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# A novel in vitro model system for studying the action of ara-C

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**Abstract** 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. While ara-C has been implicated as a potent inhibitor of mammalian cell DNA replication, the specific mechanism by which ara-C kills cells is not known. In this report we describe the development of an in vitro model system to study the molecular mechanism of ara-CMP incorporation into DNA. This model system makes use of a recently described human cell multiprotein DNA replication complex (MRC) that is competent to replicate DNA in vitro. The MRC can successfully incorporate ara-CMP into replicating DNA at internucleotide positions. These results are similar to those described for studies using intact cells. This MRC-driven in vitro replication system may therefore serve as a powerful model for the study of anticancer agents that directly affect human cell DNA synthesis.

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**Key words** Cytosine arabinoside · Anticancer drugs · Mammalian cells · Multiprotein DNA replication complex · In vitro model system

**Abbreviations** *Ara-C*, 1-β-D-arabinofuranosylcytosine · *ara-CMP*, 1-β-D-arabinofuranosylcytosine monophosphate · *ara-CTP*, 1-β-D-arabinofuranosylcytosine triphosphate · *dNTP*, deoxyribonucleotide triphosphate · *DTT*, dithiothreitol · *EDTA*, ethylenediaminetetraacetic acid · *SDS*, sodium dodecyl sulfate · *SV40*, simian virus 40

## Introduction

We have previously isolated and characterized a multiprotein form of DNA polymerase from human (HeLa) cell extracts that is fully competent to support in vitro SV40 origin-specific DNA replication [1–4]. Characterization of this isolated multiprotein form of DNA polymerase has identified several protein components of the complex. These proteins include: DNA polymerases α and δ, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), and DNA ligase I [3, 4]. In addition, the DNA replication activity mediated by the human cell multiprotein form of DNA polymerase has been shown to have many of the features observed in DNA synthesis carried out by intact cells as well as by crude cell extracts [3, 4]. A 17S multiprotein form of DNA polymerase from murine has also been recently identified and characterized in this laboratory (FM3A) cells [5] that is capable of supporting DNA replication using the polyomavirus (PyV)-based in vitro DNA synthesis system [6, 7]. It has been proposed that the isolated multiprotein form of DNA polymerase represents a mammalian multiprotein DNA Replication Complex (MRC) [4, 5]. In those reports we introduced a model to represent the MRC, which was based on the fractionation and

chromatographic profiles of the individual proteins found to copurify with the complex [4,5].

In this study the human cell MRC was examined for its ability to utilize ara-CTP as a substrate for in vitro DNA replication. The results presented here suggest that the human MRC could serve as a novel in vitro model system for studying the mechanism of action of ara-C and other anticancer drugs that directly affect DNA synthesis.

## Materials and methods

### Cell culture and harvest

Suspension cultures of HeLa cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells ($5 \times 10^5$ cells/ml medium) were harvested and washed three times with phosphate-buffered saline (PBS; 8.4 m*M* $Na_2HPO_4$, 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* $KH_2PO_4$). The cells were then pelleted by low-speed centrifugation (200 *g*, 5 min, 4°C). The cell pellets were stored at − 80°C prior to initiating the isolation of the MRC.

### Fractionation and chromatographic scheme for isolation of HeLa cell MRC

The HeLa cell MRC was isolated as described by Malkas et al. [3]. Briefly, frozen HeLa cell pellets were thawed and resuspended in 2 volumes of buffer containing 50 m*M* Tris-HCl, pH 7.5/0.25 *M* sucrose/5 m*M* $MgCl_2$/0.1 m*M* each of phenylmethyl sulfonyl fluoride (PMSF) in isopropyl alcohol, and amino acetonitrile hemisulfate (AAN), pH 7.5/1 m*M* dithiothreitol (DTT). The resuspended cells were homogenized by 30 strokes in a loose-fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 2000 rpm, and the crude nuclear and cytosolic (S-1) fractions were each collected separately.

Mitochondria were removed from the S-1 fraction by centrifugation at 15 000 rpm for 15 min, and the resultant supernatant was designated the S-2 fraction. The S-2 fraction was then subjected to centrifugation at 100 000 *g* for 60 min to remove microsomes. The postmicrosomal supernatant (S-3) was collected.

