DNA polymerase

The ability of the DNA synthesome to incorporate [^{3}H]ara-CTP into nascent DNA during in vitro DNA replication assays [34] suggests that the DNA polymerase component of the DNA synthesome is able to utilize ara-CTP more efficiently than the purified DNA polymerases studied in other laboratories [17, 22–26, 30]. To determine whether the association of DNA polymerase with its accessory factors facilitates the incorporation of ara-CTP into DNA relative to the level of incorporation supported by the simplest form of the enzyme, we assayed equivalent units of DNA polymerase elongation activity of the DNA synthesome, the DNA polymerase α_2 complex, and the DNA polymerase α-primase protein complex. The assays were performed

in the presence of either 50 μ*M* [^{3}H]dCTP (Fig. 1A) or 50 μ*M* [^{3}H]ara-CTP (Fig. 1B).

The most highly complexed form of DNA polymerase found in the 18S form of the DNA synthesome had a greater ability to incorporate dCTP and ara-CTP into DNA than either of the other two forms of DNA polymerase. Consistent with this observation, the 10S DNA polymerase α_2 complex incorporated ara-CTP more rapidly and to a greater extent than the smaller DNA polymerase α-primase complex. The initial rate of ara-CTP incorporation supported by the DNA synthesome was more closely matched by the DNA polymerase α_2 complex than the DNA polymerase α-primase complex. However, the final kinetic rate of the DNA polymerase α_2 complex more closely paralleled that of the smaller DNA polymerase α-primase complex. The difference between the final amount of ara-CTP incorporated into DNA by the DNA polymerase α_2 and DNA polymerase α-primase complexes appeared to be the result of a higher initial rate of incorporation by the DNA polymerase α_2 complex. This would suggest that the DNA synthetic activity of the DNA polymerase α_2 complex is less susceptible to initial inhibition by ara-CTP than the purified DNA polymerase α-primase complex. However, during the first 2 h from initiation of the assay, the drug appeared to inhibit both of these smaller DNA polymerase complexes equally.

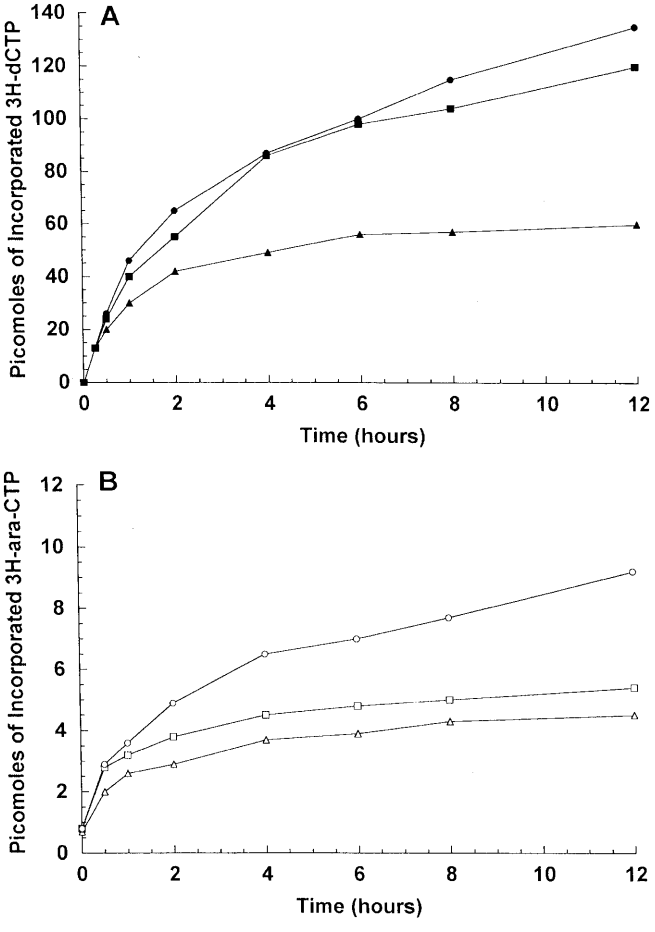


Fig. 1A,B The effect of [^{3}H]ara-CTP on the activity of DNA polymerase complexes. DNA polymerase assays were performed as described in Materials and methods. **A** Results from the assays performed in the presence of [^{3}H]dCTP; **B** results from the assays performed in the presence of [^{3}H]ara-CTP (● DNA synthesome, ■ polymerase α_2 complex, ▲ polymerase α-primase complex)

In a control experiment in which [^{3}H]dCTP was used in place of [^{3}H]ara-CTP, the initial rate of nucleotide incorporation by all three forms of the DNA polymerase appeared to be equivalent to one another. Following 12 h of inhibition by incubation with ara-CTP both the DNA synthesome and the DNA polymerase α_2 complex retained most of their initial ability to carry out DNA elongation from a primed template. This is in marked contrast to that of the smaller polymerase α-primase complex, which appeared essentially to have lost its ability to continue synthesizing DNA. The reason for the loss in activity of the purified DNA polymerase α-primase complex is uncertain. We determined, however, that the decrease in activity was not the result of protease or nonspecific nuclease activity within the polymerase α-primase complex, nor was it the consequence of prolonged incubations in reaction buffer (data not shown).

