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13. ABSTRACT ***(Maximum 200 Words)***

Gemcitabine (dFdC) and cytarabine (araC) are both analogs of deoxycytidine. In this report, we compared the effects of dFdC and araC on *in vitro* DNA synthesis mediated by the DNA synthesome with the effects of these drugs on intact MCF7 cell DNA synthesis. We also examined the effects of dFdC and AraC on their associated target protein, DNA polymerases α, and δ. Our results showed that) dFdC is a more potent inhibitor of intact cell DNA synthesis and *in vitro* SV40 DNA replication than araC; 2) the decrease in the synthetic activity of synthesome-mediated *in vitro* SV40 origin dependent DNA synthesis by dFdCTP and araCTP correlates with the inhibition of DNA polymerase α activity; and 3) the MCF7 cell DNA synthesome can serve as a unique and relevant model to study the mechanism of action of anticancer drugs that directly affect DNA synthesis.

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INTRODUCTION

The biological activity of most nucleoside antimetabolites is due to their ability to inhibit the DNA synthetic process, which is an essential function both for cell division and proliferation. 1-β-D-arabinofuranosylcytosine (araC) has been used effectively in the clinic to treat hematologic cancers [5]. 2', 2'-difluroxydeoxycytidine (gemcitabine, dFdC) is a novel deoxycytidine analog with structural and metabolic similarities to araC. AraC and gemcitabine differ in structure from the parent nucleoside, deoxycytidine, by specific modifications to the 2' carbon of the furanose ring. Clinical trials have shown that gemcitabine is effective in most solid tumors and more potent and less toxic than araC [12,13,10,18,20]. Intact cell studies have indicated that inhibition of DNA synthesis is the predominant effect of dFdC and araC [15,25,26,28]. Like araC, the major targets for dFdCTP are the DNA polymerases. It has been shown that incorporation of araCTP and dFdCTP into DNA is most likely the primary mechanism by which these drugs exert their cytotoxic effect [26]. Although inhibition of DNA synthesis has been strongly correlated with intracellular dFdCTP concentration [11], little work has been done to directly compare the effects of dFdCTP and araCTP on reducing the level of DNA replication within the cell. We have previously reported that a highly purified multiprotein form of DNA polymerase (the DNA synthesome) can be isolated from a variety of mammalian cell types and tissues [2,3a,14,17,21,33] We have shown that the DNA synthesome is fully competent to support origin-specific large T-antigen-dependent *in vitro* SV40 DNA replication [2,3a,21,33,]. Biochemical characterization of the DNA synthesome has identified several protein components of the complex that were found to be essential for DNA replication [14,23]. These proteins include the DNA polymerases α, δ, and ε, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication factor A (RPA), DNA helicase, and DNA ligase I [2,21,33]. Most importantly, in the presence of viral large T antigen and the SV40 replication origin sequence, the synthesome is fully competent to carry out all phases of the DNA replication process required to replicate an SV40 origin containing plasmid *in vitro*. We have successfully examined the action of araC and camptothecin using this model system [1,3b,9,31,32] and have now extended the results of these studies by exploring how the inhibitory effects of dFdC and araC compare with one another. Our studies compared the inhibitory effects of dFdC and araC on intact human breast cancer cell DNA synthesis and in DNA synthesis mediated by our *in vitro* DNA replication assay system. Our results demonstrated that dFdC was more potent in inhibition of *in vitro* DNA synthetic activity mediated by the DNA synthesome and in intact MCF7 cells than araC.

BODY (SUMMARY OF RESEARCH)

A Effect of dFdC and araC on intact MCF7 cell DNA synthesis:

As proposed in task 1 of my statement of work, the first goal was to generate dose-response curves to determine the correlation between concentration of araC and dFdC with extent of inhibition of MCF DNA synthesome. In order to verify that araC and dFdC affect the ability of intact MCF7 cells to carry out DNA synthesis as previously reported [1,10,15]. Exponentially growing MCF7 cells were incubated in the absence (control assigned a value of 100%) or presence of increasing concentrations of araC and dFdC. The cells were exposed for 24 hours to drug concentrations ranging from 1 to 1000μM, and the drugs were then removed by washing the cells with PBS. Fresh media was added to the cell culture and the cells were incubated with [^{3}H]-thymidine for 4 hours. The labeled cells were lysed, and the level of DNA synthesis was measured by quantifying the amount of [^{3}H]-thymidine retained in acid-insoluble material. As shown in Figure 1, intact MCF7 cell DNA synthesis was inhibited by both drugs in a concentration-dependent manner. About 10μM dFdC and 80μM araC were required to reduce MCF7 cell DNA synthesis to 50% of the control activity measured in the absence of either drug. The IC50 value for the inhibition of intact cell DNA synthesis by araC was comparable to that observed by us using the estrogen-receptor-negative breast cancer cell line; MDA MB-468 [1]. We also observed that the araC concentration required to inhibit 50% of intact HeLa cell DNA synthesis was also about 80 μM (data not shown). This value was consistent with our previously reported IC50 value for araC inhibition of intact cell DNA synthesis [22].

B Effect of dFdC and araC on intact MCF7 cell clonogenicity:

We next performed cell survival assays to compare the cytotoxic effects of dFdC and araC on the ability of MCF7 to form colonies. As shown in Figure 2, 50% of the cells lost their clonogenic capacity at concentrations of dFdC above 8 μM following a 4-hour incubation of the cells with the drug. The concentration of dFdC needed to inhibit the clonogenic survival of 50% of MCF7 cells was approximately 12-fold less than that of araC. Thus, our results from the intact DNA synthesis and clonogenicity demonstrated that dFdC was significantly more cytotoxic to intact MCF7 cells than araC.

C Isolation of DNA synthesome from human breast cancer MCF7 cells:

In order to examine the effect of the drugs on the synthesome associated DNA synthetic activity, the DNA synthesome was first isolated from human breast cancer MCF cells using our published protocol essentially as described by Malkas et al [19,21] and as outlined in Figure 3. We designated the human breast cancer MCF7 cell DNA synthesome as M7DS. In my original proposal, the DNA synthesome from MCF-10A, which is a non-malignant breast cancer cell line, was also proposed to be used as a control for comparing inhibition effect of the two drugs. However, we did not include MCF-10A cells in our studies. One reason is that the panel questioned the relevance of these experiments to the overall project was uncertain. Another reason is that MCF-10A

cells is difficult to grow and turns to be malignant after several passage during cell culture process. Since we have synthesomes from HeLa and MDA MB-468 cells as control, we therefore gave up using the synthesome from MCF-10A cells.

D Comparison of the inhibitory effects of araCTP and dFdCTP on *in vitro* SV40 DNA replication using M7DS:

We have previously shown that the DNA synthesome isolated from the HeLa cell and human breast cancer cell MDA MB-468 is capable of supporting the origin-specific T-antigen-dependent SV40 DNA replication reaction *in vitro* [1,3b,31]. These studies demonstrated the utility of the purified DNA synthesome as a relevant *in vitro* model that is useful for studying the mechanism of action of anticancer drugs such as: araC, campothecin, and VP16. In order to directly compare the anti-DNA synthetic activity of araCTP and dFdCTP, we performed *in vitro* SV40 DNA replication assays in the absence and presence of several concentrations of each of these two drugs (1μM, 10μM, 100μM and 1mM). Both drugs inhibited SV40 DNA replication in a concentration-dependent manner as measured by quantifying the amount of ^{32}P-dGTP incorporated into DNA (Figure 4). Fifty-percent inhibition of the *in vitro* DNA replication assay was achieved in presence of 10uM dCTP using approximately 8uM dFdCTP and 80uM araCTP. The results of this assay indicated that dFdCTP was able to more effectively compete with dCTP to inhibit DNA-synthesome-mediated *in vitro* DNA replication than araCTP. In consistent with the results from the intact cell, dFdCTP showed more potent in inhibiting *in vitro* SV40 DNA synthesis than araCTP. Our data also showed a close correlation between the IC50 values of both drugs for inhibiting intact cell DNA synthesis and the DNA synthesome mediated *in vitro* SV40 replication assay.

E *In vitro* replication products produced by M7DS in presence of araCTP or dFdCTP.

We further analyzed the replication products of the *in vitro* replication reaction using a 1% neutral and a 1% alkaline agarose gel (Figures 5 and 6, respectively). Our results indicated that the MCF7 cell DNA synthesome was capable of producing full-length daughter DNA as shown by the presence of form I (superhelical) DNA and form II (nicked open circular) DNA, as well as higher-order replication intermediates (Figure 5 lane 2). This reaction was also T-antigen dependent (Figure 5 lane 1). In the presence of low concentrations (1 μM) of both drugs, full-length daughter DNA molecules (form I and form II) were observed (Figure 5, lane 3 and lane 7). At higher concentrations, the production of form II DNA molecules as well as replication intermediates was inhibited in a concentration-dependent manner (note both neutral and alkaline gels (Figure 5, 6 lanes 2-10)). However, form I DNA molecules disappeared at drug concentrations higher than 1μM, indicating that dFdCTP and araCTP may impair the process to form supercoiled DNA. In the presence of both drugs, the production of short Okazaki fragments was inhibited in a concentration-dependent manner (Figure 6, lanes 3-10) and were completely inhibited at higher dFdCTP concentrations (Figure 6, lanes 5-6), suggesting that dFdCTP had a greater inhibitory effect on the initiation stage of DNA synthesis. These results were in accordance with our previously reported results using

the DNA synthesome isolated from HeLa cells and MDA MB-468 cells [1,22]. Furthermore, they correlate with other reports from this laboratory employing our DNA-synthesome-mediated *in vitro* DNA replication assay system as well as intact cells [15,27,28].

F The effects of araCTP and dFdCTP on DNA synthesome associated DNA polymerase α and DNA polymerase δ activity:

Our previous study on the inhibitory effects of araCTP on the activity of the purified DNA polymerase α and the DNA synthesome associated polymerase α provided evidence indicating that the DNA synthesome can be used as an *in vitro* model system that more closely reflects the events occurring within the intact cell than can be achieved using individually purified enzymes [4,10,32]. To further compare the activities of araC and dFdC on individual DNA replication essential proteins, we performed DNA synthesome associated DNA polymerase α and δ assays. In the polymerase α assays, we used activated calf thymus DNA as the template, and incubated the template with varying concentrations of araCTP and dFdCTP in the presence of the DNA polymerase. The polymerase α activity was measured by quantifying the amount of [^{3}H]TTP incorporated into DNA (Figure 7). Unlike *in vitro* SV40 DNA replication, the activity of the synthesome associated polymerase α was not inhibited by 1 μM of either drug. However, at approximately 80 μM dFdCTP and 100 μM araCTP the activity of the synthesome-associated DNA polymerase was inhibited by 50% relative to the activity of the control reaction performed in the absence of the drug. Our results indicated that the inhibitory effect of dFdCTP and araCTP on synthesome associated DNA polymerase α occurs at nearly equivalent concentrations. The IC50 value of araC was in good agreement with that published in our previous reports [1,10,32]. However, dFdCTP was not significantly more potent than araCTP in the DNA polymerases assay, and this contrasts with our results obtained using the *in vitro* SV40 DNA replication assay. Previously, we had reported that the inhibitory effect of araCTP was primarily through inhibition of synthesome associated DNA polymerase α and that a significant amount of inhibition of synthesome associated polymerase δ activity also occurs, but at a 4-fold higher concentration of the drug [1,10]. In this study, it was also observed that both dFdCTP and araCTP showed inhibition of DNA polymerase δ at higher concentrations than those required to inhibit DNA polymerase α. The IC50 for inhibition of DNA polymerase δ was approximately 700 μM and 750 μM for dFdCTP and araCTP, respectively (Figure 8). At a concentrations of 100 μM however, the activity of synthesome associated polymerase δ was not significantly inhibited by both drugs. This was a significant finding because the activity of DNA polymerase α was readily inhibited by apparently 7- to 9-fold less drug than DNA polymerase δ. The fact that dFdCTP was significantly more potent than araCTP at inhibiting *in vitro* SV40 replication than at inhibiting synthesome associated DNA polymerase α and δ activity strongly suggests that dFdCTP may have a greater effect on inhibiting the coordinated replication activity of an organized DNA replication multienzyme complexes than purified DNA polymerases. Furthermore, our results imply that the potent inhibitory effects of dFdC may be due to the drug targeting additional proteins during the DNA synthetic process, or inhibiting the

coordinated elongation of both strands at a replication fork by specifically slowing the ability of one of the polymerases at the replication fork to efficiently carry out DNA synthesis.

G Two-dimensional high resolution electrophoresis system:

As proposed in the statement of work, we have set up two-dimensional high-resolution electrophoresis system and are optimizing conditions to further examine the types of daughter DNA molecular produced by the M7DS.

H Kinetic studies of SV40 replication mediated by MCF7 cell DNA synthesome:

We have also started to study the kinetics of replication and coordinated DNA synthesis mediated by DNA synthesome in Specific Aim 2. We have modified the methods we proposed originally in the proposal. We have adapted a simpler approach, in which, we perform *in vitro* SV40 replication assay with or without drugs and followed by specific restriction enzymes reaction. The reaction products were then resolved by 1% neutral agarose gel and autoradiography.

FIGURES

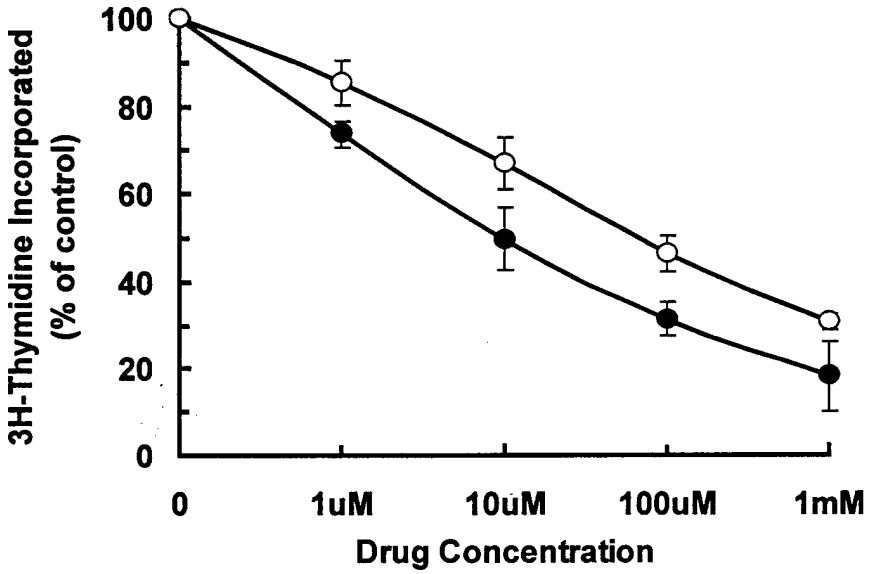


Figure 1. Effect of dFdC and araC on intact MCF7 cells DNA synthesis: MCF7 cells ($5X10^4$) were seeded onto 60mm cell culture plates and incubated for 24 hours at 37°C in Joklik's modified Eagle's medium. The cells were then exposed to one of several different concentrations of the indicated drug for 24 hours at 37°C. The cells were then labeled with ^{3}H-thymidine (1μCi/ml of medium). After a 4-hour incubation, the cells were lysed and the level of DNA synthesis was measured by quantifying the amount of ^{3}H-thymidine present in acid insoluble material (○: araC, ●: dFdC). Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.