The crude nuclear pellet was resuspended in 2 volumes of a buffer containing 50 m*M* Tris-HCl, pH 7.5/1 m*M* DTT/0.15 KCl/5 m*M* each of EDTA-$Na_3$ and EGTA-$Na_3$/0.1 m*M* each of PMSF and AAN. The resuspended nuclei were gently stirred for 2 h at 4°C. The extracted nuclei were centrifuged for 15 min at 15 000 rpm and the supernatant (NE) collected.

The NE and S-3 fractions were pooled and KCl was added to a final concentration of 2 *M*, after which polyethylene glycol (PEG) was added to a final concentration of 5% and the mixture stirred gently for 1 h at 4°C. PEG-precipitated material was pelleted by centrifugation for 30 min at 16 000 *g*, and the supernatant (PEG NE/S-3) was collected. The PEG NE/S-3 was dialyzed (to remove the PEG) for 3 h against two changes of a buffer containing 50 m*M* Tris-HCl, pH 7.5/1 m*M* DTT/1 m*M* each of EDTA-$Na_3$ and EGTA-$Na_3$/0.1 m*M* each of PMSF and AAN/0.15 *M* KCl. The dialyzate was clarified by centrifugation for 10 min at 13 000 *g*. The clarified PEG NE/S-3 fraction was layered over a 2 *M* sucrose cushion and subjected to centrifugation at 100 000 *g* for 16–18 h at 4°C. The material above the sucrose interphase was collected and designated the S-4 fraction. The sucrose interphase fraction was collected and designated P-4.

The P-4 fraction was dialyzed against 50 m*M* Tris-HCl, pH 7.5/1 m*M* DTT/1 m*M* EDTA/10% glycerol/50 m*M* KCl. The P-4 fraction was then applied to a Q-Sepharose column (Pharmacia) (25 mg protein/ml of matrix) which had been pre-equilibrated in 50 m*M* Tris-HCl, pH 7.5/1 m*M* DTT/1 m*M* EDTA/10% glycerol/50 m*M* KCl. The unbound protein was washed from the matrix with 8 volumes of 50 m*M* Tris-HCl, pH 7.5/1 m*M* EDTA/10% glycerol/50 m*M* KCl. The matrix-bound protein was eluted by an increasing KCl gradient of 50 m*M* to 1 M in 50 m*M* Tris-HCl, pH 7.5/1 m*M* DTT/1 m*M* EDTA/10% glycerol. The column fractions were dialyzed against 20 m*M* Hepes, pH 7.5/1 m*M* DTT/1 m*M* EDTA/1.5 m*M* KCl, then individually assayed for their ability to support in vitro SV40 DNA replication. The column fractions able to support in vitro DNA replication were pooled, aliquoted and then stored at -80°C. This protein fraction was designated the Q-Sepharose peak and has been previously shown to contain the DNA replication-competent MRC [3, 4]. The Q-Sepharose peak protein fraction was used in the experiments described in this report.

### Purification of SV40 large T-antigen

In vitro SV40 DNA replication requires the addition of the viral large T-antigen protein for the efficient initiation of DNA synthesis. For our studies the SV40 large T-antigen was purified as described by Malkas et al. [3].

### In vitro SV40 DNA replication assay

Assay reaction mixtures (25 µl) contained 80 m*M* Tris-HCl, pH 7.5, 7 m*M* $MgCl_2$, 1 m*M* DTT, 15–25 µg protein fraction, 1.0–2.0 µg purified SV40 large T-antigen, 50 ng plasmid $pSV0^+$ containing an insert of SV40 replication-origin DNA sequences [8], 10 µ*M* each of dTTP, dATP, rCTP, rGTP and UTP, 1 m*M* ATP, 25 µ*M* [$^{32}$P]dGTP (NEN-Boston, Mass.; 3000 Ci/mmol), 40 m*M* creatine phosphate, and 1 µg of creatine kinase. The concentration of dCTP (or ara-CTP; Sigma Chemical Co., St. Louis, Mo.) used in reactions is indicated in the Results. In reactions where [$^3$H]dCTP (NEN; 56 Ci/mmol) or [$^3$H]ara-CTP (Moravek Biochemicals, Brea, Calif.: 2 Ci/mmol) were the sole radiolabeled nucleotides, the concentration of dGTP was 10 µ*M*. The reaction was incubated at 35°C for the length of time indicated in the Results. To quantify the amount of radiolabel incorporated into the replication products Whatman DE81 filter binding was used [9].