Accessory proteins complexed to the DNA polymerase aid in the incorporation of [^{3}H]ara-CTP into internucleotide positions within nascent DNA strands

To confirm that accessory proteins for DNA polymerase increase the enzyme's efficiency to incorporate ara-CTP into nascent DNA, elongation assays were performed with each of the three forms of DNA polymerase and the reaction products were analyzed for the amount of ara-CTP incorporated into internucleotide linkages. We subjected the DNA polymerase assay products synthesized by the DNA synthesome, the DNA polymerase α_2 complex, and the purified DNA polymerase α-primase complex to nuclease digestion and subsequent HPLC resolution of the nuclease digestion products. Micrococcal nuclease and nuclease P1 were used to digest the products of the DNA polymerase assay into their individual nucleotide components. If a deoxyribonucleotide is present at a strand terminus in the DNA, then nuclease digestion of the DNA results in the generation of a deoxyribonucleoside (e.g. ara-C). However, if a deoxyribonucleotide is situated at an internucleotide position (i.e. within a DNA strand), nuclease digestion will generate a deoxyribonucleotide 3′ monophosphate (3′-ara-CMP). We showed that all three DNA polymerase species could incorporate ara-CTP into internucleotide positions.

DNA polymerase elongation assays were performed with the DNA synthesome, the DNA polymerase α_2, and the DNA polymerase α-primase using [^{3}H]ara-CTP as the only source of radiolabel in the assay. The resulting polymerase assay products were washed free of unincorporated nucleotide, digested with nuclease,

deproteinated, and then resolved by HPLC. The results of these experiments are shown in Fig. 2. Over 40% of the [^{3}H]ara-CTP incorporated into newly synthesized DNA by the synthesome was observed to be present in internucleotide linkages (Fig. 2A). When the same experiment was performed with the DNA polymerase α_2 complex the reaction resulted in a decrease (approximately 30%) in the amount of [^{3}H]ara-CTP incorporated into internucleotide linkages, while the amount of ara-CTP inserted into the 3′ termini of nascent DNA by the DNA polymerase α_2 complex was not appreciably different from that incorporated by the DNA synthesome (Fig. 2B). Furthermore, the DNA polymerase α-primase complex incorporated into internucleotide linkages even less ara-CTP than the DNA polymerase α_2 complex (i.e. it resulted in just 30% of the incorporation of ara-CTP into internucleotide linkages supported by the DNA synthesome). Over 75% of the total amount of ara-CTP incorporated into newly synthesized DNA was found at the 3′ chain terminus position (Fig. 2C). Nevertheless, all three multiprotein forms of polymerase possessed the ability to incorporate ara-CTP into internucleotide positions. However, the larger the number of accessory proteins complexed to the DNA polymerase, the greater was the ability of the polymerase to incorporate ara-C into internucleotide linkages.

The efficiency of DNA strand elongation with ara-CTP is dependent upon the form of the DNA polymerase complex

To determine whether the form of the DNA polymerase complex affected the efficiency of the DNA strand elongation reaction and the length of the reaction products formed during the reaction, we examined the DNA synthesome and DNA polymerase α-primase complex for their ability to elongate a DNA strand in the presence of ara-CTP or dCTP. Utilizing enzyme concentrations with equivalent polymerizing activity (i.e. equal numbers of units of enzyme), we performed several DNA elongation experiments. Assays containing all four of the common deoxynucleotide triphosphates (i.e. dATP, dGTP, dCTP, dTTP) served as the positive controls, while other assays containing ara-CTP along with dATP, dGTP, and dTTP, were used for this study. The reaction products were resolved on denaturing polyacrylamide gels, and the gels were dried and then exposed to Kodak XAR-5 film at −80 °C.