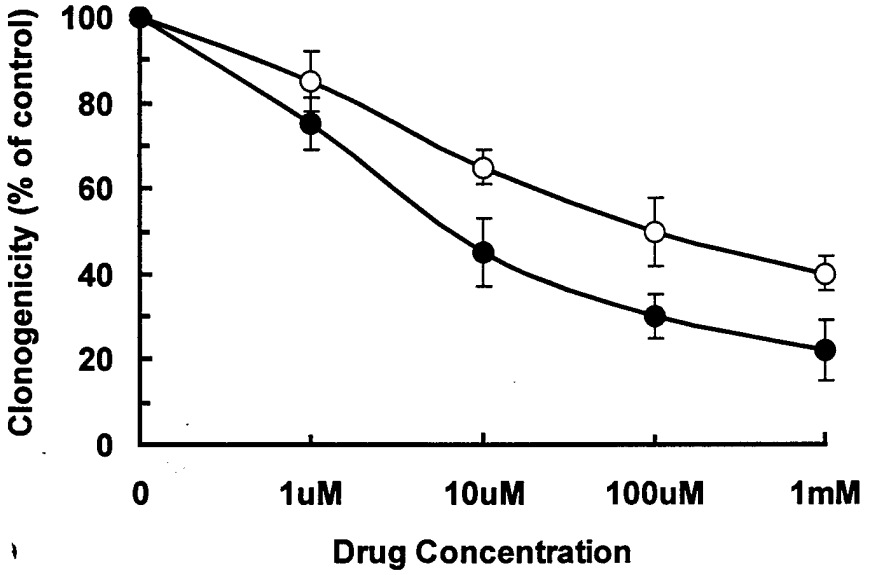
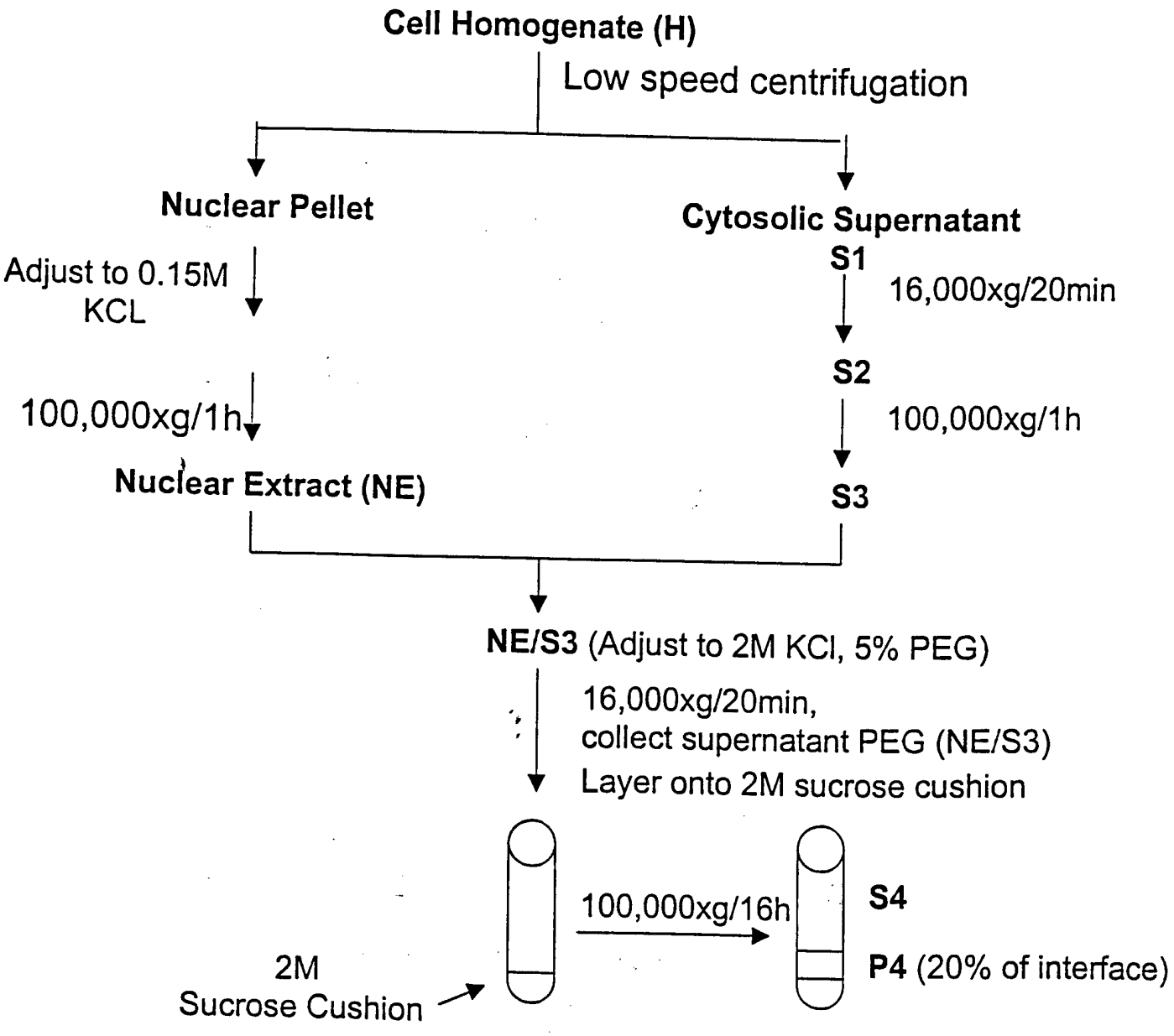


Figure 2. Effect of dFdC and araC on the clonogenicity of intact MCF7 cells: MCF7 cells (10^3) were seeded onto 60mm cell culture plates and incubated for 24 hours at 37°C in Joklik's modified Eagle's medium. Cells were then exposed to different concentrations of drugs for 4 hours followed by incubation in drug-free medium for 5 days. Colonies were fixed with 10% formaldehyde in phosphate-buffered saline and visualized by Giemsa stain and counted (○: araC,●: dFdC). Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.

Figure 3. Flow diagram of the subcellular fractionation scheme used to partial purify the DNA synthesome



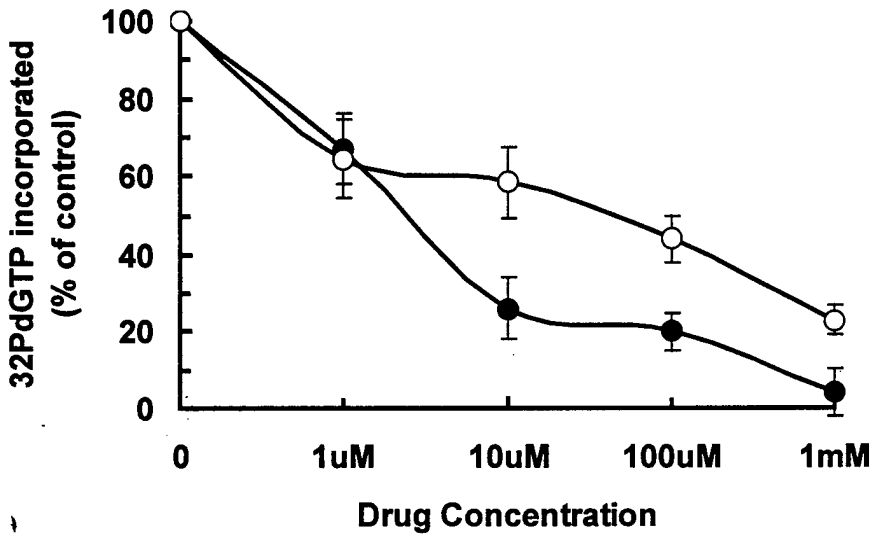


Figure 4. Effect of dFdCTP and araCTP on DNA synthesome mediated *in vitro* SV40 DNA replication. The assay was performed essentially as described in Malkas et al.[9] in the absence or presence of increasing concentration of drug. The reaction mixture (25 µl) contained 30mM HEPES (pH 7.5), 7mM $MgCl_2$, 0.5mM DTT, 2.5-3µg SV40 large T-antigen, 20µg of synthesome protein fraction, 50ng of the plasmid $pSVO^+$ containing an inserted SV40 replication origin DNA sequence, 1µCi [α-^{32}P]dGTP (New England Nuclear, 3000 Ci/mmol), 100µM each of dATP, and dTTP, 10µM each of dCTP and dGTP, 200µM each of rCTP, rGTP, and rUTP, 4mMrATP, 40mM phosphocreatine, and 1µg creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37°C for 4 hours. One microliter of the reaction mixture was spotted onto Whatman DE81 filters. The filters were then processed to quantify the amount of radiolabelled nucleotide incorporated into the DNA template [9]. Each point represents the average of three separate experiments; error bars represent standard error of the means (○: araC,●: dFdC). Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.

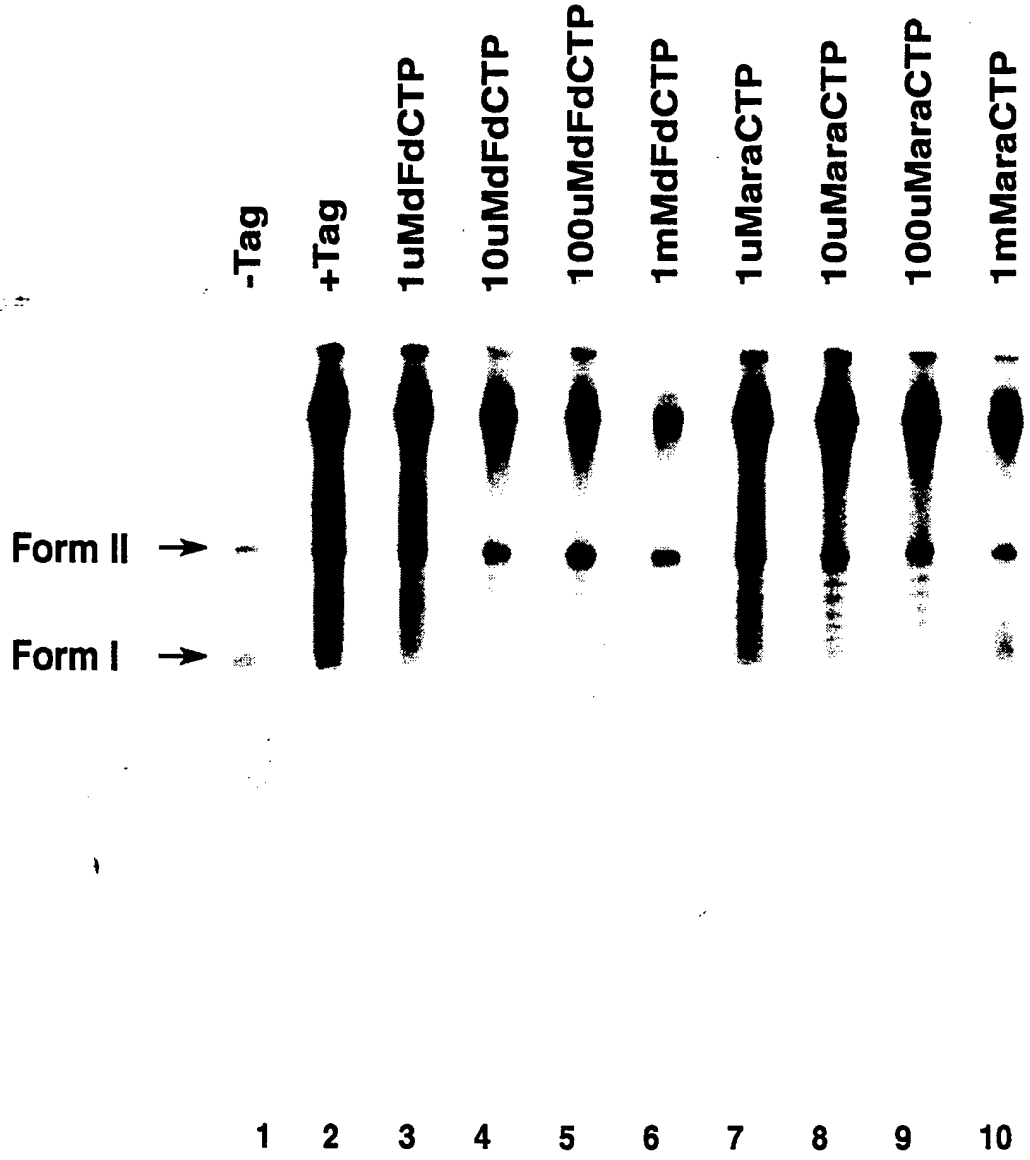


Figure 5: Neutral agarose gel analysis of the reaction products of the *in vitro* SV40 DNA replication assay. The DNA replication products formed in the *in vitro* DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2M ammonium acetate. The isolated DNA was resuspended in 10mM Tris/1mM EDTA, and the reaction products were resolved from one another using 1% agarose gels under neutral condition. The gels were dried and exposed to Kodak XAR5 films at –80°C for 8 hours.

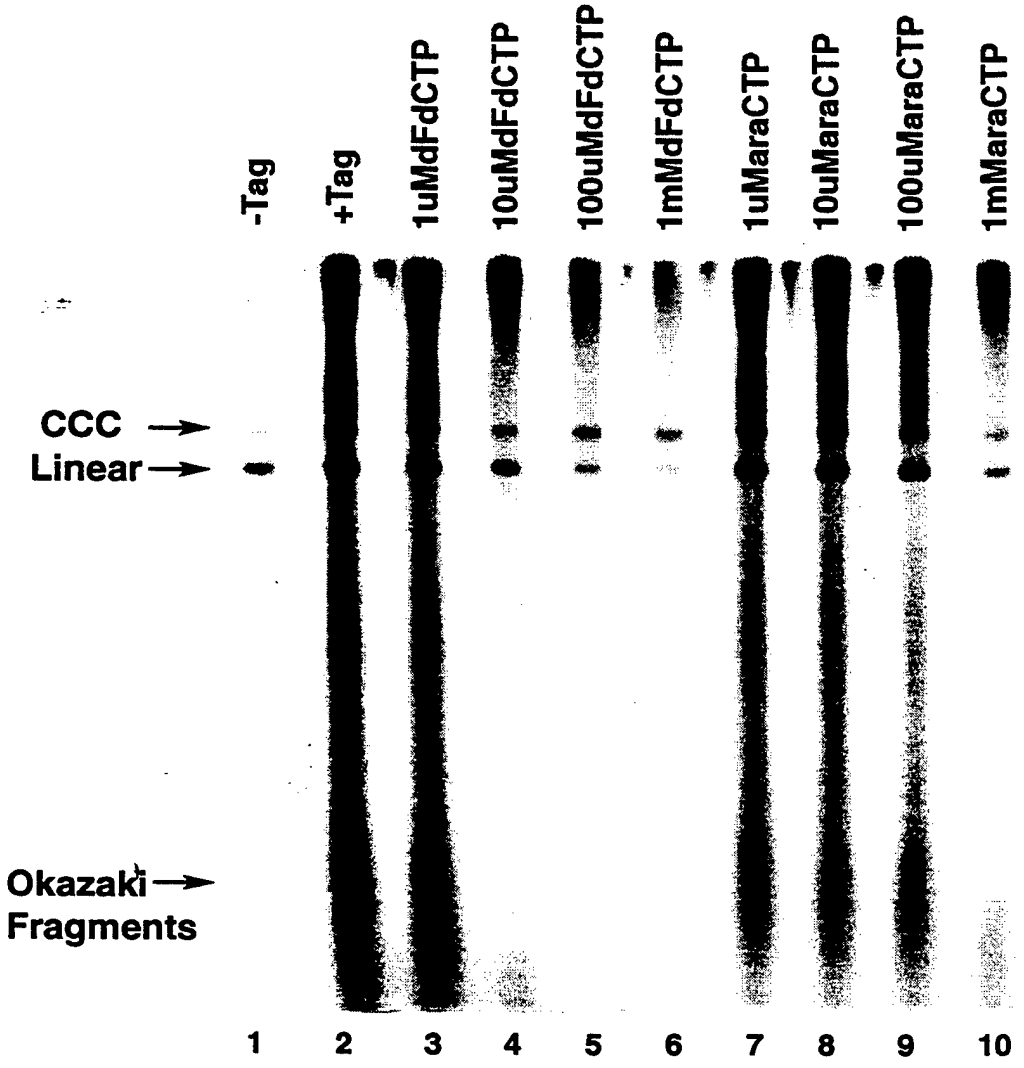


Figure 6: Alkaline agarose gel analysis of the reaction products of the *in vitro* SV40 DNA replication reaction. The DNA replication products formed in the *in vitro* DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2M ammonium acetate. The isolated DNA was resuspended in 10mM Tris/1mM EDTA, and the reaction products were resolved from one another using 1% agarose gels under alkaline condition. The gels were dried and exposed to Kodak XAR5 films at –80°C for 8 hours.

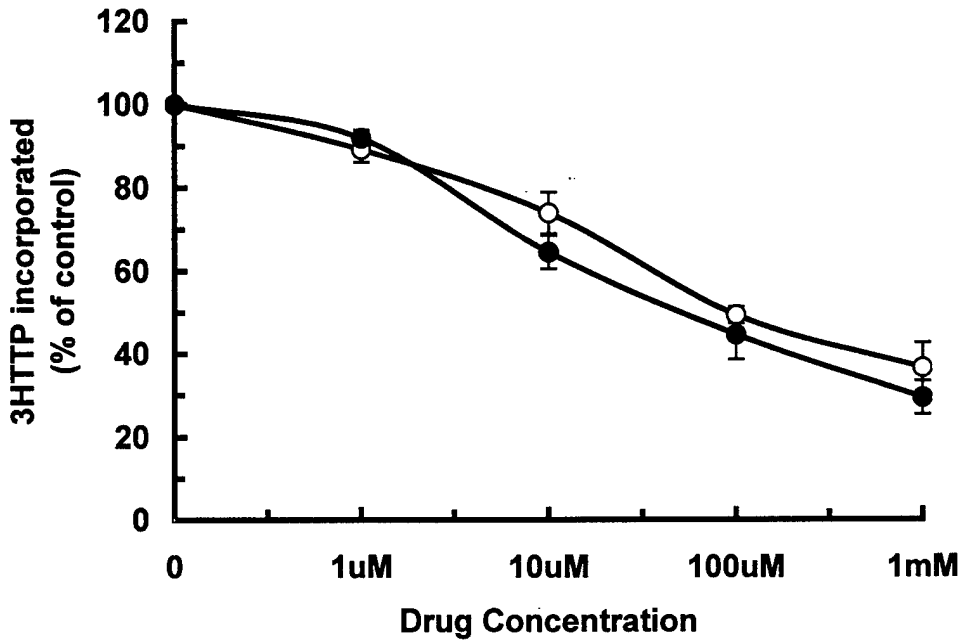


Figure 7. Effect of dFdCTP and araCTP on DNA synthesome-associated DNA polymerase α activity: The assays were performed using activated calf thymus DNA as the template. Reaction mixtures were incubated with different concentrations of drugs as described in the Materials and Methods (○: araC,●: dFdC). The amount of [^{3}H]dTTP incorporated into DNA was determined by Whatman DE81 filter binding [21]. Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared.