For neutral DNA agarose gel electrophoretic analysis of the DNA products, the DNA replication reaction was stopped by adding 100 µg of carrier RNA and 0.1% SDS. The DNA replication assay reaction products were then extracted with phenol/chloroform and further processed for analysis on a 1% DNA agarose gel in 89 m*M* Tris borate/89 m*M* boric acid/2 m*M* EDTA (TBE) [9]. The agarose gels were dried and subjected to autoradiography to visualize the replication products [9].

### Nuclease digestion and high-pressure liquid chromatographic (HPLC) analysis of DNA replication products

Prior to subjecting the in vitro DNA replication products to nuclease digestion and HPLC analysis, unincorporated radiolabeled dNTP was removed from the in vitro DNA replication reactions by filtering the mixtures through a Centricon 30 membrane (Amicon, W.R. Grace, Danvers, Mass.) with 10 m*M* Tris-HCl, pH 8.0/1 m*M* EDTA (TE) buffer and, finally, 10 m*M* Tris-HCl, pH 8.0. Calcium chloride was then added to a final concentration of 2 m*M*. The DNA replication products were then digested with 50 units of micrococcal nuclease (Sigma) at 37°C for 45 min as described previously [10]. The pH was then adjusted to 6 with 2 *N* HCl. This was followed by

two rounds of digestion each with 5 units of spleen phosphodiesterase II at 37°C each for 30 min. Authentic deoxyribonucleotides and deoxyribonucleosides were added to these digested samples to serve as standards for HPLC analysis as reported previously [10]. The samples were then filtered through a Centricon (Amicon) membrane to remove proteins prior to HPLC analysis.

The nuclease-digested in vitro DNA replication products were subjected to HPLC analysis as described previously [10]. The resolution and efficiency of the HPLC separation has been reported previously [10]. Briefly, using a reverse-phase C18 column (Waters) and a mobile phase containing 0.01 *M* $KH_2PO_4$, pH 5.5, a linear gradient of 0–15% methanol (80%) was run over 30 min at a flow rate of 1.5 ml per min. Fractions were collected at 30-s intervals and counted on a Beckman LS6000 Beta-counter. The tritiated deoxyribonucleotides/sides eluted were identified by coelution with authentic standards, as monitored by absorption at a wavelength of 254 nm.

## Results

### The effect of ara-CTP on MRC-mediated DNA replication.

Experiments were initiated that were designed to examine the effect of ara-CTP on the DNA synthetic reaction carried out by the MRC. We have shown previously that the MRC-driven DNA synthesis reaction is dependent only on exogenously added dNTPs, indicating that newly synthesized DNA molecules produced by the human MRC only contain dNMP residues that are added to the in vitro DNA replication reaction mixture [4]. To determine whether ara-CTP would affect the human MRC's ability to support DNA synthesis, MRC-driven SV40 in vitro DNA replication reactions containing 10 μ*M* dCTP with increasing concentrations of ara-CTP were performed (Materials and methods). The in vitro SV40 DNA replication reactions were incubated at 35°C for 1 h. After incubation the level of in vitro DNA synthesis in the reactions was measured by quantifying the amount of radiolabeled nucleotide incorporated into macromolecular material during the reaction (Materials and methods). The results of these experiments are shown in Fig. 1. A logarithmic relationship was observed between the inhibition of MRC-mediated DNA synthesis and the concentration of ara-CTP present in the in vitro DNA replication reaction. MRC-driven DNA synthesis was inhibited by 50% when the ara-CTP to dCTP ratio was 10 to 1.