The results of these experiments are shown in Fig. 3, and clearly demonstrate the profound inhibitory effect of ara-CTP on the ability of each of the two DNA polymerase complexes to elongate a primed DNA template. The majority of the products generated during a 20-h incubation by both DNA polymerase complexes in the presence of ara-CTP ranged in size from 36 to 43 nucleotides in length. The first site for insertion of ara-CTP encountered by the DNA polymerases in this assay was located at base 36. The next ara-CTP location was found at base 39, followed by two consecutive sites at bases 42 and 43. Extension past this double insertion

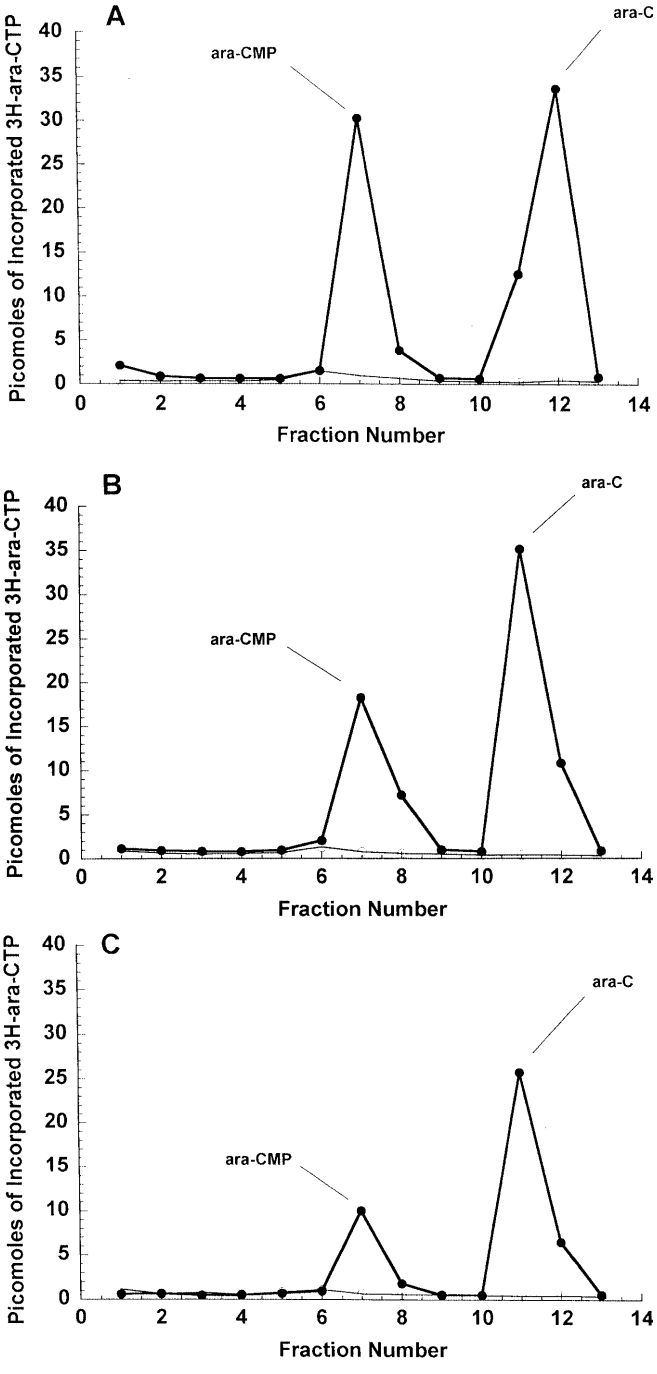


Fig. 2A–C HPLC elution profile of nuclease-treated DNA polymerase assay products. The incorporation of [^{3}H]ara-CTP into DNA over time by the three DNA polymerase complexes was analyzed by enzymatic digestion and HPLC elution as described in Materials and methods. The elution profiles for the 3′ terminal ara-C nucleotides and the internucleotide ara-CMP residues are depicted. **A** Results of the analysis of assay products formed by the DNA synthesome; **B** results of the analysis of assay products formed by the DNA polymerase α_2 complex; **C** results of the analysis of the assay products formed by the DNA polymerase α-primase complex. Examinations were performed in each assay at 0 h (○) and 4 h (●)