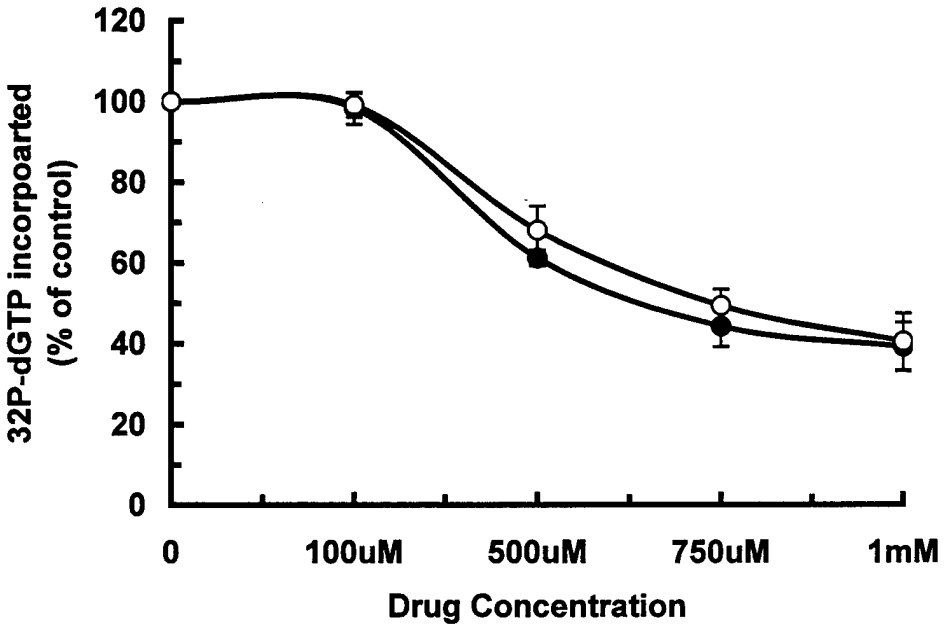


Figure 8. Effect of dFdCTP and araCTP on the synthesome-associated DNA polymerase δ activity: The assay was performed using a poly(dGdC) template as described in the Materials and Methods (○: araC,●: dFdC). The amount of [α-^{32}P]dGTP incorporated into DNA was determined by Whatman DE81 filter binding [9]. Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared.

KEY RESEARCH ACCOMPLISHMENT

- **dFdC was more potent than araC at inhibiting intact MCF7 cell DNA synthesis and clonogenicity.** [^{3}H]-Thymidine incorporation was inhibited by 50% at a dFdC concentration of 10uM, which was about 10-fold lower than the concentration of araC required to inhibit intact cell DNA synthesis by the same amount. As examined by clonogenicity assay, dFdC was also significantly more cytotoxic than araC after a 24-hour incubation.

- **dFdCTP was more potent than araCTP at inhibiting *in vitro* SV40 DNA synthesis mediated by DNA synthesome derived form MCF7 cells.** In the presence of 10uM dCTP, 8uM dFdCTP and 80uM araCTP were required to inhibit *in vitro* SV40 DNA synthesis by 50%.

- **dFdCTP and araCTP affected the formation of MCF7 DNA synthesome replication intermidiates.** *In vitro* SV40 replication assays demonstrated that the formation of full-length DNA along with replication intermediates were inhibited by dFdCTP in a concentration-dependent manner. However, full-length DNA was produced in the *in vitro* DNA replication assay even when the dFdCTP was incubated in the assay at concentrations of up to 1 mM.

- **The inhibitory effect of dFdCTP and araCTP was primarily through inhibition of synthesome associated DNA polymerase α** Although dFdCTP is more potent than araCTP at inhibiting *in vitro* SV40 DNA synthesis, there is no significant difference between the inhibitory effect of these two drugs on the activity of the MCF7 synthesome associated DNA polymerases α and δ. It was found that the drug concentrations required to inhibit 50% of the synthesome associated DNA polymerase δ activity were much higher than that of DNA polymerase α for both dFdCTP and araCTP.

- **The MCF7 cell DNA synthesome can serve as a relevant model to study the mechanism of action of anticancer drugs that directly affect DNA synthesis.**

- **We have also further purified the MCF7 cell DNA synthesome and proved that the synthesome is able to mediate all aspects of SV40 replication *in vitro*.**

REPORTABLE OUTCOMES

Manuscripts:

1. Jiang HY, Hickey RJ, Tom TD, Wills, PW, Abdel-Aziz, WF. and Malkas, LH (1999): DNA replication is mediated by a discrete multiprotein complex. J. Biol. Chem. Submitted
2. Jiang HY, Hickey RJ, Abdel-Aziz, WF, and Malkas, LH (1999): Effects of gemcitabine and araC on *in vitro* DNA synthesis mediated by the human breast cell DNA synthesome. Cancer Chemother. Pharmacol. In press
3. Abdel-Aziz WF, Hickey RJ, Jiang HY, Malkas LH (1999): AraC affects the formation of cancer cell DNA synthesome replication intermediates. Cancer Chemother. Pharmacol.

Abstract:

1. Jiang HY, Hickey RJ, Tom TD, Wills, PW, Abdel-Aziz, WF. and Malkas, LH: DNA replication is mediated by a discrete multiprotein complex. Graduate Student Research Day, University of Maryland. April, 1999
2. Jiang HY, Hickey RJ, Will PW, Tom TD, Abdel-Aziz WF, Malkas, LH: The DNA Synthesome: Extensive purification of a model for studying the mechanism of action of anticancer drugs. American Association for Cancer Research 90th Annual Meeting, Philadelphia, PA, April, 1999
3. Abdel-Aziz WF, Hickey RJ, Jiang HY, Malkas LH (1999): AraC affects the formation of cancer cell DNA synthesome replication intermediates. American Association for Cancer Research 90th Annual Meeting, Philadelphia, PA, April, 1999
4. Han S, Hickey RJ, Jiang HY, Malkas LH: Defining the mechanism of action of araC using the human cell DNA synthesome as an in vitro system. American Association for Cancer Research 90th Annual Meeting, Philadelphia, PA, April, 1999

Degree obtained:

Doctor of Philosophy
Pharmacology and Experimental Therapeutics, School of Medicine, University of Maryland Baltimore, 1999

CONCLUSIONS AND DISCUSSION

Gemcitabine is a novel deoxycytidine analog with both structural and metabolic similarities to araC. AraC is one of the most effective drugs available today for the treatment of acute leukemia and other hematopoietic malignancies. Gemacitabine is also effective against leukemia [14,21], but it has proven to be effective against a variety of solid tumors. Like araCTP, dFdCTP inhibits DNA synthesis mainly through the inhibition of the activity of the DNA polymerases. A considerable body of work has accumulated in which the effects of these two drugs on intact cell DNA synthesis in a variety of cancer cell lines. Using a cell survival assay, Heinemann et al. [11,12] discovered, that Chinese hamster ovary cells are significantly more sensitive to dFdC than araC after both a 4- and an 18-hour incubation. Our study using the human breast cancer cell line MCF7 in a clonogenic assay also showed that dFdC is approximately 15-fold more cytotoxic than araC following a 24-hour incubation with the drug. Gemcitabine inhibits [^{3}H]thymidine uptake approximately 10-fold more than araC in intact MCF7 cells. Accumulation of dFdCTP was observed to be cell line dependent, with the cell lines that are more sensitive to the drug accumulating higher amounts of dFdCTP in cultures [16,25]. This may explain the difference between the IC50 values obtained in our experiments, and these obtained by other investigators who performed similar studies with CHO cells [11], human T-lymphoblastoid CCRF-CEM cells [15] and HL60 cells [29].

DNA synthesis is the most prominent activity inhibited by dFdC in cultured cells [11]. Huang et al. [16] directly investigated the molecular mechanism of action of dFdC and araC *in vitro* on DNA synthesis using purified DNA polymerase α and ε. However, the use of purified DNA polymerases may not adequately reflect the DNA synthetic process as it occurs within the intact cell. In the intact cell, DNA synthesis involves the coordinated activity of DNA polymerases α and δ along with that of several other enzymes and factors. In this report, we describe studies performed with intact MCF7 cells and the DNA synthesome isolated from these cells. In our report we directly compared the effectiveness of dFdCTP and araCTP as inhibitors of the DNA synthetic process. Our results showed that dFdCTP is significantly more potent than araCTP in the *in vitro* SV40 DNA replication assay. Full-length DNA was produced in the presence of very low levels of both drugs, suggesting that incorporation of dFdCTP and araCTP did not stop the polymerases from elongating the DNA template. Our results were in agreement with those of Ross et al. [29], who demonstrated that dFdC was progressively incorporated into nascent DNA of increasing size in intact HL60 cells. In contrast, studies of dFdCTP incorporation using *in vitro* primer extension assays by purified DNA polymerase α and δ demonstrated that after incorporation of dFdCTP to the 3′ terminus of the elongating DNA strand, one more deoxynucleotide can be added before the DNA polymerases are unable to continue elongating the nascent strand. Therefore, dFdC appears to act as a chain terminator [16]. Although dFdCTP appears to be a more potent inhibitor of SV40 DNA synthesis *in vitro* than araC, the inhibitory effects of dFdCTP and araCTP on the activity of the DNA synthesome associated DNA polymerase α and δ are similar. The IC50 values of dFdCTP and araCTP for DNA polymerase α are 80 μM and

100 μM, respectively. These values are comparable to the intracellular dFdCTP concentration, which was reported to range from 64 μM to 362 μM in the leukemia cells of patients undergoing dFdCTP therapy [7,8]. Taken together, our data and the data reported by others suggest that DNA polymerase α is a major target for dFdCTP and araC. Inhibition of DNA synthesome associated polymerase δ occurred, but at substantially higher drug concentrations than required to inhibit DNA polymerase α to the same extent. About 300 μM of dFdCTP was required to inhibit DNA polymerase δ by 10%. Therefore, it is most likely that the inhibitory effect of dFdCTP and araCTP on the SV40 origin dependent DNA replication process is mediated primarily through inhibition of DNA polymerase α. This conclusion agrees with our previous reports describing the analysis of the mechanism of action of araCTP in this same assay [1,10,32,33]. The fact that dFdCTP was significantly more potent than araCTP suggests that dFdCTP may have more impact on the organized multiprotein DNA replication complex (i.e., the DNA synthesome) found in intact cells than would be suggested from studies employing purified individual DNA polymerases. Furthermore, studies from intact cells and *in vitro* assays had shown that addition of dCTP could not completely restore DNA synthesis to the level observed in assays performed without dFdCTP [16]. This observation indicates that the inhibition of DNA polymerase α activity by dFdC was not simply competitive with dCTP. Primer extention assays have shown that the 3′→5′ exonuclease activity of purified DNA polymerase ε was essentially unable to excise nucleotide from DNA containing dFdCMP at either the 3' terminus or from an internal position within the DNA; araCMP, however, was reported to be removed from 3′-terminus of DNA [16], indicating that dFdC may have more impact on DNA repair than previously thought.

However, the greater anticancer activity of dFdC is not only attributed to the inhibition of DNA polymerase α activity, but may be derived from damage to the DNA into which gemcitabine is incorporated. Gemcitabine is more potent than araC for several reasons. First, the chemical addition of fluorine atom to the 2′ position of the furanose ring of the drug makes dFdC more lipophilic and therefore more permeable to cells than araC. Second, deoxycytidine kinase has a higher affinity for dFdC than araC which leads to higher levels of dFdCTP than araCTP in cells. Third, dFdC inhibits ribonucleotide reductase which causes depletion of the cellular pools of deoxynucleoside triphosphate, particularly, the levels of the competing metabolite, dCTP. Thus, the ratio of cellular dCTP to dFdCTP favors the inhibition of DNA synthesis by dFdCTP [6,12]. In contrast, araC has no effects on dNTP pools and is not known to act as an inhibitor of ribonucleotide reductase which helps maintain higher levels of active metabolite within the cell. It has been reported that the cellular dCTP level was 3.5 μM in K562 cells [5]. Since the concentration of dCTP (10 μM) was used in our *in vitro* assays, it is possible that the differential effects of dFdC and araC on the DNA synthesis might be more dramatic if true intracellular dCTP concentrations were used. Finally, the elimination of cellular dFdCTP is slower than araCTP [16]. All of these characteristics of dFdCTP contribute to the higher intra-cellular concentration of dFdCTP as compared to araCTP. dFdCTP is found at from 9- to 20-fold higher concentrations in treated cells than is in cells treated with equivalent concentrations of araC [11]. However, the increase in the

intracellular concentration of dFdCTP does not completely account for the greater cytotoxicity of dFdCTP in intact cells, which has been reported to be 180-fold more toxic than araC [6].

In conclusion, in this report we have demonstrated that dFdCTP is significantly more potent than araCTP at inhibiting the DNA synthetic process in our cell-free SV40 origin dependent *in vitro* DNA replication assay system employing the human MCF7 cell DNA synthesome. The results of this study verify that the DNA synthesome can serve as a relevant *in vitro* model system for studying the mechanism of action of anticancer drugs that directly affect DNA synthesis, and that the mechanisms through which these drugs inhibit *in vitro* DNA synthesis closely parallel the inhibitory effects of these drugs in intact cells [1,4,10,32,33]. We have demonstrated in our laboratory that the DNA synthesome is able to incorporate araCTP into internucleotide linkages, and that this incorporation of araC into internucleotide linkages more closely resembles the molecular events occurring in intact cells than can be achieved using purified DNA polymerases [32,33]. Continued analysis of the mechanisms by which dFdCTP mediates its cytotoxic effects will uncover the effects dFdC has on the initiation, elongation and termination stages of the DNA synthesis process. Thus, our *in vitro* model system, which utilizes the DNA synthesome to mediate the DNA synthetic reaction is anticipated to be of substantial value for gaining insight into the mechanism(s) of action of dFdCTP and other anticancer drugs that directly inhibit cellular DNA synthesis. The continued validation of the *in vitro* DNA replication model system employing the DNA synthesome is therefore expected to be of considerable value in the search for more effective anticancer drugs than conventional model systems that only employ highly purified individual enzymes such as DNA polymerase or topoisomerase II.

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APPENDIX

#2002 Balance between apoptotic (AI) and proliferative (PI) indices in gemcitabine-treated HCT116 colon carcinoma mouse xenografts. Wexler, E.J., Czerniak, P.M., Slee, A.M., and Kerr, J.S. *General Pharm. DuPont Pharmaceuticals, Wilmington, DE 19898.*

Tumor growth is dependent on the balance between apoptosis and proliferation. How therapeutic agents affect this balance is often unknown. Gemcitabine, a new anticancer drug with novel metabolic properties, has antitumor effects that vary with dosing schedule. These properties make it a useful tool to characterize the apoptotic/proliferative balance in tumors. When dosed Q1D×5 at 3.5 mg/kg/day and Q3D×5 at 120mg/kg/day, tumor growth in the HCT116 xenograft was reduced 73% and 92% ($p<0.05$), respectively. AI was determined using Tunel immunohistochemistry *in situ*, and PI was measured by bromodeoxyuridine (BrdU) incorporation. Composite images of tumor sections were quantitated for areas of necrosis, proliferation and viability using an M2 MCID Image Analysis System (Imaging Resources). AI in controls was 2.4%+/-0.13 (Mean+/−SEM), and significantly ($p<0.05$) increased with both dosing regimens, 5.7%+/−0.06 with Q1D×5, and 3.5%+/−0.02 in the Q3D×5 group. PI in controls was 28.1%+/−1.9 with no change in PI with daily dosing (27.6%+/−1.9) but Q3D×5 dosing led to a 26% reduction in PI to 20.8% +/−3.5 ($p<0.05$). Although tumors from animals treated with gemcitabine were significantly smaller than controls, the percent of viable cells normalized for total tumor area was increased by 22–35%. These data demonstrate that AI and PI may be used to characterize the intratumor dynamics associated with different dosing regimens.