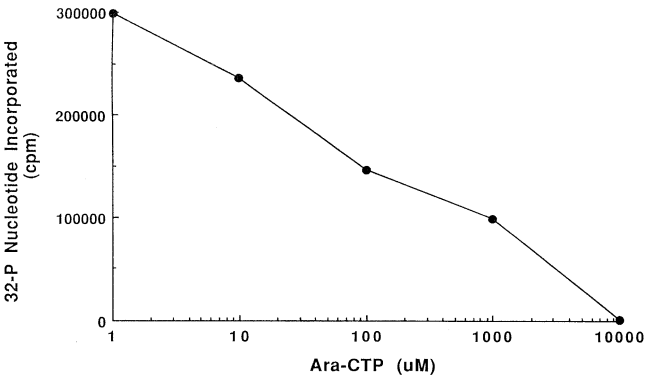


**Fig. 1.** Effect of ara-CTP on MRC-mediated DNA replication in vitro. MRC-mediated SV40 in vitro DNA replication reactions were performed in the presence of 10 μ*M* dCTP and increasing concentrations of ara-CTP. The DNA replication reactions were incubated for 1 h at 35°C, the reactions were stopped by spotting the reaction mixture onto Whatman DE81 filters, and the amount of $^{32}$P-dGTP incorporated into DNA during the reaction was determined (as described by Sambrook et al. [9]

### Full-length DNA products are made by the MRC in the presence of ara-CTP

In order to determine the types of DNA molecules produced by the MRC in the presence of ara-CTP, MRC-driven in vitro DNA replication assays containing either 10 μ*M* dCTP or 100 μ*M* ara-CTP plus 10 μ*M* dCTP were performed (Materials and methods). The in vitro DNA replication reactions were incubated at 35°C for 4 h. The level of incorporation of radiolabeled nucleotide into newly synthesized DNA molecules by the MRC was approximately two- to three-fold lower in the presence of ara-CTP than in the presence of dCTP alone (Fig. 2). Neutral DNA agarose gel electrophoresis analysis (Fig. 3) (Materials and methods) showed that full-length daughter DNA molecules (both relaxed circles and supercoiled plasmid) were produced during the DNA synthesis reactions even though ara-CTP inhibited the DNA replication process orchestrated by the MRC. The full-length daughter DNA molecules were resistant to DpnI endonuclease digestion (data not shown), indicating that they were the product of semiconservative DNA replication [3, 5]. These results suggest that, even though ara-CTP inhibits the in vitro DNA synthetic process of the human MRC, full-length daughter molecules can eventually be produced by the MRC.

### The human MRC can incorporate ara-CTP into DNA at internucleotide positions

In order to determine whether the MRC was capable of actually incorporating ara-CTP into newly synthesized DNA, we performed in vitro DNA replication assays containing either $^3$H-ara-CTP (100 μ*M*) or $^3$H-dCTP (10 μ*M*) as the sole radiolabeled nucleotide in the assay (Materials and methods). As can be seen in Fig. 4, $^3$H-ara-CTP and $^3$H-dCTP were incorporated into DNA by the MRC over the entire course of the reaction, but the level of $^3$H-ara-CTP incorporation was approximately three fold lower than the level of $^3$H-dCTP incorporation.

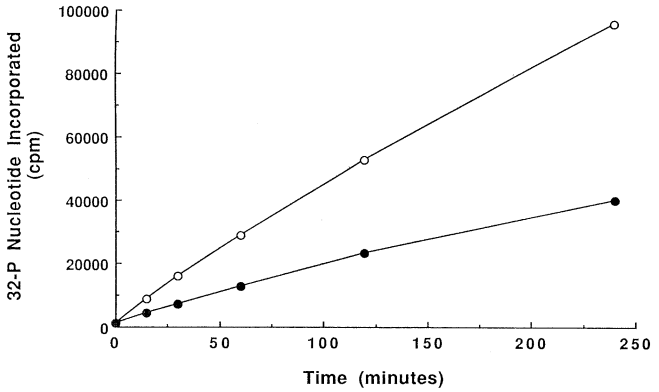


**Fig. 2.** MRC-mediated in vitro DNA replication in the absence or presence of ara-CTP. MRC-driven in vitro replication reactions were carried out in the absence of ara-CTP (○) or in the presence of 100 μ*M* ara-CTP (●) under the conditions described in the text. Incorporation of $^{32}$P-dGTP into newly replicated DNA was monitored at intervals throughout the assays by spotting 2 μl of the reaction mixture onto Whatman DE81 filters at the indicated time intervals. The amount of $^{32}$P-dGTP retained on the filters was determined as described by Sambrook et al. [9]