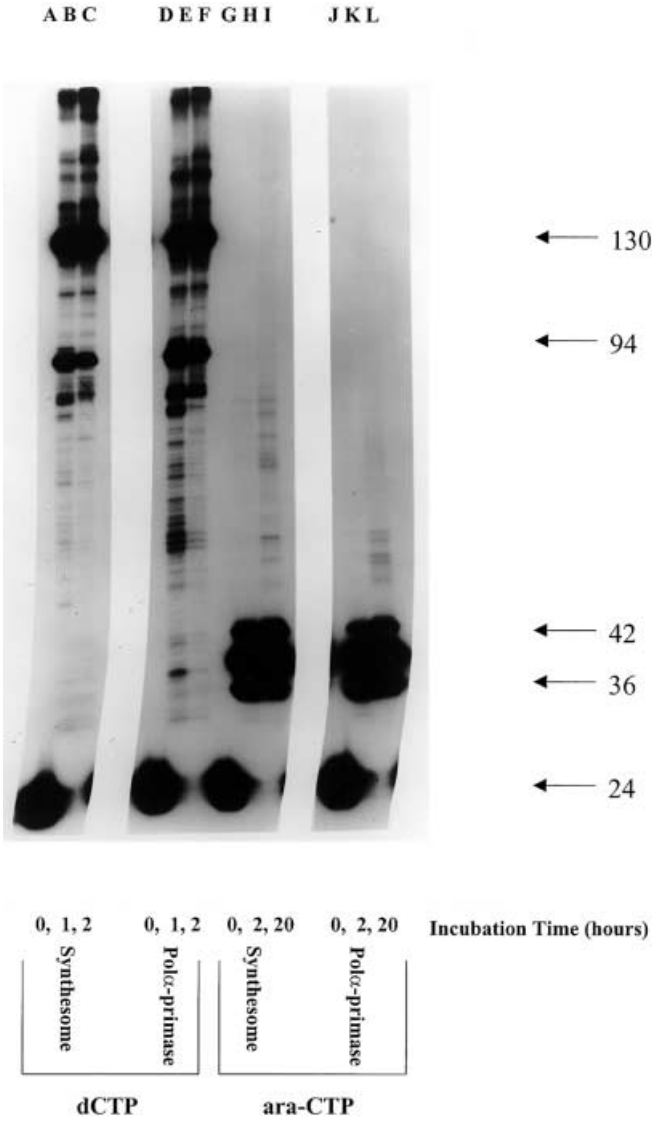


Fig. 3 The effect of ara-CTP on the efficiency of the multiprotein form of DNA polymerase in elongating a DNA strand. DNA polymerase elongation assays were performed as described in Materials and methods. The assay products were resolved by electrophoresis through a 6% denaturing polyacrylamide sequencing gel. *Lanes A*, *B*, *and C* reaction products formed during the assay performed by the DNA synthesome in the presence of all four deoxyribonucleotide triphosphates. The reaction was carried out under standard elongation assay conditions for 0, 1, and 2 h, respectively. *Lanes D*, *E*, *and F* reaction products from the assays performed with the purified DNA polymerase α-primase complex in the presence of all four deoxyribonucleotide triphosphates. The reactions were conducted under standard elongation assay conditions for 0, 1, and 2 h, respectively. *Lanes G*, *H*, *and I* similar to *lanes A*, *B*, *and C* except that the reaction was performed in the presence of ara-CTP instead of dCTP, and the reaction was carried out for periods of 0, 2, and 20 h, respectively. *Lanes J*, *K*, *and L* are similar to *lanes D*, *E*, *and F* except that the reaction was performed in the presence of ara-CTP instead of dCTP, and the reaction was carried out for periods of 0, 2, and 20 h, respectively. The relative size of the nucleotide products formed during the reaction is indicated on the right side of the autoradiogram. The placement of these markers was assigned by running in four adjacent lanes sequencing reactions of the M13mp18 template utilizing the same 24-nucleotide primer employed during the assay (Materials and methods)

was supported by the DNA synthesome. Moreover, the DNA synthesome produced in this assay a small amount of nascent DNA that was over 75 nucleotides in length and required the insertion of 14 ara-CTPs. In addition, there was a relatively abundant elongation product that was 57 nucleotides in length with eight ara-CTP insertion sites. In contrast, the DNA polymerase α-primase complex was considerably less successful than the DNA synthesome in extending the DNA strand past the second ara-CTP insertion site.

The site of ara-C incorporation differentially affects the ability of the DNA polymerase complexes to elongate a primed DNA strand

The majority of the DNA products formed by the two DNA polymerase complexes in the elongation assay were terminated by the consecutive insertion of two ara-CTP molecules. This observation raises the possibility that the incorporation of ara-CTP within a specific sequence context might differentially affect the ability of one or both of these polymerase complexes to extend the nascent DNA. To test this possibility we utilized six different DNA primers containing one or two ara-C nucleotides at either an internal or the 3′ terminal position (Fig. 4A). We then compared the ability of the replication-competent DNA synthesome and the purified DNA polymerase α-primase complex to utilize these primers in a DNA polymerase elongation assay. The elongation activity of both forms of DNA polymerase was profoundly inhibited by the presence of ara-C at the 3′ terminus of the primer (Fig. 4B,C). Furthermore, the presence of a second ara-C nucleotide preceding the 3′ terminal ara-C consistently increased this inhibition over a single ara-C nucleotide at the 3′ terminus, but the increase was not dramatic. In both of these cases, the level of inhibition was similar for the two DNA polymerase complexes. Interestingly, the DNA synthesome which had been previously shown to incorporate ara-CTP and produce full-length DNA products with a higher efficiency than the smaller DNA polymerase α-primase complex was more sensitive to inhibition by ara-C nucleotides located at internal positions within the DNA primer than the DNA polymerase α-primase complex.