#2003 Analogs of phenylbutyrate as mechanistic probes of differentiation. Ludeman, S.M., Carducci, M.A., Springer, J.B., Kruszewski, M., Shulman-Roskes, E.M., and Colvin, O.M. *Duke Comprehensive Cancer Center, Durham, NC 27710 and The Johns Hopkins Oncology Center, Baltimore, MD 21205.*

Phenylbutyrate (PB) has emerged in phase 1 trials as a promising differentiation agent. Most literature reports refer to PB as a prodrug for phenylacetate (PA) with the conversion occurring through β-oxidation. To investigate this hypothesis, a series of PB analogs were synthesized which were incapable of undergoing a β-oxidation mechanism. In addition, one analog did not have an acid functionality but in its place had a group which was electronically similar to a carboxylate moiety. Each of these analogs (benzyloxyacetate, 2,2-difluoro-4-phenylbutyrate and 1,1,1-trifluoro-5-phenyl-2-pentanone) were screened for cell growth inhibition against five lines of human prostate cancer and each had activity that either paralleled or was better than that of PB. Relative to PB, significantly lower IC_{50} values were also achieved for the analogs against multiple cell lines. These preliminary results suggest that (1) β-oxidation is not intricate to PB activity and (2) PB may have a mechanism of action which is unique in comparison with that of PA and/or butyrate.

#2004 Selection and characterization of a CHO cell line resistant to bis(ethyl) polyamine analogues. McCloskey, D.E., and Pegg, A.E. *Dept. of Cellular and Molecular Physiology, Penn State University College of Medicine, Hershey, PA 17033.*

A CHO cell line that is resistant to bis(ethyl) polyamine analogues has been selected using N^1,N^{12}-bis(ethyl)spermine [BE3-4-3]. The parental CHO cell line used was the C55.7 CHO cell line which is auxotrophic for putrescine because of a point mutation which rendered ornithine decarboxylase inactive [Somat Cell Genet. 1985, 11(1):11–23]. Cells were exposed to BE3-4-3 for 120 h and then placed in drug-free medium and allowed to recover exponential growth. Four cycles of treatment with 10 μM, 10 μM, 100 μM, and 10 μM BE3-4-3, respectively, were carried out. The resulting cells (C55.7Res) were resistant to at least 100 μM BE3-4-3 with growth in the presence of the polyamine analogue similar to that of the parental cells cultured in drug-free medium. Characterization of the C55.7Res cells indicate that they are cross resistant to N^1,N^{11}-bis(ethyl)norspermine [BE3-3-3] with IC_{50} values of 7 μM and 80 μM, respectively for C55.7 and C55.7Res. The resistance to BE3-3-3 has remained stable through 20 passages in drug-free medium, indicating a heritable alteration in the C55.7Res cells. The C55.7Res cells are not cross-resistant to methylglyoxal bis(guanylhydrazone), adoxorubicin or cisplatin. Intracellular accumulation of BE3-3-3 is similar to that of the parental cell line, indicating that the resistance is not a result of altered drug transport. Preliminary studies indicate that the polyamine pools of the C55.7Res cells are altered and that spermidine/spermine N^1-acetyl transferase activity is not induced by BE3-3-3 concentrations up to 25 μM whereas there is >250 fold induction in C55.7 cells at 25 μM. Further characterization of this cell line may lead to better understanding of the mechanisms of action of the polyamine analogues. Support by GM-26290.

#2005 A novel DNA minor groove binder MS-247: Design, synthesis and antitumor activity against human cancer xenografts. Matsunaga A., Komatsu H., Kohno H., Edatsugi H., Matsuba Y., Nakanishi O., Sato S., Yamori T., Tsuruo T. *Life Sciences Laboratory, Mitsui Chemicals Inc., Chiba, Japan; Institute of Biological Science, Mitsui Pharmaceuticals, Inc., Chiba, Japan; Japanese Foundation for Cancer Research, Tokyo, Japan.*

MS-247 (2-[[N-[1-methyl-2-[5-[N-[4-[N.N-bis(2-chloroethyl)amino]phenyl]carbamoyl]-1H-benzimidazol-2-yl]pyrrol-4-yl]carbamoyl]ethyldimethylsulfonium di-p-toluenesulfonate) is a new synthetic anticancer agent with a novel DNA binding moiety, based on the structure of typical DNA minor groove binder Netropsin, and a DNA alkylating phenyl mustard residue. MS-247 showed significant cytotoxicity in several murine tumor cell lines and showed strong *in vivo* antitumor activity against murine tumor model systems. MS-247 was also effective against 38 human cancer cell lines. We further examined the antitumor activity of MS-247 against a panel of 17 human cancer xenografts, including lung, colon, stomach, breast and ovarian cancers. Antitumor activities of MS-247 were significant and higher than those of adriamycin and cisplatin in all 17 xenografts. Among these cancers, a marked antitumor activity of MS-247 was observed in lung cancers (NCI-H23: T/C 4%, DMS114: T/C 19%, DMS273: T/C 2%). MS-247 was also effective against a paclitaxel-insensitive cancer, HCT15, and CPT-11-insensitive cancers, A549, HBC-4 and SK-OV-3. These results suggest that MS-247 can be a candidate for clinical investigation.

#2006 The DNA synthesome: Extensive purification and serve as a model for studying the mechanism of anticancer drugs. HaiYan Jiang, Robert J. Hickey, Philip W. Wills, Timothy, D. Tom and Linda H. Malkas. *Department of Pharmacology and Experimental Therapeutics, University of Maryland, Baltimore, School of Medicine.*

DNA replication enzymes and factors have been found involved in cell cycle control and cancer formation. The concept that many enzymes and factors involved in the mammalian DNA replication function together as an organized multiprotein complex has been supported by increasing evidence. We have purified a discrete multiprotein complex containing DNA polymerase α from human cancer HeLa and MCF7 cell lines. An enrichment of this complex was seen through the purification steps and this DNA polymerase α containing complex copurifies with peak DNA polymerase α *and in vitro* simian virus 40(SV40) DNA replication activity. We have designated this complex the DNA synthesome. Finally, we purified the DNA synthesome by electroeluting this complex from native polyacrylamide gel. Further analysis of the DNA synthesome showed it contains DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RPA), topoisomerse I and topoisomerse II, which are essential components for DNA replication. Taken together our evidence indicates that the DNA synthesome represents the fundamental DNA replication unit of the human cell. Finally, The DNA synthesome was used as *in vitro* model system to investigate the mechanisms of action of cytarabine(ara-C) and gemcitabine(dFd-C), which directly affect cellular DNA replication. This work was supported in part by grant #CA57350 and CA73060 to LHM and CA74904 to RJH. JHY is a recipient of DOD Breast Cancer Research Fellowship.

PREVENTION/BASIC SCIENCE AND CLINICAL STUDIES 3: Breast and Prostate Cancer Chemoprevention

#2007 Altered urinary excretion of 2-OH-estrone to 16-OH-estrone in women after soya isoflavone consumption. Cree, M., Josyula S., Anderson, K.E., Nagamani, M. and Lu, L.-J.W., *The University of Texas Medical Branch, Galveston, TX 77555.*

Women consuming a traditional Asian diet high in soy have a lower rate of breast cancer than women consuming a western diet low in soy content. We investigated the effect of soya isoflavones on a putative breast cancer risk marker, i.e., urinary ratios of anticarcinogenic 2-OH-estrone (2OHE1) to carcinogenic 16-OH-estrone (16OHE1). Eight premenopausal women in a metabolic unit were placed on an isocaloric, soya diet containing 150 mg isoflavones daily for one complete menstrual cycle and 4 months later on another soya diet without isoflavones. Overnight 12 hour urine was analyzed for 2OHE1 and 16OHE1 using a commercial ELISA kit. Results showed that a diet rich in isoflavones increases the levels of 2OHE1 (16.96 ± 2.96 nmol/12 h with isoflavones, 11.61 ± 2.03 without isoflavones, $p < 0.01$), had no effect on the formation of 16OHE1 (7.72 ± 1.25 nmol/12 h with isoflavones, 6.99 ± 1.14 without isoflavones, $p < 0.2$). Urinary ratios of 2OHE1 to 16OHE1 were higher while subjects were on the isoflavone-containing soya diet (2.57 ± 0.35) than on the isoflavone-free soya diet (1.99 ± 0.32, $p < 0.005$). In summary, soya isoflavones may prevent breast cancers by increasing the levels of anti-carcinogenic metabolites of 17β-estradiol. Supported by USPHS CA65628, CA56273, CA45181, NIH NCRR GCRC MO1 RR00073, and AICR 95B119.

#2008 Reduction of estrone sulfate levels in premenopausal women after soya consumption. Lu, L.-J.W., Anderson, K.E., Grady, J.J., and Nagamani, M. *The Univ. of Tex. Med. Branch, Galveston, TX 77555.*

Soybean (soya) consumption is associated with reduced rates of breast cancer; this oncoprotective effect may be due in part to reduced circulating ovarian steroids (CEBP 5: 63-70, 1996). We further determined that these reductions were observed for the entire cycle and are not mediated through the interaction of phytoestrogen isoflavones with gonadotropins. We examined the effect of soya diet on the levels of estrone sulfate which is the most abundant circulating estrogen and is a good index for estrogenicity. Eleven premenopausal women ingested 36-oz portions of soymilk containing ~150 mg of daidzein and genistein daily for one menstrual cycle. Their daily serum estrone sulfate levels were analyzed by specific radio-immunoassay. Approximately 30% and 40% de-

Resistance to methotrexate (MTX), an important cause of treatment failure for children with acute lymphoblastic leukaemia (ALL), can result from reduced intracellular drug accumulation. Cloning of the Reduced Folate Carrier (*RFC*) gene, encoding a putative MTX-transport protein, has allowed characterization of molecular changes leading to defective MTX transport. The structure of the *RFC* gene in transport-defective MTX-resistant pediatric leukaemia cells, CEM/MTX R, was compared to parental CCRF-CEM cells. Sequencing of the entire coding region identified a single point mutation (G → A) in CEM/MTX R cells resulting in a Glu → Lys substitution. Western analysis of CEM/MTX R membrane preparations indicated greatly reduced levels of RFC by comparison with wild-type cells. The full length *RFC* cDNA was electroporated into CEM/MTX R cells, and transfectant cells over-expressing the wild-type *RFC* gene were characterized. By comparison with the vector-only control, there was a 125-fold reversal of MTX resistance in the transfected cells, while response to the lipophilic antifolate, trimetrexate, was unaffected. Surprisingly, there was no reversal of resistance to tomudex, another antifolate transported by RFC, in the transfectant cells. The results indicate that alterations in the *RFC* gene may contribute to transport-related MTX resistance in childhood ALL, but that additional mechanisms can mediate resistance to antifolate compounds.

#4459 Characterization of RNA binding by mutant variants of human dihydrofolate reductase. McPherson, J.P., Sauerbrey, A., Meehl, M., Russo, A., Skacel, N., and Bertino, J.R. *Program of Molecular Pharmacology and Therapeutics, Memorial Sloan Kettering Cancer Center, New York, NY 10021.*

Previous studies have shown *in vitro* that human dihydrofolate reductase (hDHFR) regulates its own synthesis through negative feedback at the level of protein translation. The autoregulatory nature of this mechanism has been postulated to be due to the specific binding of the enzyme to its own mRNA. Both translational inhibition and RNA binding by hDHFR are attenuated in the presence of the antifolate inhibitor methotrexate (MTX). In order to understand the role of antifolates in RNA binding and translational inhibition, we have compared the RNA binding of native hDHFR to that of mutants with impaired binding to MTX. Native hDHFR and mutant variants with substitutions at leu22 and/or phe31 were purified as hexahistidine fusion proteins by Nickel affinity chromatography and RNA binding was examined using electrophoretic mobility-shift assays. Native hDHFR protein (nM) bound avidly to its mRNA (pM) and this binding was decreased in the presence of nM concentrations of MTX and was nearly abolished at μM concentrations. In contrast, μM concentrations of 5-fluorouracil only slightly impaired RNA binding. Mutant variants with substitutions at leu22 and/or phe31 were observed to retain RNA binding in the absence of drug. However, MTX-dependent attenuation of RNA binding by these mutant enzymes was found to require 10- to 100-fold higher concentrations of MTX compared to native hDHFR. Ongoing studies will determine whether these mutants also demonstrate differences in their ability to regulate DHFR synthesis.

#4460 Ara-C affects the formation of cancer cell DNA synthesome replication intermediates. Abdel-Aziz, W.; Jiang, H.Y.; Hickey, R.J.; and Malkas, L.H. *Department of Pharmacology and Experimental Therapeutics, UMB, Baltimore, MD 21201.*

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex (DNA synthesome) from human as well as from murine mammary carcinoma cells. DNA synthesome supports the *in vitro* replication of DNA containing the papovavirus origin of replication in the presence of the viral large T-antigen. We are currently using the *in vitro* replication assay to study the mechanism of action of anticancer drugs affecting DNA replication. In order to directly validate that the *in vitro* interaction of ara-C with the DNA synthesome is representative of the molecular events occurring in intact cells, the types of daughter DNA molecules produced by the synthesome in the presence and absence of ara-CTP were analyzed and compared with papovavirus replication intermediates isolated from virus-infected cells exposed to ara-C. Our results using different concentrations of ara-CTP (1μM, 10μM, 100μM, and 1mM) demonstrated that full length daughter DNA molecules were obtained in the presence of 1 and 10μM ara-CTP, while at higher concentrations (100μM and 1mM), there was an inhibition of full length daughter DNA synthesis. Our data suggest that the initiation phase of DNA synthesis is inhibited by ara-CTP since the production of the short Okazaki fragments was inhibited at all concentrations of the drug. We have also found that increasing ara-C concentration was accompanied by a gradual decrease in ^{3}H-thymidine incorporation in intact cells. The IC_{50} of ara-C for inhibition of *in vitro* DNA replication was comparable to that required for inhibition of intact cell DNA synthesis. Ara-CTP also inhibited the synthesome-associated DNA polymerases α and δ.

#4461 Defining the mechanism of action of Ara-C using the human cell DNA synthesome as an in vitro model system. Han, S.; Hickey, R.J.; Jiang, H.Y.; Malkas, L.H. *Department of Pharmacology and Experimental Therapeutics, UMB, Baltimore, MD 21201.*

The antimetabolite 1-β-D-arabinofuranosylcytosine (ara-C) has been used as a highly effective agent for the treatment of leukemia. It has been shown to be a potent inhibitor of intact cell DNA synthesis, and its active metabolite (ara-CTP) is also a strong inhibitor of the activity of isolated DNA polymerases α, δ and ε *in vitro*. We have recently observed that the DNA polymerases α, δ, and ε are components of the DNA synthesome and the activities of these DNA polymerases are differentially inhibited by ara-CTP. In this study, we further invested how this anticancer drug disrupts the growth of daughter DNA molecules by interacting with the synthesome associated DNA polymerases. By using oligonucleotide-primed templates, we examined the ability of the synthesome associated DNA polymerases α and δ to insert ara-CTP into DNA and to bind to, (and to extend from) a DNA strand containing an ara-CMP 3' terminus. We found that the synthesome associated DNA polymerase α could incorporate ara-CTP into the internucleotide positions but serves as a poor substrate for the addition of the next deoxynucleotide.