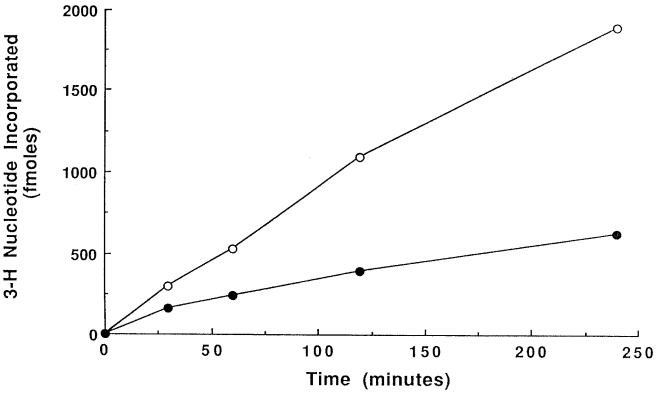


**Fig. 4** [$^3$H]ara-CTP or [$^3$H]dCTP incorporation into DNA by the MRC. MRC-mediated SV40 in vitro DNA replication reactions were performed as described in the text. The amount of either $^3$H-ara-CTP or $^3$H-dCTP incorporated into DNA was determined by Whatman DE81 filter binding [9]. (○ reaction containing $^3$H-dCTP, ● assay performed with $^3$H-ara-CTP as the sole radiolabeled nucleotide)

To determine whether the MRC incorporated ara-CTP into either a DNA chain terminal position or internucleotide positions within the newly synthesized DNA, MRC-mediated DNA replication reactions were performed in the presence of $^3$H-ara-CTP, and the resulting tritium-labeled DNA products were subjected to digestion with spleen phosphodiesterase II and micrococcal nuclease (Materials and methods). Digestion of the DNA products with these two nucleases results in the hydrolysis of the 5′-phosphodiester bond between adjacent deoxyribonucleotides which results in the generation of 3′-monophosphate deoxyribonucleotides. Due to the 5′ to 3′ nature of DNA synthesis, any base occupying a DNA strand at the 3′ terminus position will be converted to its corresponding deoxyribonucleoside by this nuclease digestion, while a base at an internucleotide position in the DNA will be digested to a 3′-dNMP [11]. The nuclease-treated DNA products were then resolved by HPLC (Materials and Methods). The result of this experiment is shown in Fig. 5. The data indicate that a significant proportion (approximately 75%) of the $^3$H-ara-CMP residues were located within internucleotide positions in the DNA products formed by the MRC, while only approximately 25% of the ara-C nucleotides occupied a DNA chain terminus position. Taken together, these results demonstrate that the isolated MRC is capable of utilizing ara-CTP as a substrate, and incorporating the ara-CTP into internucleotide positions in newly synthesized DNA.