When the ara-C nucleotide was located only four nucleotides from the 3′ terminus of the primer (primer F), the elongation activity of the DNA synthesome was inhibited twice as much as that of the DNA polymerase α-primase complex. An ara-C nucleotide located only two nucleotides from the 3′ terminus of the primer produced an inhibition of both DNA polymerase complexes. This inhibition was greater than that produced by primers containing ara-C nucleotides at more internal positions within the primer, but less than that produced by primers containing ara-C nucleotides at their 3′ terminus. In addition, the difference in the level of inhibition between the two DNA polymerase complexes

Fig. 4A–C The effect incorporation of ara-C at internal and terminal positions of a DNA primer has on the elongation of the primer by the DNA synthesome. DNA primer elongation assays were performed as described in Materials and methods. The figure shows the relative efficiency of elongating DNA primers containing ara-C nucleotides at either the 3′ termini of the primer or within the DNA primer. The elongation of ara-C-containing primer is compared to that of a control primer that lacks ara-C. **A** shows the different primers utilized in this set of assays. *Boldfaced* and *underlined C* indicates the location of ara-C residues. **B** shows the assay performed with the DNA synthesome, and **C** shows the assay performed with the DNA polymerase α-primase complex

A

Normal 20mer

Primer B: 5' d(AGAGTACCTTTAATTGCTCC) 3'

20mers with terminal ara-C

Primer C: 5' d(AGAGTACCTTTAATTGCTC**C**) 3'

Primer D: 5' d(AGAGTACCTTTAATTGCT**CC**) 3'

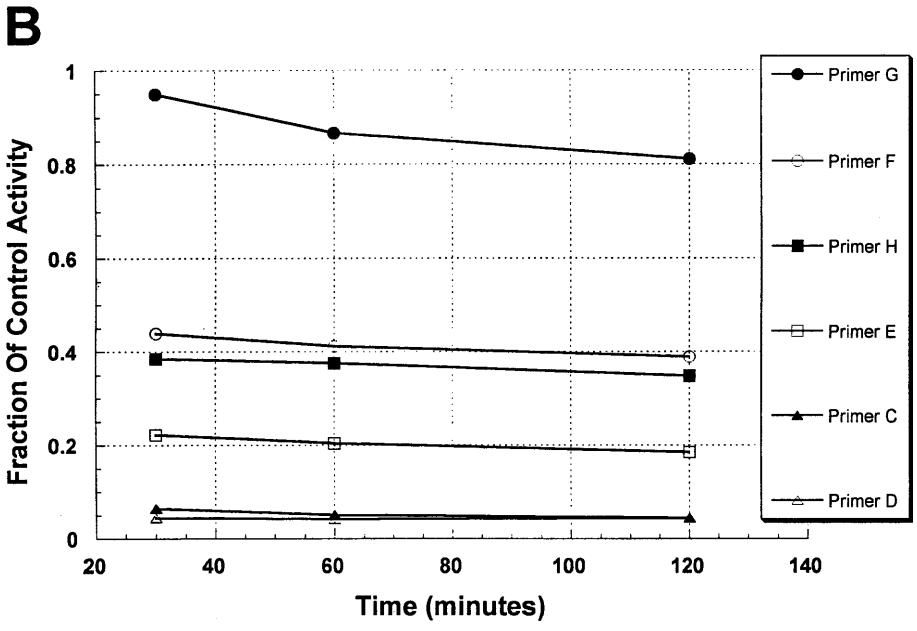
20mers with internal ara-CMP

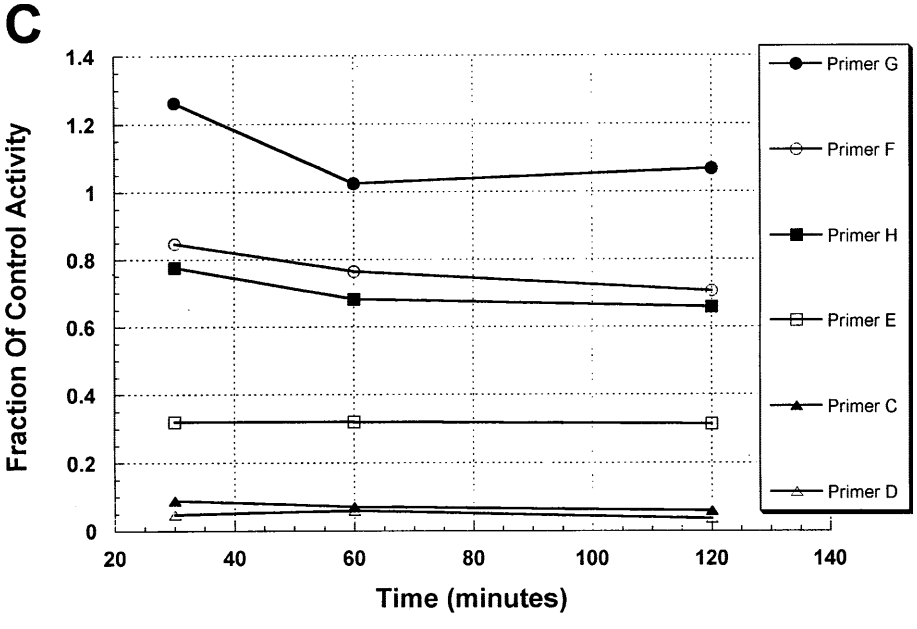
Primer E: 5' d(AGAGTACCTTTAATTGCT**C**C) 3'