#4462 Comparison of arabinosylcytosine and dideoxycytidine induced drug resistance in H9 cells. Fernandez, M., Han, T., Yo, P., Wang, W., and Agarwal, R.P. *University of Miami, School of Medicine, Miami, FL 33101.*

Our laboratory has been involved with the studies of host cell factors causing resistance to antiviral and anticancer agents. This report compares resistance induced by the deoxycytidine analogs, dideoxycytidine (ddC, an antiviral agent), and arabinosylcytosine (araC, an anticancer agent). H9-ddC0.5w, H9-ddC5.0w, H9-araC0.05w, and H9-araC0.5w cells were selected by propagating human lymphocytic H9 cells in the presence of 0.5 μM ddC, 5.0 μM ddC, 0.05 μM araC and 0.5 μM araC, respectively. In the resistant cells, the deoxycytidine kinase (dCK) activity was 48.5%, 35.9%, 54% and 31.1%, ddCTP concentrations 20.4%, 2.1%, 11.5%, and 0.4%; and araCTP concentrations 28.2%, 16.4%, 2.7% and 0.4% of control. The ddC resistant cells were 5.4-fold and 15.7-fold resistant, and H-9 araC0.05w and H9-araC0.05w cells were 4.6-fold and 5.4-fold resistant to ddC. Whereas araC resistant cells were significantly resistant to araC (27.8-fold and >294-fold) the ddC resistant cells were negligibly resistant to araC (1.2-fold and 1.1-fold). Differences were noted in the phosphorylation of ddCDP and araCDP to their triphosphates. araCDP was phosphorylated to araCTP more efficiently than ddCDP to ddCTP. These observations suggest that, even though ddC and araC are analogs, there are differences in metabolism and toxicity in ddC resistant and araC resistant cells and conclusions drawn from one cell line may not be applicable to the other. (Supported by NIAID Grant # AI 29155)

#4463 Ara-C resistance in leukemic lines confers cross-resistance or collateral sensitivity to other classes of anti-leukemic drugs. Martin-Aragon, S., Fu, C., Solorzano, M., Ardi, V., and Avramis, V.I. *Division of Hematology/Oncology, School of Medicine, USC, Childrens Hospital LA, Los Angeles, CA 90027.*

The major limitation of antimetabolite drugs is that they produce drug-resistant clones *in vitro* and in patients who either do not respond or relapse soon after response has been documented. Leukemia patients in relapse are much more difficult to treat with other classes of antileukemic drugs. In order to understand this phenomenon of cross resistance, we used CEM/O and 5 CEM/ara-C-resistant leukemic clones to treat with drugs or radiation. 6-TG or 6-TGuo are cross resistant with ara-C from 1.1- to 15.8-fold using either MTT or a biology assay. Vincristine (VCR) showed cross-resistance with ara-C from 200- to 1E4-fold and only one resistant cell line, CEM/ara-C/G, was 3-fold more sensitive than CEM/O. This clone expresses p53 and does not overexpress bcl-2 protein. Taxotere, another mitotic inhibitor, showed cross-resistance, which was not dose dependent. Dexamethasone (DXM) also showed a significant degree of cross-resistance from 274- to 1E7-fold. The CEM/ara-C/G showed a 4.85E5-fold cross resistance of DXM with ara-C. Gamma radiation treatments up to 12.3 Gy showed a dose-dependent cross-resistance with ara-C from 1.43- to 2.42E5-fold. Only Idarubicin was collaterally sensitive with ara-C from 4.6- to 218-fold in these cell lines. Other classes of anti-neoplastic drugs are being tested to further evaluate this phenomenon. It is apparent that ara-C resistance confers cross-resistance to many other classes of drugs and radiation, probably due to lack of apoptosis. This phenomenon may be significant in its adverse effects on the treatment of patients with relapsed leukemias. Efforts in determining the molecular mechanisms of cross-resistance in these cell lines are under investigation.

#4464 Correlated overexpression of TS and FR-α in 5-FU resistant H630 cell lines. Gmeiner, W.H., Kolar, C., Liu, J., and Lawson, T. *University of Nebraska Medical Center, Omaha, NE 68198-6805.*

Thymidylate synthase (TS) inhibition is central to cancer chemotherapy. TS is catalytically active as part of a ternary complex that includes the substrate dUMP and reduced folate. Thus, for TS overexpression to successfully overcome inactivation of normal levels of TS, the malignant cell must also have available increased levels of reduced folate. RT-PCR was used to amplify folate receptor (FR-α) mRNA from H630, H630-1, and H630-10 cell lines. These are human colorectal cell lines and the −1 and −10 refer to the levels of 5-FU (in micromolar) to which they have been adapted in culture medium. JAR choriocarcinoma cells were included in this study because they had been previously shown to express FR-α. The results indicate that TS overexpression and FR-α overexpression positively correlate in the 5-FU resistant cell lines with levels ~5-fold higher in H630-1 and ~30-fold higher in H630-10 cell lines relative to H630 cells. Novel multimeric FdUMP (FdUMP[10]) agents have been developed in our laboratory and these have been conjugated with folic acid and show increased cytotoxicity to H630, H630-1 and H630-10 cell lines, relative to unconjugated multimers.

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Complementation Between Polyomavirus Large T Mutants Affected in Viral DNA Replication and Transactivation of E2F Promoters

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There is a N-terminal domain common to all 3 polyomavirus T antigens that appears to be required for various aspects of cellular transformation. The biochemical activity associated with this domain is unclear but it may function as a DNA J domain interacting with members of the 70-kD heat shock protein family. We have generated a series of mutations affecting the putative J domain and analyzed the biological activities of the 3 T antigens. The mutations do not affect the properties of the middle and small T antigens but impair most of the properties of the large T antigen. This includes immortalization, transactivation of E2F-responsive promoters and viral DNA replication. Although the mutants are inactive in transactivation, they can bind to pRb. Interestingly, the transactivation defect can be complemented by dl-141, another large T mutant unable to transactivate and defective in pRb binding. We propose a model to explain the requirement for a DNA J domain in large T mediated viral DNA replication.

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Purification and Detailed Characterization of DNA Synthesome Using Combined Low Pressure Chromatography and Electroelution

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Strong evidence has been accumulated that most the enzymes and factors involved in the replication of mammalian DNA function together as an organized multiprotein complex. We have previously reported that a highly purified multiprotein form of DNA polymerase can be isolated from a variety of mammalian cell types and tissues. Native polyacrylamide gel electrophoresis of this purified multiprotein complex from HeLa cells revealed the presence of several high molecular weight multiprotein species. One of these complexes was readily recognized in Western blot analysis by a monoclonal antibody against the DNA replication essential protein DNA polymerase α. In this report, we describe the most extensive purification of the HeLa cell DNA synthesome using combined low pressure chromatography and electroelution from native polyacrylamide gel. This most purified DNA synthesome showed DNA polymerase α, δ, and high specific *in vitro* simian virus 40 (SV40) origin dependent DNA replication activity. Furthermore, Western blot showed that this DNA synthesome contains all of the enzymes required for DNA replication.

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Defining the Mechanism of Action of Anticancer Drugs Using the Human Cell DNA Synthesome as an *in Vitro* Model

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The multi-protein DNA replication complex (DNA synthesome) from human breast cancer cells can serve as a unique and novel *in vitro* system to investigate the mechanism of action of anticancer drugs. The antimetabolite 1-β-arabinofuranosylcytosine (ara-CTP) has been used as a highly effective agent for the treatment of leukemia . It has been shown to be a potent inhibitor of intact cell DNA synthesis and the *in vitro* DNA polymerase activity of the isolated DNA polymerases α, δ and ε. Using the highly purified DNA synthetic apparatus (i.e, the DNA synthesome), we have shown that DNA polymerase α, δ, and ε are components of the synthesome and that their activity is differentially inhibited by ara-CTP. Furthermore, the inhibition of the DNA synthesome associated DNA polymerase α activity increase in a concentration dependent manner, and correlated closely with the inhibition of SV40 origin dependent in vitro DNA replication. In contrast to the effect on polymerase α, DNA synthesome associated polymerase δ and ε were not significantly inhibited by ara-CTP. We also have shown that the synthesome associated DNA polymerase α could incorporate ara-CTP into the internucleotide positions, but ara-CTP serves as a poor substrate for the addition of the next deoxynucleotide. Our results suggest that inhibition of the activity of the mammalian cell DNA synthesome by ara-CTP is primarily due to primarily the inhibition of synthesome associated polymerase α. The work was supported in part by grants from the National Institute of Health/NCI: # CA 57350 and CA73060 to L.H.M, and CA74904 to R.J.H.

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ORIGINAL ARTICLE

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Effects of gemcitabine and araC on in vitro DNA synthesis mediated by the human breast cell DNA synthesome

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Abstract *Purpose*: Gemcitabine (dFdC) and cytarabine (araC) are both analogs of deoxycytidine. Gemcitabine is a relatively new drug that has been shown in both clinical trials and in vitro systems to have more potent antitumor activity than araC. We have previously isolated a fully functional multiprotein DNA replication complex from human cells and termed it the DNA synthesome. Using the DNA synthesome, we have successfully examined the mechanism of action of several anticancer drugs that directly affect DNA synthesis. In this study, we compared the effects of dFdC and araC on in vitro DNA synthesis mediated by the DNA synthesome with the effects of these drugs on intact MCF7 cell DNA synthesis. *Methods*: We examined the effects of dFdC and araC on intact MCF7 cell DNA synthesis and clonogenicity. We also performed in vitro SV40 replication assays mediated by the MCF7 cell-derived DNA synthesome in presence of dFdCTP and araCTP. The types of daughter molecules produced in the assay were analyzed by neutral and alkaline agarose gel electrophoresis. Finally, we examined the effects of dFdCTP and araCTP on the synthesome-associated DNA polymerase α and δ activities. *Results*: Our results showed that dFdC was more potent than araC at inhibiting intact MCF7 cell DNA synthesis and clonogenicity. [^{3}H]Thymidine incorporation was inhibited by 50% at a dFdC concentration of 10 μM, which was about tenfold lower than the concentration of araC required to inhibit intact cell DNA synthesis by the same amount. As examined by clonogenicity assay, dFdC was also significantly more cytotoxic than araC after a 24-h incubation. In vitro SV40 replication assays using the DNA synthesome derived from MCF7 cells demonstrated that the formation of full-length DNA along with replication intermediates were inhibited by dFdCTP in a concentration-dependent manner. Full-length DNA was produced in the in vitro DNA replication assay even when the dFdCTP was incubated in the assay at concentrations of up to 1 mM. We observed that in the presence of 10 μM dCTP, 3 μM dFdCTP and 60 μM araCTP were required to inhibit in vitro SV40 DNA synthesis by 50%. Although dFdCTP is more potent than araCTP at inhibiting in vitro SV40 DNA synthesis, there was no significant difference between the inhibitory effect of these two drugs on the activity of the MCF7 synthesome-associated DNA polymerases α and δ. It was found that the drug concentrations required to inhibit 50% of the synthesome-associated DNA polymerase δ activity were much higher than those required to inhibit 50% of DNA polymerase α activity for both dFdCTP and araCTP. *Conclusion*: Taken together, our results demonstrated that: (1) dFdC is a more potent inhibitor of intact cell DNA synthesis and in vitro SV40 DNA replication than araC; (2) the decrease in the synthetic activity of synthesome-mediated in vitro SV40 origin-dependent DNA synthesis by dFdCTP and araCTP correlates with the inhibition of DNA polymerase α activity; and (3) the MCF7 cell DNA synthesome can serve as a unique and relevant model to study the mechanism of action of anticancer drugs that directly affect DNA synthesis.

Key words AraC · Gemcitabine · In vitro · DNA replication · DNA synthesome

Abbreviations *ara-C* 1-β-D-arabinofuranosylcytosine · *ara-CTP* 1-β-D-arabinofuranosylcytosine triphosphate · *dFdC* 2′,2′-difluorodeoxycytidine, gemcitabine ·

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dFdCTP gemcitabine triphosphate · *DTT* dithiothreitol · *EDTA* ethylenediaminetetraacetic acid · *EGTA* ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *HEPES* *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) · *PBS* phosphate-buffered saline · *PMSF* phenylmethyl sulfonyl fluoride · *SV40* simian virus 40 · *SDS* sodium dodecyl sulfate · *Tris* tris(hydroxymethyl) aminoethane

Introduction

Nucleoside antimetabolites comprise one of the most effective classes of drugs used in the treatment of viral diseases and specific types of malignancy. The biological activity of most nucleoside antimetabolites is due to their ability to inhibit the DNA synthetic process, which is an essential function both for cell division and proliferation. 1-β-D-Arabinofuranosylcytosine (araC) has been used effectively in the clinic to treat hematologic cancers [6]. 2′,2′-Difluorodeoxycytidine (gemcitabine, dFdC) is a novel deoxycytidine analog with structural and metabolic similarities to araC. Both of these compounds are prodrugs which are transported into the cell where they are activated following phosphorylation by deoxycytidine kinase. AraC and dFdC differ in structure from the parent nucleoside, deoxycytidine, by specific modifications to the 2′ carbon of the furanose ring. Clinical trials have shown that dFdC is effective in most solid tumors and more potent and less toxic than araC [11, 13, 14, 19, 21]. Studies of intact cells have indicated that inhibition of DNA synthesis is the predominant effect of dFdC and araC [16, 17, 26, 27, 29]. Like araC, the major targets for dFdCTP are the DNA polymerases. It has been shown that incorporation of araCTP and dFdCTP into DNA is most likely the primary mechanism by which these drugs exert their cytotoxic effects [27]. Using in vitro DNA primer extension assays employing purified DNA polymerases, dFdCTP and araCTP have shown qualitative and quantitative differences in their molecular actions on DNA synthesis [16]. Studies comparing dFdC and araC have shown that dFdC is transported more rapidly and is a better substrate for deoxycytidine kinase than araC [11]. Furthermore, dFdC inhibits ribonucleotide reductase and thus causes depletion of intracellular nucleotide pools [12]. However, araC is not known to inhibit the activity of ribonucleotide reductase [12]. Finally, dFdCTP also shows a slower elimination rate than araCTP [11]. All of these characteristics of dFdC result in the development of higher intracellular concentrations of active metabolite (i.e. dFdCTP). Although inhibition of DNA synthesis has been strongly correlated with intracellular dFdCTP concentration [12], little work has been done to directly compare the effects of dFdCTP and araCTP on reducing the level of DNA replication within the cell.

We have previously reported that a highly purified multiprotein form of DNA polymerase (the DNA synthesome) can be isolated from a variety of mammalian cell types and tissues [2, 3, 15, 18, 22, 31, 34]. We have shown that the DNA synthesome is fully competent to support origin-specific large T antigen-dependent in vitro SV40 DNA replication [2, 3, 22, 34]. Biochemical characterization of the DNA synthesome has identified several protein components of the complex that are essential for DNA replication [15, 23]. These proteins include the DNA polymerases α, δ, and ε, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen, replication factor C, replication factor A, DNA helicase, and DNA ligase I [2, 24, 34]. Most importantly, in the presence of viral large T antigen and the SV40 replication origin sequence, the synthesome is fully competent to carry out all phases of the DNA replication process required to replicate an SV40 origin-containing plasmid in vitro. We have successfully examined the action of araC and camptothecin using this model system [1, 4, 10, 32, 33] and have now extended the results of these studies by exploring how the inhibitory effects of dFdC and araC compare with one another.

Our studies compared the inhibitory effects of dFdC and araC on intact human breast cancer cell DNA synthesis and in DNA synthesis mediated by our in vitro DNA replication assay system. In comparison with araC, dFdC was shown to more strongly inhibit the in vitro DNA synthetic activity of the DNA synthesome and the intact MCF7 cells DNA synthesis. We also demonstrated that the inhibition of in vitro DNA synthesis was preferentially mediated by the action of dFdCTP and araCTP on DNA polymerase α and not DNA polymerase δ.

Materials and methods

Materials

AraC and araCTP were purchased from Sigma Chemical Co. (St. Louis, Mo.). dFdC and dFdCTP were kindly supplied by Eli Lilly & Co. (Indianapolis, Ind.). AraC and dFdC were dissolved in water. AraCTP and dFdCTP were dissolved in 10 m*M* HEPES, pH 7.5. The stock solutions were aliquoted and stored at −80 °C. [α-^{32}P]dGTP (3000 Ci/mmol, 10 mCi/ml), [methyl-^{3}H]thymidine (90 Ci/mmol; 2.5 mCi/ml) and [^{3}H]dTTP (72.6 Ci/mmol) were obtained from Dupont New England Nuclear (Boston, Mass.).

Cell culture

Suspension cultures of MCF7 cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of calf and fetal bovine serum. Exponentially growing cells were harvested and washed three times with phosphate-buffered saline (PBS). The cells were then pelleted by low-speed centrifugation. The cell pellets were stored at −80 °C prior to initiating subcellular fractionation.