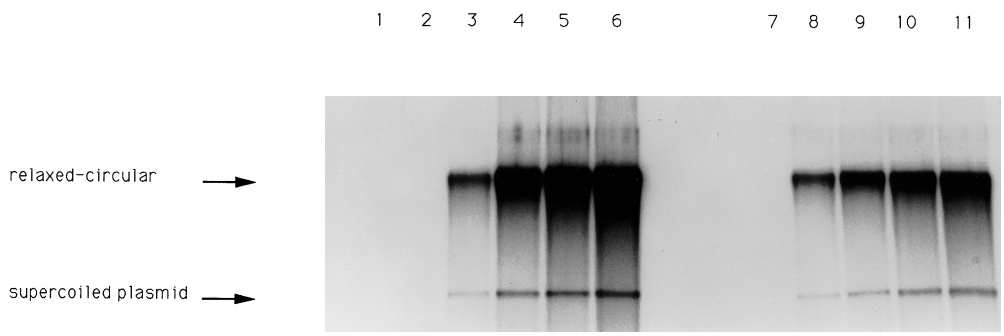


**Fig. 3** Neutral agarose gel analysis of the MRC-driven in vitro DNA replication products formed in the absence or the presence of ara-CTP. The DNA replication products were isolated by phenol/chloroform extraction of 10μl aliquots of reaction mixture taken at 0 (*lanes 2, 7*), 30 (*lanes 3, 8*), 60 (*lanes 4, 9*), 120 (*lanes 5, 10*), and 240 (*lanes 6, 11*) min after initiating the DNA synthesis reaction. The extracted DNA was precipitated with 2 *M* ammonium acetate in the presence of 2 volumes of isopropanol. The precipitated DNA was resuspended in Tris/EDTA (10 m*M*/1 m*M*) and loaded onto 1% agarose gels containing TBE (lane *1* DNA products isolated from a reaction mixture lacking T-antigen, *lanes 2–6* reaction products isolated from a reaction performed in the absence of ara-CTP, *lanes 7–11* reaction products formed in the presence of 100 μ*M* ara-CTP). The positions of relaxed circular and supercoiled plasmid DNA are indicated

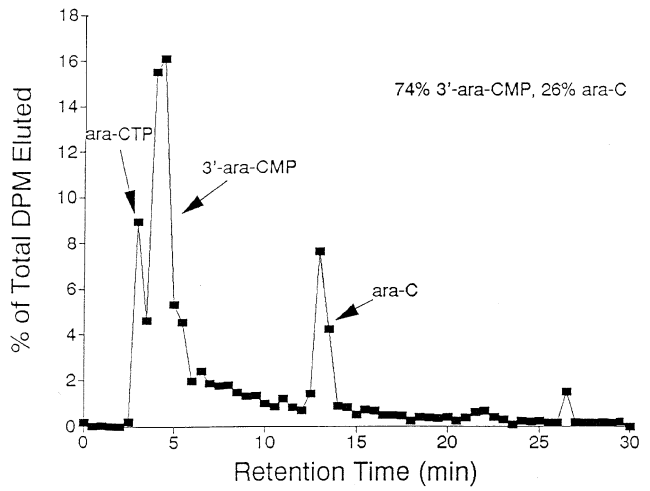


**Fig. 5** HPLC elution profile of nuclease-treated MRC DNA replication products. MRC-mediated SV40 in vitro DNA replication reactions were performed as described in the text. To determine the position of ara-CMP in newly synthesized DNA the reactions used $^{3}$H-ara-CTP in place of dCTP. The DNA replication products were subjected to nuclease digestion and HPLC analysis as described in the text. The elution positions of the $^{3}$H-ara-C nucleoside, the 3′ monophosphate nucleotide and the triphosphate nucleotide are indicated and were assigned based on their coelution with authentic standards

## Discussion

Although ara-C is one of the most effective agents used in the treatment of acute leukemia in humans [12], the precise mechanism(s) responsible for ara-C-induced cytotoxicity remains unresolved. Early studies suggested that ara-CTP, the active metabolite of ara-C, competes with dCTP for binding to DNA polymerase, resulting in the inhibition of cellular DNA synthesis [13–15]. Later kinetic studies, however, indicated that ara-CTP is only a weak competitive inhibitor of DNA polymerase, and that a simple competitive inhibition of the enzyme does not explain the effects of this agent on cellular DNA replication and cytotoxicity [16]. Additional studies have suggested that as ara-CMP residues are incorporated into DNA, they act as relatively potent terminators of growing daughter DNA strands [17–22]. The biochemical studies of Mikita and Beardsley [21] indicate that the presence of an ara-CMP residue at the 3′ terminus of a newly synthesized DNA strand results in the profound inhibition of further chain elongation by purified human DNA polymerase α enzyme. Recently, Perrino and Mekosh [22], in studies using an immunopurified human DNA polymerase α, have found the extension of a growing daughter DNA strand from an ara-CMP 3′ terminus by the next complementary nucleotide to be approximately 2000-fold less efficient than extension from a correctly base paired 3′ terminus. These studies have given rise to the current perception that once ara-CMP is incorporated into a growing daughter DNA strand it acts as an inhibitor of DNA synthesis by terminating further DNA chain elongation.