Primer F: 5' d(AGAGTACCTTTAATTG**C**TCC) 3'

Primer G: 5' d(AGAGTAC**C**TTTAATTGCTCC) 3'

Primer H: 5' d(AGAGTAC**C**TTTAATTG**C**TCC) 3'





extending a primer containing ara-C one nucleotide 5′ of the terminal dC (primer E) was not as dramatic as the difference in the level of inhibition exhibited by the two DNA polymerase complexes with primer F. The DNA synthesome-associated polymerase was inhibited 76% with primer E, while the polymerase α-primase complex was inhibited 68%.

The DNA polymerase complexes have differential abilities to repair ara-C lesions

After having examined the ability of both DNA polymerase complexes to incorporate and elongate primers containing ara-C residues, we examined the DNA synthesome and DNA polymerase α_2 complex for the presence of an intrinsic DNA repair activity that could mediate excision and repair of an ara-C nucleotide located at an internal position within nascent DNA. Since the polymerase α-primase complex has not been reported to participate in DNA repair and our own preliminary experiments confirmed this (data not shown), the DNA polymerase α-primase complex was not examined in this analysis. Both the DNA synthesome and the DNA polymerase α_2 complex have 3′–5′ exonuclease activity [28] and therefore have the potential to participate in the excision and repair of ara-C nucleotides incorporated into DNA.

To determine whether either of these two forms of the DNA polymerase exhibit repair activity following incorporation of ara-C into DNA, we utilized two of the primers from the previous analysis, one containing an ara-C at the 3′ terminal position (primer C) and the other an ara-C nucleotide at an internal position (primer G). Both primers were annealed to a 28-bp template in separate reactions. We then measured the ability of the two DNA polymerase complexes to incorporate [^{3}H]dCTP into reaction products formed in the presence of primers C and G and compared the results of this analysis with those of a control assay that employed a primer that contained dCMP in place of ara-CMP (primer B). The control assay enabled us to normalize the results of this study for the potential exchange of [^{3}H]dCTP with any of the deoxycytidine nucleotides present in the template. This study enabled us to quantify the polymerase-mediated rate of exchange of [3H]dCTP with the ara-CMP contained in primer C and G.

The DNA synthesome consistently excised and replaced the ara-C nucleotide that was intrinsic to the primer template with a higher efficiency than the smaller DNA polymerase α_2 complex. Interestingly, the DNA synthesome possessed a significantly enhanced ability to repair ara-C nucleotides present within the primer as compared to the polymerase α_2 complex, and the difference between the amount of repair activity exhibited by the two DNA polymerase complexes was greater when ara-C nucleotides were contained within the primer (threefold) than when it was present at the 3′ terminus of the primer (twofold) (Fig. 5). The extent to which ara-C nucleotides present in a double-stranded DNA could be repaired by the two DNA polymerase complexes was determined by subtracting the polymerase-mediated incorporation of [^{3}H]dCTP into control DNA (primer B/template) from the incorporation of [^{3}H]dCTP into either of the two ara-C-nucleotide-containing templates (primers C and G annealed to template).