Isolation of the MCF7 DNA synthesome

The DNA synthesome-containing protein fraction was purified essentially as described by Lin et al. [20] and Malkas et al. [24] and as outlined in Fig. 1. Briefly, frozen cell pellets from exponentially grown MCF7 cells were thawed and resuspended in three volumes of the homogenization buffer containing 50 m*M* HEPES (pH 7.5),

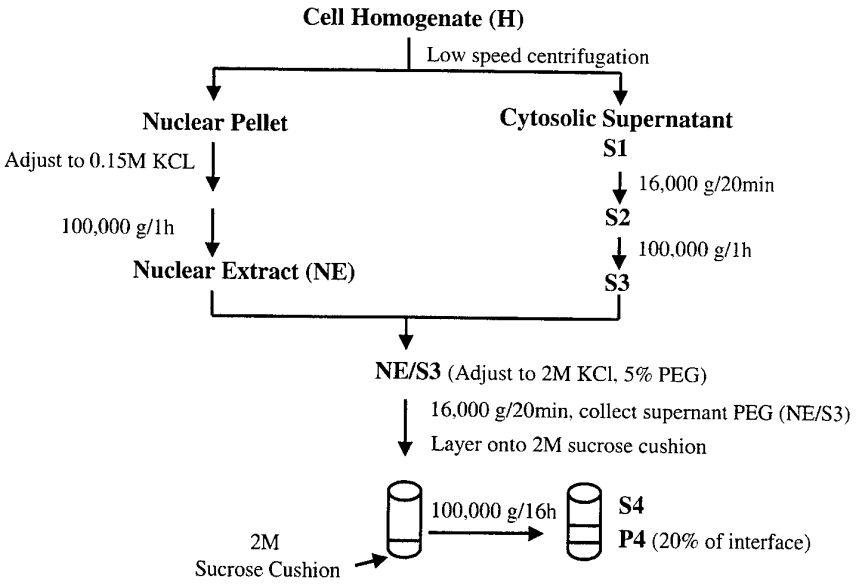

Fig. 1 Purification scheme for the MCF7 cell DNA synthesome

200 m*M* sucrose, 5 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* DTT and 0.1 m*M* PMSF. The pellets were homogenized using 30 strokes of a loose-fitting Dounce homogenizer, and the homogenate was centrifuged at 3000 *g* for 15 min to separate the nuclear pellet and cytosolic supernatant (S1). The nuclear pellet was resuspended in two volumes of a buffer containing 50 m*M* HEPES (pH 7.5), 400 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EDTA, 2 m*M* DTT, and 0.1 m*M* PMSF and rocked for 2 h at 4 °C followed by centrifugation at 100,000 *g* for 1 h at 4 °C, and the supernatant (NE) was collected. EDTA and EGTA were added to a concentration of 5 m*M* to the S-1 fraction, and the S1 fraction was centrifuged at 16,000 *g* for 20 min. The supernatant (S2) was centrifuged at 100,000 *g* for 1 h to prepare the S3 fraction.

The NE and S-3 fractions were combined, and both KCl and polyethylene glycol (PEG 8000) were added to the combined NE/S3 to a final concentration of 2 *M* and 5%, respectively. The mixture was rocked for 1 h at 4 °C followed by centrifugation at 16,000 *g* for 15 min. The supernatant was dialyzed for 2 h against a buffer containing 50 m*M* HEPES (pH 7.5), 250 m*M* sucrose, 1 m*M* DTT, 150 m*M* KCl, 0.1 m*M* PMSF, and 1 m*M* each of EDTA and EGTA. The dialyzed fraction was clarified by centrifugation at 15,000 *g* for 15 min, and the resulting supernatant was layered onto a 2 *M* sucrose cushion and centrifuged at 100,000 *g* for 18 h at 4 °C. Following centrifugation, 20% of the interface of the sample above the sucrose cushion was collected and designated as P4. The upper 80% of the solution was designated the S4 fraction. The P4 fraction was dialyzed in a buffer containing 20 m*M* HEPES (pH 7.5), 5 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1 m*M* DTT, centrifuged at 4 °C for 10 min at 3000 *g* and stored in aliquots at −80 °C until needed.

Measurement of intact cell DNA synthesis

Exponentially growing MCF7 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then exposed for 24 h to increasing concentrations of drug prior to labeling with [^{3}H]thymidine. After 4 h of incubation, the cells were lysed and the level of DNA synthesis was measured in terms of the amount of [^{3}H]thymidine retained in acid-insoluble material.

Clonogenicity assay

Cells (10^3) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then exposed to increasing concentrations of drug for 4 h. After incubation, the medium was removed and the cells were washed with PBS and then incubated for 5 days in fresh drug-free medium. Colonies were visualized by staining with Giemsa stain, and the number of colonies formed on each plate was quantified by counting the stained colonies with a diameter ≥1 mm.

DNA polymerase α assay

Using our previously published procedure [24], DNA polymerase α activity was assayed in the absence or presence of increasing concentrations of drug using an activated calf thymus DNA as the template (Sigma Co.). Briefly, 20 µl reaction mixture contains 20 µg of synthesome fraction, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 0.1 m*M* each of dATP and dGTP, 0.01 m*M* dCTP, 2 µg activated calf thymus DNA template, and 0.1 µCi [^{3}H]dTTP. The reaction was started by incubating the reaction mixture in the absence or presence of increasing concentrations of dFdCTP or araCTP for 1 h at 37 °C. The amount of [^{3}H]dTTP incorporated was quantified by liquid scintillation counting after spotting the reaction mixture on DE81 filters as described by Malkas et al. [24].

DNA polymerase δ assay

DNA polymerase δ activity was detected using a poly(dGdC) template. A 12.5-µl volume of assay mixture contained 12.5 ng of template, 5% glycerol, 2 mg bovine serum albumin, 25 m*M* HEPES (pH 5.9), 10 m*M* $MgCl_2$, 10 µ*M* dCTP, 0.25 µCi [α-^{32}P]dGTP (3000 Ci/mmol), and 5 µg synthesome protein. The reactions were carried out at 37 °C with increasing concentration of drugs for 15 min. The whole reaction mixture was spotted onto Whatman DE81 filters. The filters were then processed to quantify the amount of radiolabeled nucleotide incorporated into the DNA template [10].

In vitro SV40 replication assay

The assay was performed essentially as described previously [24, 32] in the absence or presence of increasing concentrations of drug. The reaction mixture (25 µl) contained 30 m*M* HEPES (pH 7.5), 7 m*M* $MgCl_2$, 0.5 m*M* DTT, 2 µg SV40 large T antigen, 20 µg of synthesome protein fraction, 50 ng of the plasmid pSVO$^+$

containing an inserted SV40 replication-origin DNA sequence, 1 μCi [α-^{32}P]dGTP (3000 Ci/mmol), 100 μ*M* each of dATP, and dTTP, 10 μ*M* each of dCTP and dGTP, 200 μ*M* each of rCTP, rGTP, and rUTP, 4 m*M* rATP, 40 m*M* phosphocreatine, and 1 μg creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37 °C for 4 h. The reaction mixture was spotted onto Whatman DE81 filters and quantified by liquid scintillation counting. For gel analysis of the replication products, the reaction was stopped by adding 100 μg yeast RNA in 1% SDS followed by digestion for 1 h at 37 °C with 2 μg proteinase K. DNA replication products formed in the assay were then isolated by extracting the digestion mixture twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The extracted DNA was then precipitated in the presence of 2 *M* ammonium acetate with 2-propanol and the DNA pellet collected by centrifugation and resuspended in 10 m*M* Tris/1 m*M* EDTA. The products were analyzed using either a 1% neutral agarose gel containing TBE (90 m*M* Tris/90 m*M* boric acid/2 m*M* EDTA) or a 1% alkaline gel containing 50 m*M* NaOH/1 m*M* EDTA. The gels were dried and autoradiograms of the dried gels were produced by exposing the dried gels to Kodak film at −80 °C.

Purification of the SV40 large T antigen

T antigen was purified as previously described [24, 30].

Results

Effect of dFdC and araC on intact MCF7 cell DNA synthesis

In order to verify that araC and dFdC affect the ability of intact MCF7 cells to carry out DNA synthesis as previously reported [1, 11, 16], exponentially growing MCF7 cells were incubated in the absence (control, assigned a value of 100%) or presence of increasing concentrations of araC and dFdC. The cells were exposed for 24 h to drug concentrations ranging from 0.1 to 1000 μ*M*, and the drugs were then removed by washing the cells with PBS. Fresh medium was added to the cell culture and the cells were incubated with [^{3}H]thymidine for 4 h. The labeled cells were lysed, and the level of DNA synthesis was determined by quantifying the amount of [^{3}H]thymidine retained in acid-insoluble material. As shown in Fig. 2, intact MCF7 cell DNA synthesis was inhibited by both drugs in a concentration-dependent manner. About 10 μ*M* dFdC and 80 μ*M* araC were required to reduce MCF7 cell DNA synthesis to 50% of the control activity measured in the absence of either drug. The IC_{50} value for the inhibition of intact cell DNA synthesis by araC was comparable to that observed by us using the estrogen-receptor-negative breast cancer cell line, MDA MB-468 [1]. We also observed that the araC concentration required to inhibit 50% of intact HeLa cell DNA synthesis was also about 80 μ*M* (data not shown). This value was consistent with our previously reported IC_{50} value for araC inhibition of intact cell DNA synthesis [22].

We next performed cell survival assays to compare the cytotoxic effects of dFdC and araC on the ability of MCF7 to form colonies (Fig. 3). at concentrations of

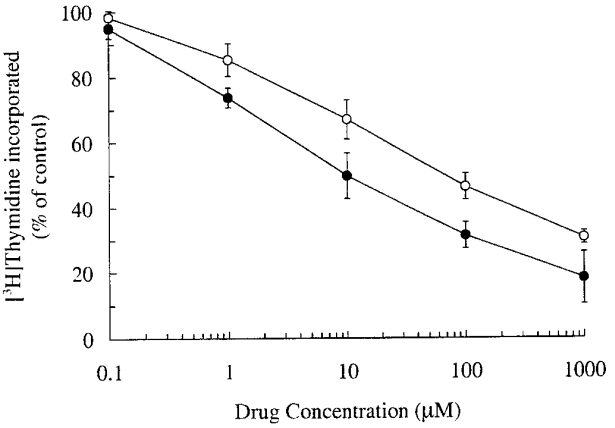

Fig. 2 Effect of dFdC and araC on intact MCF7 cell DNA synthesis. MCF7 cells (5×10^4) were seeded onto 60 mm cell culture plates and incubated for 24 h at 37 °C in Joklik's modified Eagle's medium. The cells were then exposed to one of several different concentrations of the indicated drug for 24 h at 37 °C. The cells were then labeled with [^{3}H]thymidine (1 μCi/ml of medium). After a 4-h incubation, the cells were lysed and the level of DNA synthesis was determined by quantifying the amount of [^{3}H]thymidine present in acid-insoluble material (○ araC, ● dFdC). Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared

dFdC above 8 μ*M* 50% of the cells lost their clonogenic capacity following a 4-h incubation with the drug. The concentration of dFdC needed to inhibit the clonogenic

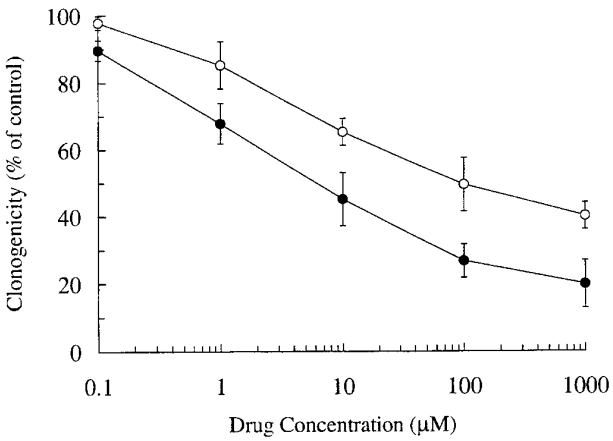

Fig. 3 Effect of dFdC and araC on the clonogenicity of intact MCF7 cells. MCF7 cells (10^3) were seeded onto 60 mm cell culture plates and incubated for 24 h at 37 °C in Joklik's modified Eagle's medium. Cells were then exposed to different concentrations of drugs for 4 h followed by incubation in drug-free medium for 5 days. Colonies were fixed with 10% formaldehyde in PBS and visualized by Giemsa stain and counted as described in Materials and methods (○ araC, ● dFdC). Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared

survival of 50% of MCF7 cells was approximately 12-fold less than that of araC. Thus, our results from the intact DNA synthesis and clonogenicity demonstrated that dFdC was significantly more cytotoxic to intact MCF7 cells than araC.

Comparison of the inhibitory effects of araCTP and dFdCTP on in vitro SV40 origin-dependent DNA replication using the MCF7 cell DNA synthesome

We have previously shown that the DNA synthesome isolated from the HeLa cell and human breast cancer cell MDA MB-468 is capable of supporting the origin-specific T antigen-dependent SV40 DNA replication reaction in vitro [1, 4, 32]. These studies demonstrated the utility of the purified DNA synthesome as a relevant in vitro model that is useful for studying the mechanism of action of anticancer drugs such as araC, camptothecin, and VP16. In order to directly compare the anti-DNA synthetic activity of araCTP and dFdCTP, we performed in vitro SV40 origin-dependent DNA replication assays in the absence and presence of several concentrations of each of these two drugs. Both drugs inhibited SV40 origin-containing DNA replication in a concentration-dependent manner as measured by quantifying the amount of [^{32}P]dGTP incorporated into DNA (Fig. 4A). A 50% inhibition of the in vitro DNA replication assay was achieved in presence of 10 μM dCTP using approximately 3 μM dFdCTP and 60 μM araCTP. The results of this assay indicated that dFdCTP was able to more effectively compete with dCTP to inhibit synthesome-mediated in vitro DNA replication than araCTP. Our data showed a close correlation between the IC_{50} values of both drugs for inhibiting intact cell DNA synthesis and the DNA synthesome-mediated in vitro SV40 replication assay.

We further analyzed the replication products of the in vitro replication reaction using a 1% neutral and a 1% alkaline agarose gels (Fig. 4B,C). Our results indicated that the MCF7 cell DNA synthesome was capable of ▶

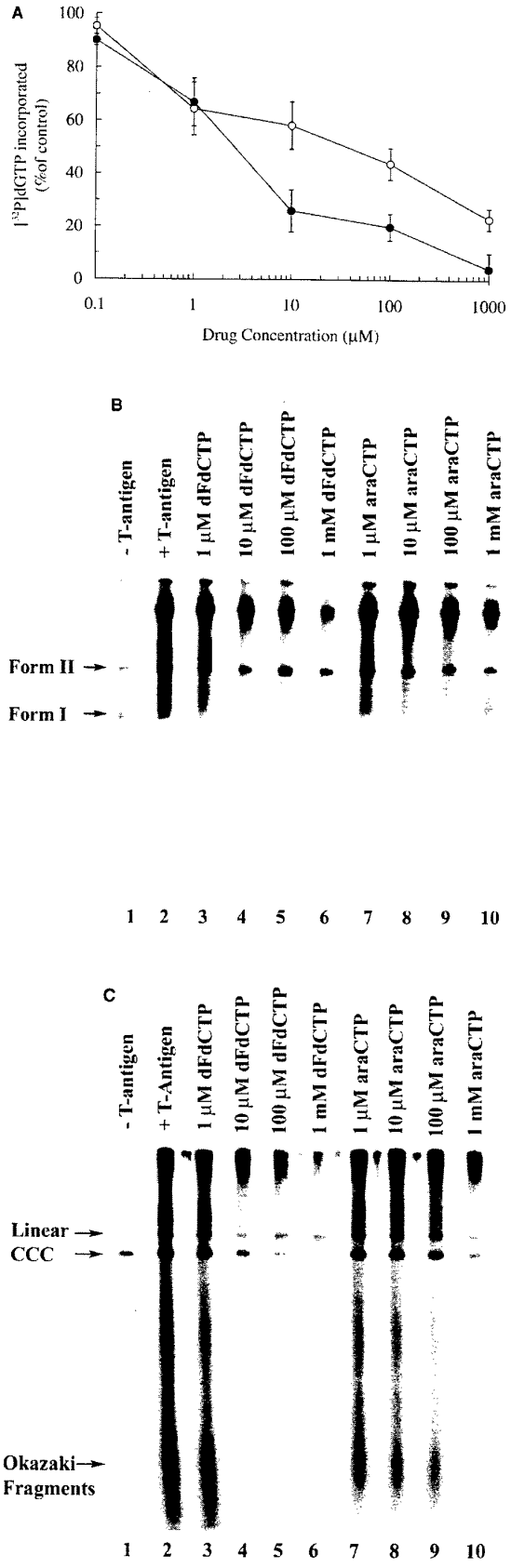

Fig. 4A–C Effect of dFdCTP and araCTP on DNA synthesome-mediated in vitro SV40 DNA replication. **A** Inhibition of dFdCTP and araCTP on the synthesome-mediated in vitro SV40 DNA synthesis as a function of drug concentrations (○ araCTP, ● dFdCTP). The assays were performed as described in Materials and methods. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%). **B** Neutral agarose gel analysis of the reaction products of the in vitro SV40 DNA replication assay. **C** Alkaline agarose gel analysis of the reaction products of the in vitro SV40 DNA replication reaction. The DNA replication products formed in the in vitro DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA, and the reaction products were resolved using 1% agarose gels under either neutral or alkaline conditions (Materials and methods). The gels were dried and exposed to Kodak XAR5 films at −80 °C for 8 h (*CCC* covalently closed circular DNA)

producing full-length daughter DNA as shown by the presence of form I (superhelical) DNA and form II (nicked open circular) DNA, as well as higher-order replication intermediates (Fig. 4B, lane 2). This reaction was also T antigen-dependent (Fig. 4B, compare lanes 1 and 2). In the presence of low concentrations (1 μM) of both drugs, full-length daughter DNA molecules (form I and form II) were observed (Fig. 4B, lanes 3 and 7). At higher concentrations, the production of form II DNA molecules as well as replication intermediates was inhibited in a concentration-dependent manner (note both neutral and alkaline gels, Fig. 4B,C, lanes 2–10). However, form I DNA molecules disappeared at drug concentrations higher than 1 μM, indicating that dFdCTP and araCTP may impair the process to form supercoiled DNA. In the presence of both drugs, the production of short Okazaki fragments was inhibited in a concentration-dependent manner (Fig. 4C, lanes 3–10) and was completely inhibited at higher dFdCTP concentrations (Fig. 4C, lanes 5 and 6), suggesting that dFdCTP had a greater inhibitory effect on the initiation stage of DNA synthesis.