The concept of ara-C acting solely as a growing daughter DNA chain terminator has been challenged, however, following studies utilizing intact cells [14, 23–25]. Graham and Whitmore [14] analyzed high-molecular-weight DNA from mouse L cells that had been labeled with $^{3}$H-ara-CMP. They found only a small percentage of the $^{3}$H-ara-CMP at the 3′ termini of the DNA; most was located at internucleotide positions. In another study a pH-step alkaline elution technique was used to investigate the effects of ara-C on DNA replication in intact HL-60 cells [23], and it was shown that, although ara-C slows DNA replication, it eventually becomes incorporated into genomic length DNA. In addition, ara-CMP incorporation into a growing DNA strand was observed not to cause absolute chain termination since the ara-CMP incorporated into DNA was found predominantly in internucleotide linkages [23]. These results agree with those of Dijkwel and Wanka [24] who studied the effects of ara-CMP incorporation into the newly synthesized DNA of calf liver cells. Their experiments revealed that short, newly synthesized DNA strands containing ara-CMP are eventually converted to full-size DNA molecules [24]. Thus 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 addition of dNTPs to the 3′-ara-CMP occurs, resulting in ara-CMP being found in internucleotide linkages. Therefore, the current findings regarding the action of ara-C on intact cell DNA synthesis suggest that the incorporation of ara-CMP into cellular DNA results in the inhibition of DNA replication by the slowing of the replication process. Eventually, however, deoxynucleotide monophosphate residues are added to the 3′ terminus of the incorporated ara-CMP, and full-length daughter DNA molecules are produced. 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 [17–22].

The results described in this report indicate that the human MRC can utilize the active metabolite of ara-C (i.e. ara-CTP) as a substrate for in vitro DNA synthetic reactions. Our results are in accord with those of Graham and Whitemore, Ross et al., and Dijkwel and Wanka [10, 14, 23, 24] who demonstrated, using intact cells, that $^{3}$H-ara-C is incorporated into short DNA fragments initially, and that, as the experiments proceed, the radiolabeled ara-C moves into larger DNA fragments. Our results, which were obtained in a cell-free system, are also in agreement with those of Magnusson et al. [26], who studied the effects of ara-C on PyV DNA replication in intact cells. These workers demonstrated the incorporation of radiolabeled

ara-CMP at internucleotide linkages in the progeny PyV form I DNA produced in infected cells, implying that ara-C (i.e. ara-CTP) can act as a substrate for DNA polymerase present in intact cells, and that ara-C only slows the growth of newly replicating daughter DNA strands in intact cells. We also observed that ara-CTP slows the DNA replication process orchestrated by the MRC. However, the synthesis of daughter form I plasmid DNA in the presence of ara-CTP during MRC-mediated in vitro SV40 DNA replication reactions was observed. These results suggest that, even though ara-CTP inhibits the in vitro DNA synthetic process of the human MRC, full-length daughter molecules are eventually produced by the MRC.

Collectively, our results suggest that the human MRC can be used as an effective in vitro model system for studying the effects of ara-CTP on DNA replication. Our results imply that the MRC is able to carry out the replication reactions with ara-CTP in a manner that is analogous to that observed in intact cells. This conclusion is based on our observation that newly replicated DNA can be isolated from MRC-mediated DNA replication reactions containing ara-CMP in internucleotide linkages. Our results are also consistent with observations of other investigators who have found that isolated nascent DNA, replicated in the presence of ara-C in intact cells, contains at least 70% of the ara-CMP in internucleotide positions [10, 26].

Thus, the in vitro model system based on the human-cell-derived MRC should prove valuable in facilitating the identification and analysis of compounds that affect cellular DNA synthesis. It is our belief that the MRC represents an avenue for deciphering the mechanism of action of these anticancer drugs, and its utilization may lead to improved rational drug design.

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