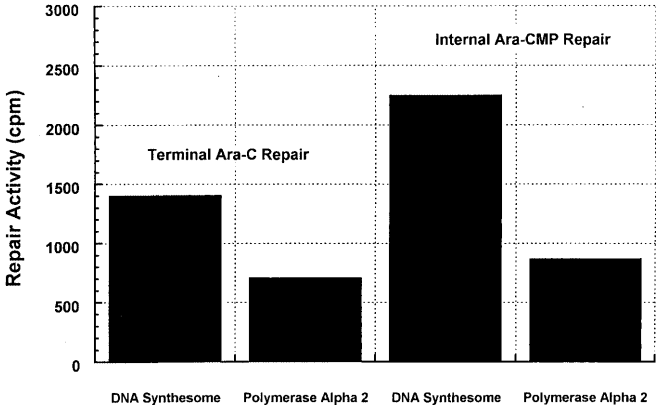


Fig. 5 The intrinsic repair activity of the DNA synthesome and DNA polymerase α_2 complex induced by the presence of ara-C at either the 3′ terminus or within a double-stranded DNA molecule. DNA repair assays were performed as described in Material and methods. The extent to which [^{32}P]dCTP is substituted for ara-C located at either the internal or the terminal position of the DNA template is shown. Repair was measured as the amount of [^{32}P]dCTP incorporated into double-stranded DNA containing ara-C. The spontaneous incorporation of [^{32}P]dCTP into the primer template lacking ara-CTP (i.e. containing deoxycytidine nucleotides) was determined as described in the Results and subtracted from the incorporation of [^{32}P]dCTP into templates containing ara-C nucleotides

Discussion

Defining the mechanism(s) by which ara-C exerts its cytotoxic effects on cells has proven to be difficult. Molecular analyses with purified DNA polymerase α seemed to reveal a clear mechanistic picture. Numerous biochemical and enzymatic investigations have clearly shown that the drug severely inhibits DNA strand elongation once ara-CTP is incorporated at the 3′ end of the nascent DNA strand. This is presumably due to the inability of the DNA polymerase to extend the nascent DNA strand from an ara-C terminus [17, 22–26, 30]. However, the results of studies utilizing intact cells have failed to support this conclusion. Instead, the intact cell studies have demonstrated that, while ara-C profoundly inhibits cellular DNA replication, the drug is incorporates primarily into internucleotide positions [6, 16, 19, 20, 27].

In attempting to resolve this apparent conflict in the data, we suggested that the enzymatic results reported [16, 22–26, 30] do not confirm the intact cell data [6, 16,

19, 20, 21] because most of the enzymatic studies relied on purified DNA polymerases that lacked one or more of the accessory factors normally found complexed to the DNA polymerase in intact cells. We have known for a considerable time that DNA replication requires the concerted action of a number of proteins and enzymes that act in concert to efficiently replicate DNA from defined origins, creating semiconservatively replicated DNA (reviewed in reference 11). It has been shown that mammalian cell DNA replication minimally requires the concerted activity of the following proteins: DNA polymerase α-primase, replication protein A, replication factor C, DNA ligase I, topoisomerases I and II, DNA polymerase δ and PCNA [29, 31]. In addition, recent evidence strongly suggests that these proteins work coordinately with a high rate of interaction and interdependence [7, 11, 29, 31]. Consequently, even the simplest model for studying DNA polymerization requires the inclusion of eight essential replication proteins if a physiologically meaningful picture of the process is to emerge.

Yet, the simple model for examining the elongation activity of purified DNA polymerase does not fully duplicate what occurs within the nucleus of the cell. Over the past decade, several studies have strongly demonstrated the existence of large protein complexes from eukaryotic-cell extracts that are capable of participating in DNA replication. These replication complexes range in size from the DNA polymerase α_2 complex purified by Vishwanatha et al. [33] (which has a sedimentation coefficient of 10S) to the much larger 100–150S megacomplexes isolated by Tubo and Berezney [32] (which also have DNA polymerase-primase activities). Interestingly, the large 100–150S megacomplex is substantially unstable outside its nuclear environment, and readily dissociates into smaller components that have sedimentation coefficients of 10S and 17S. These sizes correspond to the apparent size of the DNA polymerase α_2 complex and the murine-derived DNA synthesome [35], respectively. Jazwinski and Edelman have also reported the isolation of a 2-MDa complex from yeast which apparently supports yeast 2 μm DNA replication in vitro [12]. Characterization of the 2-MDa complex from yeast has revealed that it contains DNA polymerase I, DNA primase, DNA ligase, and topoisomerase II. Finally, a multiprotein form of DNA polymerase that maintains a sedimentation coefficient of 17S for the murine-derived complex and 18S for the human cell-derived synthesome has been isolated in sucrose gradients throughout its purification. We have shown that the purified complex can efficiently replicate DNA in vitro [2, 21, 35], and we have determined that DNA polymerase α-primase, DNA polymerase δ, PCNA, topoisomerase I, DNA ligase, and DNA helicases all copurify with this sedimentable form of DNA polymerase.