These results are in accordance with our previously reported results using the DNA synthesome isolated from HeLa cells and MDA MB-468 cells [1, 22]. Furthermore, they correlate with the results of other studies carried out in this laboratory employing our DNA synthesome-mediated in vitro DNA replication assay system as well as intact cells [16, 28].

The effects of araCTP and dFdCTP on DNA synthesome-associated DNA polymerase α and DNA polymerase δ activity

Our previous study on the inhibitory effects of araCTP on the activity of the purified DNA polymerase α and the DNA synthesome-associated polymerase α provided evidence indicating that the DNA synthesome can be used as an in vitro model system that more closely reflects the events occurring within the intact cell than can be achieved using individually purified enzymes [32, 33].

To further compare the activities of araC and dFdC on individual DNA replication essential proteins, we performed DNA synthesome-associated DNA polymerase α and δ assays. In the polymerase α assays, we used activated calf thymus DNA as the template, and incubated the template with various concentrations of araCTP and dFdCTP in the presence of the DNA synthesome (Materials and methods). The polymerase α activity was determined by quantifying the amount of [^{3}H]TTP incorporated into DNA (Fig. 5). Unlike in vitro SV40 DNA replication, the activity of the synthesome-associated polymerase α was not inhibited by 1 μM of either drug. However, at approximately 80 μM dFdCTP and 100 μM araCTP the activity of the synthesome-associated DNA polymerase was inhibited by 50% relative to the activity of the control reaction performed in the absence of the drug.

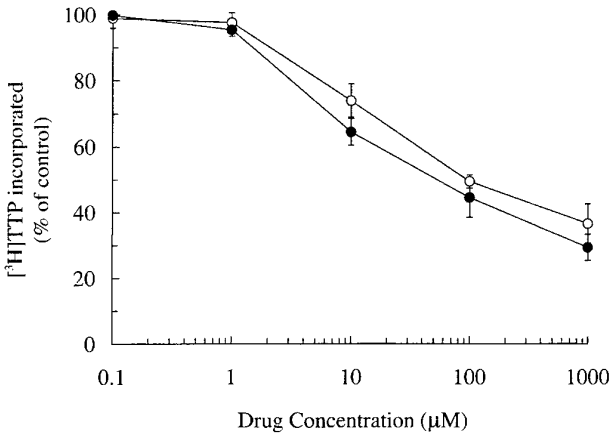

Fig. 5 Effect of dFdCTP and araCTP on DNA synthesome-associated DNA polymerase α activity. The assays were performed using activated calf thymus DNA as the template. Reaction mixtures were incubated with different concentrations of drugs as described in Materials and methods (○ araCTP, ● dFdCTP). The amount of [^{3}H]dTTP incorporated into DNA was determined in terms of binding to Whatman DE81 filters [21]. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%)

Our results indicate that the inhibitory effect of dFdCTP and araCTP on synthesome-associated DNA polymerase α occurs at nearly equivalent concentrations. The IC_{50} value of araC was in good agreement with that previously reported [1, 10, 32]. However, dFdCTP was not significantly more potent than araCTP in the DNA polymerase assay, and this contrasts with our results obtained using the in vitro SV40 DNA replication assay.

We have previously reported that the inhibitory effect of araCTP is primarily through inhibition of synthesome-associated DNA polymerase α and that a significant amount of inhibition of synthesome-associated polymerase δ activity also occurs, but at a fourfold higher concentration of the drug [1, 10]. In this study, it was also observed that both dFdCTP and araCTP showed inhibition of DNA polymerase δ at higher concentrations than those required to inhibit DNA polymerase α. The IC_{50} values of dFdCTP and araCTP for inhibition of DNA polymerase δ were approximately 700 μM and 750 μM, respectively (Fig. 6). However, the activity of synthesome-associated polymerase δ was not significantly inhibited by either drug at a concentration of 100 μM. This was a significant finding because the activity of DNA polymerase α was readily inhibited by apparently seven- to ninefold less drug than DNA polymerase δ.

The fact that dFdCTP was significantly more potent than araCTP at inhibiting in vitro SV40 replication than at inhibiting synthesome-associated DNA polymerase α and δ activity strongly suggests that dFdCTP may have a greater effect on inhibiting the coordinated replication activity of an organized DNA replication multienzyme complex than purified DNA polymerases. Furthermore,

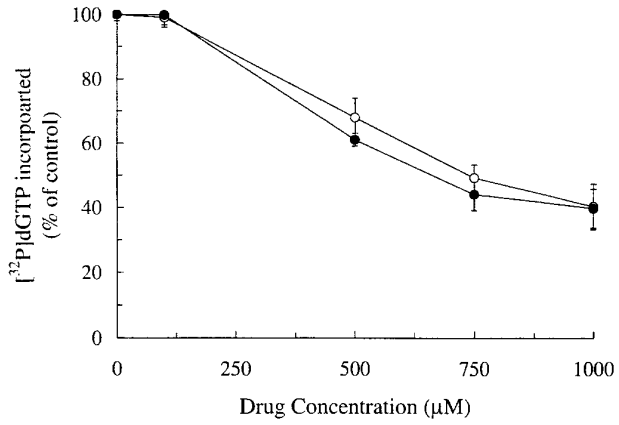

Fig. 6 Effect of dFdCTP and araCTP on the synthesome-associated DNA polymerase δ activity. The assay was performed using a poly(dGdC) template as described in Materials and methods (○ araCTP, ● dFdCTP). The amount of [α-^{32}P]dGTP incorporated into DNA was determined in terms of binding to Whatman DE81 filters [9]. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%)

our results imply that the potent inhibitory effects of dFdC may be due to the drug targeting additional proteins during the DNA synthetic process, or inhibiting the coordinated elongation of both strands at a replication fork by specifically slowing the ability of one of the polymerases at the replication fork to efficiently carry out DNA synthesis.

Discussion

dFdC is a novel deoxycytidine analog with both structural and metabolic similarities to araC. AraC is one of the most effective drugs available today for the treatment of acute leukemia and other hematopoietic malignancies. dFdC is also effective against leukemia [14, 21], and has also proven to be effective against a variety of solid tumors. Like araCTP, dFdCTP inhibits DNA synthesis mainly through the inhibition of the activity of the DNA polymerases. A considerable body of work has accumulated on the effects of these two drugs on intact cell DNA synthesis in a variety of cancer cell lines. Using a cell survival assay, Heinemann et al. [11, 12] have discovered that Chinese hamster ovary cells are significantly more sensitive to dFdC than to araC after both a 4- and an 18-h incubation. Our study using the human breast cancer cell line MCF7 in a clonogenic assay also showed that dFdC is approximately 15-fold more cytotoxic than araC following a 24-h incubation with the drug. dFdC inhibits [^{3}H]thymidine uptake approximately tenfold more than araC in intact MCF7 cells. Accumulation of dFdCTP has been observed to be cell line-dependent, with the cell lines that are more sensitive to the drug accumulating higher amounts of dFdCTP in cultures [16, 25]. This may explain the difference between the IC_{50} values obtained in our experiments, and those obtained by other investigators who have performed similar studies with CHO cells [11], human T-lymphoblastoid CCRF-CEM cells [15] and HL60 cells [29].

DNA synthesis is the most prominent activity inhibited by dFdC in cultured cells [11]. Huang et al. [16] have directly investigated the molecular mechanism of action of dFdC and araC in vitro on DNA synthesis using purified DNA polymerase α and ε. However, the use of purified DNA polymerases may not adequately reflect the DNA synthetic process as it occurs within the intact cell. In the intact cell, DNA synthesis involves the coordinated activity of DNA polymerases α and δ along with that of several other enzymes and factors. In this report, we describe studies performed with intact MCF7 cells and the DNA synthesome isolated from these cells. We directly compared the effectiveness of dFdCTP and araCTP as inhibitors of the DNA synthetic process. dFdCTP was significantly more potent than araCTP in the in vitro SV40 DNA replication assay. Full-length DNA was produced in the presence of very low levels of both drugs, suggesting that incorporation of dFdCTP and araCTP did not stop the polymerases from elongating the DNA template.

Our results are in agreement with those of Ross et al. [29], who have demonstrated that dFdC is progressively incorporated into nascent DNA of increasing size in intact HL60 cells. In contrast, studies of dFdCTP incorporation using in vitro primer extension assays by purified DNA polymerase α and δ have demonstrated that after incorporation of dFdCTP to the 3′ terminus of the elongating DNA strand, one more deoxynucleotide can be added before the DNA polymerases are unable to continue elongating the nascent strand. Therefore, dFdC appears to act as a chain terminator [16]. Although dFdCTP appears to be a more potent inhibitor of SV40 DNA synthesis in vitro than araC, the inhibitory effects of dFdCTP and araCTP on the activity of the DNA synthesome-associated DNA polymerase α and δ are similar. The IC_{50} values of dFdCTP and araCTP for DNA polymerase α are 80 μM and 100 μM, respectively. These values are comparable to the intracellular dFdCTP concentration, which has been reported to range from 64 μM to 362 μM in the leukemia cells of patients undergoing dFdCTP therapy [7, 8].

Taken together, our results and those reported by others [6, 7] suggest that DNA polymerase α is a major target for dFdCTP and araC. Inhibition of DNA synthesome-associated polymerase δ occurred, but at substantially higher drug concentrations than required to inhibit DNA polymerase α to the same extent. About 300 μM of dFdCTP was required to inhibit DNA polymerase δ by 10%. Therefore, it is most likely that the inhibitory effect of dFdCTP and araCTP on the SV40 origin-dependent DNA replication process is mediated primarily through inhibition of DNA polymerase α. This conclusion agrees with the findings of our previous studies analyzing the mechanism of action of araCTP in this same assay [1, 10, 32, 33].

The fact that dFdCTP was significantly more potent than araCTP suggests that dFdCTP may have more impact on the organized multiprotein DNA replication complex (i.e. the DNA synthesome) found in intact cells than would be suggested from studies employing purified individual DNA polymerases. Furthermore, studies from intact cells and in vitro assays have shown that addition of dCTP cannot completely restore DNA synthesis to the level observed in assays performed without dFdCTP [16]. This observation indicates that the inhibition of DNA polymerase α activity by dFdC is not simply a result of competition with dCTP. Primer extension assays have shown that the $3' \rightarrow 5'$ exonuclease activity of purified DNA polymerase ε is essentially unable to excise nucleotide from DNA containing dFdCMP at either the 3′ terminus or from an internal position within the DNA. AraCMP, however, has been reported to be removed from the 3′ terminus of DNA [16], indicating that dFdC may have more impact on DNA repair than previously thought.

However, the greater anticancer activity of dFdC is not only attributed to the inhibition of DNA polymerase α activity, but may be derived from damage to the DNA into which dFdC is incorporated. dFdC is more potent than araC for several reasons. First, the chemical addition of fluorine atom to the 2′ position of the furanose ring of the drug makes dFdC more lipophilic and therefore more permeable to cells than araC. Second, deoxycytidine kinase has a higher affinity for dFdC than araC which leads to higher levels of dFdCTP than araCTP in cells. Third, dFdC inhibits ribonucleotide reductase which causes depletion of the cellular pools of deoxynucleoside triphosphate, particularly the levels of the competing metabolite, dCTP. Thus, the ratio of cellular dCTP to dFdCTP favors the inhibition of DNA synthesis by dFdCTP [6, 12]. In contrast, araC has no effects on dNTP pools and is not known to act as an inhibitor of ribonucleotide reductase which helps maintain higher levels of active metabolite within the cell.

It has been reported that the cellular dCTP level is 3.5 μM in K562 cells [5]. Since the concentration of dCTP used in our in vitro assays was 10 μM, it is possible that the differential effects of dFdC and araC on DNA synthesis might be more dramatic if true intracellular dCTP concentrations were used. Finally, the elimination of cellular dFdCTP is slower than that of araCTP [16]. All of these characteristics of dFdCTP contribute to the higher intracellular concentration of dFdCTP as compared to araCTP. dFdCTP is found at from 9- to 20-fold higher concentrations in treated cells than in cells treated with equivalent concentrations of araC [11]. However, the increase in the intracellular concentration of dFdCTP does not completely account for the greater cytotoxicity of dFdCTP in intact cells, which has been reported to be 180-fold more toxic than araC [12, 16, 26, 27].

In conclusion, in this study we demonstrated that dFdCTP is significantly more potent than araCTP at inhibiting the DNA synthetic process in our cell-free SV40 origin-dependent in vitro DNA replication assay system employing the human MCF7 cell DNA synthesome. The results of this study indicate that the DNA synthesome can serve as a relevant in vitro model system for studying the mechanism of action of anticancer drugs that directly affect DNA synthesis, and that the mechanisms through which these drugs inhibit in vitro DNA synthesis closely parallel the inhibitory effects of these drugs in intact cells [1, 4, 10, 32, 33]. We have demonstrated in our laboratory that the DNA synthesome is able to incorporate araCTP into internucleotide linkages, and that this incorporation of araC into internucleotide linkages more closely resembles the molecular events occurring in intact cells than can be achieved using purified DNA polymerases [32, 33].

Continued analysis of the mechanisms by which dFdCTP mediates its cytotoxic effects will uncover the effects dFdC has on the initiation, elongation and termination stages of the DNA synthesis process. Thus, our in vitro model system, which utilizes the DNA synthesome to mediate the DNA synthetic reaction is anticipated to be of substantial value for gaining insight into the mechanism(s) of action of dFdCTP and other anticancer drugs that directly inhibit cellular DNA synthesis. Continued validation of the in vitro DNA replication model system employing the DNA synthesome is therefore expected to show the system to be of considerably more value in the search for more effective anticancer drugs than conventional model systems that only employ highly purified individual enzymes such as DNA polymerase or topoisomerase II.

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ORIGINAL ARTICLE

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Ara-C affects formation of cancer cell DNA synthesome replication intermediates

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Abstract *Purpose*: An intact and fully functional multiprotein DNA replication complex (DNA synthesome) from human as well as from murine mammary carcinoma cells was first isolated and characterized in our laboratory. The human cell synthesome supports the in vitro origin-specific simian virus 40 (SV40) DNA replication reaction in the presence of the viral large T-antigen using a semiconservative mechanism and has been shown to contain all the proteins and enzymes required to support DNA synthesis. We are currently using the DNA synthesome as a unique model for analyzing the mechanism of action of anticancer drugs affecting DNA replication. The purpose of this study was to further investigate the mechanism of action of ara-C using the DNA synthesome isolated from the human breast cancer cell line MDA MB-468. *Methods*: Synthesome-mediated SV40 DNA replication was performed in the presence of various concentrations of ara-CTP (the active metabolite of ara-C) and the types of daughter DNA molecules produced were analyzed using neutral and alkaline gel electrophoresis. We also examined the effect of ara-C on intact MDA MB-468 cell DNA synthesis and on cell proliferation. In addition, we studied the effect of ara-CTP on the activity of some of the synthesome target proteins (the DNA polymerases α and δ). *Results*: Full-length daughter DNA molecules were obtained in the presence of low concentrations of ara-CTP while at higher concentrations, there was an inhibition of full-length daughter DNA synthesis. The findings suggest that specifically the initiation phase of DNA synthesis was inhibited by ara-CTP since the production of the short Okazaki fragments was suppressed at all concentrations of the drug above 10 μ*M*. In addition, it was found that the IC_{50} of ara-CTP for inhibition of synthesome-mediated in vitro DNA replication was comparable to that required to inhibit intact cell DNA synthesis. Further experimentation has shown that ara-CTP preferentially inhibits the activity of the synthesome-associated DNA polymerase α enzyme while the DNA polymerase δ seems to be resistant to the inhibitory effect of that drug. *Conclusions*: Our results indicate that ara-C's action on DNA replication is mediated primarily through DNA polymerase α and suggest that this enzyme plays a key role in DNA synthetic initiation events. The results also provide definitive support for the use of the DNA synthesome as a unique and powerful model for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

Key words Ara-C · DNA synthesome · DNA replication · In vitro

Abbreviations *ara-C* 1-β-D-arabinofuranosylcytosine · *ara-CMP* 1-β-D-arabinofuranosylcytosine monophosphate · *ara-CTP* 1-β-D-arabinofuranosylcytosine triphosphate · *DTT* dithiothreitol · *EDTA* ethylenediaminetetraacetic acid · *EGTA* ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid ·

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HEPES *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) · *PBS* phosphate-buffered saline · *PEG* polyethylene glycol · *PMSF* phenylmethyl sulfonyl fluoride · *SV40* simian virus 40 · *Tris* Tris(hydroxymethyl)aminoethane

Introduction

Eukaryotic cell DNA replication is a highly complex process that involves the precise interactions of many proteins with one another and with the DNA template being replicated [23]. In the past few years, a general picture has emerged in which the DNA synthesizing machinery in mammalian cells is clearly organized into a multiprotein complex that is competent to coordinate the synthesis of both strands of the DNA template [2, 17, 18, 29–31, 36].