While each of these complexes [2, 12, 21, 32, 33, 35] contains many, if not all, of the proteins reported to be minimally essential for DNA replication [11], they also possess other proteins which may not be essential for replication but which appear to facilitate elongation of nascent DNA strands during the DNA synthetic process. These auxiliary proteins clearly have an effect on the DNA polymerases required for the elongation of a DNA strand. Lamothe et al. have demonstrated that the activity of the DNA polymerase α_2 complex is greatly enhanced by two auxiliary proteins, C1 and C2 [18], which are components of the DNA polymerase α_2 complex. Also, the auxiliary proteins gp44, gp62, and gp45 have been shown to significantly stimulate T4 phage DNA polymerase activity through the formation of a multiprotein complex [14]. Studies with the DNA pol III holoenzyme of *E. coli* have illustrated that, although the DNA polymerase activity minimally resides in a core complex consisting of three subunits, high processivity during DNA synthesis can be achieved with the addition of a fourth subunit to the core complex. Furthermore, the incorporation of six other subunits to this holoenzyme dramatically enhances the catalytic activity of the core polymerase complex [14].

Since the activity of DNA polymerases can be altered by their interaction with accessory proteins, we theorized that the ability of human cell DNA polymerase to utilize and efficiently incorporate antimetabolite drugs such as ara-CTP into DNA might also depend on the interaction of specific accessory factors with the DNA polymerase. Specifically, we hypothesized that drugs that are known to inhibit DNA replication by directly interacting with and inhibiting the activity of specific proteins required for DNA replication (e.g. DNA polymerase) would differentially affect the activity of the isolated DNA polymerase and the enzyme when it is present within a multiprotein complex.

In the study reported here we examined the ability of three distinct multiprotein complex forms of the human DNA polymerase to synthesize DNA in the presence of ara-CTP. The results of this investigation demonstrate that the auxiliary proteins in the DNA synthesome and the DNA polymerase α_2 complex facilitate the incorporation of ara-CTP into internucleotide positions within newly synthesized DNA. While the DNA polymerase α_2 complex was more resistant to inhibition by ara-CTP than the smaller DNA polymerase α-primase complex, the larger DNA synthesome complex was even more resistant than the DNA polymerase α_2 complex to inhibition by ara-CTP. Both the DNA polymerase α_2 and the DNA synthesome appear to continue to elongate a DNA strand with high efficiency following the incorporation of ara-CTP into nascent DNA. Interestingly, the enhanced ability of the DNA synthesome to incorporate ara-C into internucleotide positions is not indicative of the development of a mutator phenotype. Our results indicate that the DNA synthesome is actually more sensitive to the presence of ara-C within a DNA primer than the DNA polymerase α-primase complex and also exhibits an enhanced repair activity in comparison to the polymerase α_2 complex for ara-C nucleotides present at both the 3′ termini and internal

positions within a DNA strand. Nevertheless, the enhanced activities of the DNA synthesome as compared to purified DNA polymerase α-primase complex in the in vitro polymerase elongation assays does not fully account for the remarkable efficiency of the DNA synthesome to incorporate ara-C into internucleotide positions in the in vitro SV40 DNA replication assays [34].

The differences in the efficiency with which these two assays incorporate ara-CTP into DNA might help us better understand the apparently conflicting evidence gathered from biochemical and intact cell studies. Enzymatically, ara-CTP profoundly inhibits DNA chain elongation by purified DNA polymerase, effectively blocking DNA strand elongation and the incorporation of ara-CTP into internucleotide linkages [17, 23–26, 30]. However, these studies utilized purified DNA polymerase α in its simplest form. Inside the cell, however, DNA polymerase α interacts with various other enzymes and accessory proteins which can greatly affect the processivity of this enzyme. Indeed, our results indicate that the DNA synthesome (i.e. DNA polymerase in its highly complexed form) can incorporate more ara-CTP molecules into internucleotide positions within newly synthesized DNA than either the smaller DNA polymerase α_2 complex or the still smaller DNA polymerase α-primase complex.

While these findings partly explain the discrepancy between in vitro and in vivo experiments, the DNA synthesome in simple polymerase elongation assays was not able to fully mimic the results of intact cell studies [6, 16, 19, 20, 27] or our own in vitro DNA replication assays [34]. This suggests that in vitro DNA polymerase elongation assays are not as reflective of true cellular DNA replication as are in vitro SV40 DNA replication assays. We believe that the most logical explanation for this difference would be that a primer elongation assay does not fully simulate the mechanistic complexities of a moving replication fork. The DNA synthesome coordinately synthesizes both leading and lagging strand DNA synthesis at a replication fork, while purified DNA polymerase cannot physically coordinate synthesis of both strands. It is quite possible that the coordination of leading and lagging strand DNA synthesis by one DNA synthesome complex would result in a more substantial protein-DNA interaction. Potentially, this interaction might better stabilize the complex during the replication process and facilitate the incorporation of ara-C molecules. Nevertheless, results from this and other studies with the DNA synthesome and ara-C [1, 10, 13, 34] indicate that the mechanism(s) by which ara-C exerts its cytotoxic effect on cells are complex and are therefore best studied in a system that takes into account the complex interactions between all of the proteins necessary to efficiently carry out the DNA replication process.

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