An intact, stable, and functional multiprotein DNA replication complex (designated DNA synthesome) from human [1, 5, 20, 26, 31] and murine mammary carcinoma cells [43] was first successfully isolated, purified and characterized in our laboratory. The human cell DNA synthesome is fully competent to support the SV40 origin-specific and large T-antigen-dependent in vitro DNA replication reaction. This reaction uses a semiconservative DNA replication mechanism to produce daughter DNA products which include form I (supercoiled) DNA, form II (nicked open circle) DNA, and the higher order topological intermediates [31]. We have shown that the DNA synthesome contains all the proteins and enzymes required to support DNA synthesis, including DNA polymerases α, δ, and ε, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen, replication factor C (RF-C), replication protein A (RP-A), DNA helicase, and DNA ligase [1, 5, 20, 26].

We are currently using the DNA synthesome as a unique model for analyzing the mechanism of action of anticancer drugs affecting DNA replication. Coll et al. [4] have found that there is a close correlation between the IC_{50} value of camptothecin (a topoisomerase I poison) required to inhibit intact HeLa cell DNA synthesis and that required to suppress synthesome-mediated in vitro DNA synthesis. In a related study, Wills et al. [41] have demonstrated that the DNA synthesome has the ability to successfully incorporate ara-CMP into the replicating DNA at internucleotide positions in a manner similar to that observed using intact cells.

In this study, we used the DNA synthesome isolated from the human breast cancer cell line MDA MB-468 to further investigate the mechanism of action of ara-C. The synthesome-mediated in vitro SV40 DNA replication reaction was performed in the presence of increasing concentrations of ara-CTP, and the types of daughter DNA molecules produced were analyzed using neutral and alkaline gel electrophoresis. In addition, we determined, for ara-CTP, the IC_{50} value required to inhibit the in vitro SV40 DNA replication reaction and compared this IC_{50} value to that of ara-C required for the inhibition of intact MDA MB-468 cell DNA synthesis. Furthermore, the effect of ara-CTP on the activity of the synthesome-associated DNA polymerases α and δ was studied. Our results provide definitive support for the use of the DNA synthesome as a unique and powerful model for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

Materials and methods

Materials

Ara-C, ara-CTP, activated calf thymus DNA, and nonradioactive nucleotides were purchased from the Sigma Chemical Company (St. Louis, Mo.). Poly (dG-dC) poly (dG-dC) template was obtained from Pharmacia Biotec, and [α-^{32}P]dGTP, methyl ^{3}H-thymidine, and ^{3}H-TTP were obtained from NEN (Boston, Mass.).

Cell culture and harvest

Suspension cultures of MDA MB-468 cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml medium) were harvested and washed three times with PBS comprising 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.8 m*M* Na_2HPO_4. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 min, 4 °C) and the cell pellets were stored at −80 °C until fractionated.

Isolation and purification of the DNA synthesome from MDA MB-468 cells

The DNA synthesome was isolated according to our published procedures [5, 31]. Briefly, MDA MB-468 cell pellets (10 g) were thawed and resuspended in two volumes of the homogenization buffer containing 50 m*M* HEPES (pH 7.5), 200 m*M* sucrose, 5 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* DTT and 0.1 m*M* PMSF. The pellets were homogenized by 30 strokes of a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 5000 rpm for 15 min to separate the cytosol (S-1) from the nuclei. The nuclear pellets were resuspended in two volumes of a buffer containing 50 m*M* HEPES (pH 7.5), 400 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EDTA, 2 m*M* DTT, and 0.1 m*M* PMSF and rocked for 2 h at 4 °C. The nuclear pellets were then separated by centrifugation at 40,000 rpm for 1 h and the supernatant (NE) was collected. EDTA and EGTA (5 m*M* final concentration of each) were added to the S-1 fraction and the mitochondria were then removed by centrifugation at 15,000 rpm for 15 min. The supernatant (S-2) was subjected to centrifugation at 40,000 rpm for 1 h to remove the microsomes, and the postmicrosomal fraction was collected and designated S-3.

The NE and S-3 fractions were combined, adjusted to 2 *M* KCl and 5% PEG (PEG 8000), and rocked for 1 h at 4 °C. The NE/S-3 fraction was then centrifuged at 15,000 rpm for 15 min to remove the PEG-precipitated materials and the supernatant was dialyzed for 2 h against a buffer containing 50 m*M* HEPES (pH 7.5), 250 m*M* sucrose, 1 m*M* DTT, 150 m*M* KCl, 0.1 m*M* PMSF, and 1 m*M* each of EDTA and EGTA. The dialyzed fraction was clarified by centrifugation at 15,000 rpm for 15 min and the resulting supernatant was layered onto a 2 *M* sucrose cushion and subjected to centrifugation at 40,000 rpm for 18 h at 4 °C. The supernatant solution (S-4) and the sucrose interphase (P-4) were removed. The P-4 fraction, which has the replication activity, was dialyzed against a buffer containing 20 m*M* HEPES (pH 7.5), 5 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1 m*M* DTT. The dialyzed P-4 fraction was

subjected to centrifugation at 5000 rpm for 10 min and stored in aliquots at −80 °C.

Purification of SV40 large T-antigen

T-antigen was purified as described by Simanis and Lane [38] and it is the only virally encoded protein required for SV40 origin-dependent DNA replication in vitro [7].

Effect of Ara-C on MDA MB-468 cell growth

MDA MB-468 cells (5×10^4) were seeded in 60-mm cell culture plates and incubated for 48 h at 37 °C. The cells were then exposed to increasing concentrations of ara-C. After 24 h, the medium was removed, the cells were washed twice with PBS, and allowed to grow in drug-free medium for 5 days. The cells were then treated with trypsin/EDTA and quantified using a Coulter cell counter.

In vitro SV40 DNA replication assay

The reaction was carried out according to the procedures of Malkas et al. [31] with some modifications. The reaction mixture (25 µl) contained 30 m*M* HEPES (pH 7.5), 7 m*M* $MgCl_2$, 0.5 m*M* DTT, 1.5–3 µg SV40 large T-antigen, 50 µg synthesome protein fraction, 50 ng of the plasmid $pSVO^+$ containing a 200-bp insert of the SV40 replication origin DNA sequence [39], 1 µCi [α-^{32}P]dGTP (3000 Ci/mmol), 100 µ*M* each of dATP, and dTTP, 10 µ*M* each of dCTP and dGTP, 200 µ*M* each of rCTP, rGTP, and rUTP, 4 m*M* rATP, 40 m*M* phosphocreatine, and 1 µg creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37 °C for 4 h. To determine the amount of radiolabel incorporated into the daughter DNA molecules, the reaction mixture was spotted on Whatman DE81 filters and quantified by liquid scintillation counting. For gel analysis of the replication products, the reaction was stopped by adding 100 µg yeast RNA in 1% sodium dodecyl sulfate followed by digestion for 1 h at 37 °C with 2 µg proteinase K. DNA replication products were then isolated by phenol/chloroform extraction followed by precipitation with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA and electrophoresed in 1% agarose gels containing either TBE (90 m*M* Tris/90 m*M* boric acid/2 m*M* EDTA) or alkaline (50 m*M* NaOH/1 m*M* EDTA) buffers. Gels were dried and exposed to Kodak films at −80 °C followed by autoradiography.

Measurement of intact MDA MB-468 cell DNA synthesis

Exponentially growing MDA MB-468 cells (5×10^4) were incubated at 37 °C with increasing concentrations of ara-C for 24 h. The cells were then labeled by the addition of methyl ^{3}H-thymidine (1 µCi/ml) to the medium. After a 4-h incubation, the cells were lysed and the amount of radiolabel incorporated into DNA was measured by quantifying the amount of ^{3}H in acid-insoluble materials.

DNA polymerase α assay

The assay was carried out according to the procedure described by Malkas et al. [31]. Briefly, the reaction mixture (20 µl) contained 20 µg of synthesome fraction, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* each of dATP and dGTP, 0.01 m*M* dCTP, 2 µg activated calf thymus DNA template (Sigma), and 0.11 µCi ^{3}H-TTP (72.6 Ci/mmol). The reaction was started by incubating the reaction mixture in the absence or presence of increasing concentrations of ara-CTP (1, 10, 50, 100, 200, 800 and 1000 µ*M*) for 1 h at 37 °C. The amount of ^{3}H-TTP incorporated was quantified by liquid scintillation counting after spotting the reaction mixture on DE81 filters as described by Sambrook et al. [37].

DNA polymerase δ assay

The assay was carried out according to the method of Lee et al. [25] with some modifications. The reaction mixture (25 µl) contained 20 µg of synthesome fraction, 25 m*M* HEPES (pH 5.9), 10 m*M* $MgCl_2$, 0.2 mg/ml bovine serum albumin, 0.01 m*M* dCTP, 2 U/ml of poly (dG-dC) poly (dG-dC) template (Pharmacia Biotec), 5% glycerol, and 0.25 µCi of [α-^{32}P]dGTP (3000 Ci/mmol). The DNA template was denatured by heating for 5 min at 100 °C and chilled in ice. The reaction mixture was incubated in the absence or presence of 100, 200, 400, and 800 µ*M*, and 1 m*M* ara-CTP for 15 min at 37 °C. It was then spotted on DE81 filters and the amount of ^{32}P-dGTP retained on the filter was measured as described by Sambrook et al. [37].

Results

Effect of Ara-C on MDA MB-468 cell proliferation

To determine the effect of ara-C on the growth of MDA MB-468 cells, the drug was added for 24 h to exponentially growing cells at concentrations ranging from 10 n*M* to 1 m*M*. The medium was then removed and the cells were allowed to grow in drug-free medium for 5 days at which time the number of growing cells was quantified. Figure 1 shows that a logarithmic increase in ara-C concentration was accompanied by a steady decrease in the ability of cells to proliferate. The concentration of ara-C required to inhibit cell growth by 50% was approximately 1 µ*M*. This result agrees

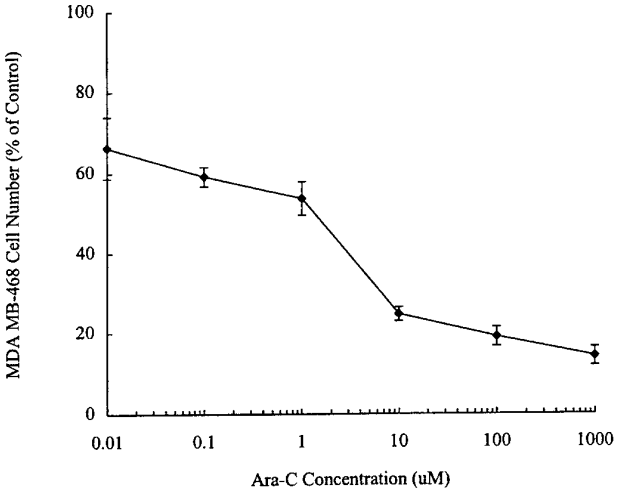

Fig. 1 Effect of ara-C on MDA MB-468 cell growth. MDA MB-468 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 48 h at 37 °C. The cells were exposed to increasing concentrations of ara-C. After 24 h, the medium was removed and the cells were allowed to grow in drug-free medium for 5 days. The cells remaining in the plate were then treated with trypsin/EDTA and the viable cells were counted. Each point on the graph represents the mean ± SEM (standard error of the mean) of three to five independent experiments. Cells grown in the absence of ara-C provided the control cultures, with which the number of cells in plates containing ara-C were compared

clearly with the previously reported data for HL-60 cells [27].

Ara-C inhibits both intact MDA MB-468 cell DNA synthesis and the synthesome-mediated in vitro SV40 DNA replication reaction

To validate the usefulness of the synthesome as an in vitro model system for studying the mechanism of action of ara-C, we determined whether the concentration of ara-C that effectively inhibits MDA MB-468 intact cell DNA synthesis was comparable to the concentration of ara-CTP required to suppress the synthesome-driven in vitro SV40 DNA replication reaction. Exponentially growing MDA MB-468 cells were exposed to increasing concentrations of ara-C for 24 h and then labeled with ^{3}H-thymidine for 4 h. The IC_{50} of ara-C required to inhibit intact cell DNA synthesis was 115 μM (Fig. 2). For the in vitro study, the synthesome-mediated in vitro SV40 DNA replication reaction was carried out in the absence or presence of increasing concentrations of ara-CTP. The reaction mixture was incubated at 37 °C for 4 h and the level of DNA synthesis was determined by quantifying the amount of ^{32}P-dGTP incorporated into the replication products. The concentration of ara-CTP required to cause 50% inhibition of in vitro DNA synthesis was 65 μM (Fig. 3). This result indicates that there was a reasonable correlation between the IC_{50} of ara-CTP for inhibition of the synthesome-mediated in vitro DNA

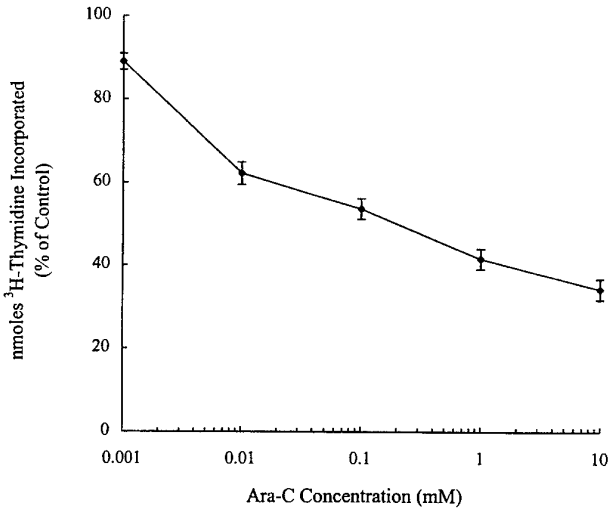

Fig. 2 Effect of ara-C on intact MDA MB-468 cell DNA synthesis. MDA MB-468 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were exposed to increasing concentrations of ara-C for 24 h and then labeled with ^{3}H-thymidine (1 μCi/ml of medium). After a 4-h incubation, the cells were lysed and the level of DNA synthesis was determined by quantifying the amount of ^{3}H-thymidine present in acid-insoluble material. Each point on the graph represents the mean ± SEM of three to five independent experiments. Controls consisted of plates of cells grown in the absence of ara-C for 24 h prior to labeling with ^{3}H-thymidine

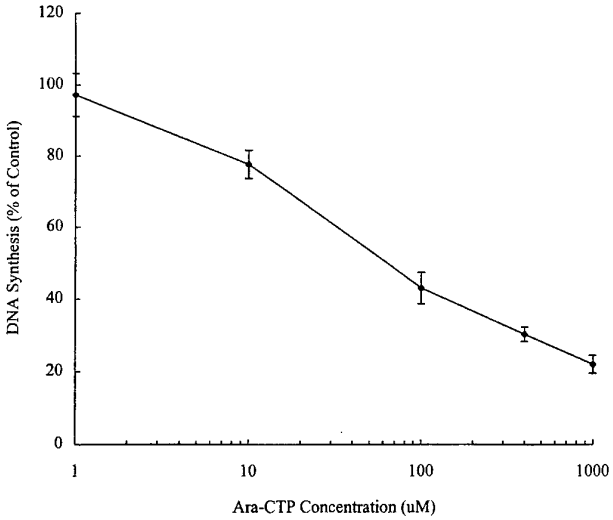

Fig. 3 Effect of increasing concentrations of ara-CTP on the synthesome-mediated in vitro SV40 DNA replication reaction. The replication reaction was initiated by incubating the reaction mixture at 37 °C for 4 h in the absence or presence of increasing concentrations of ara-CTP under the conditions described in the text. To determine the amount of radiolabeled ^{32}P-dGTP incorporated into the daughter DNA molecules, the reaction mixture was spotted on Whatman DE81 filters which were then washed, and the amount of radiolabeled nucleotide retained on the each filter quantified by liquid scintillation counting [37]. One unit of in vitro SV40 DNA synthesis activity is equivalent to 1 nM of total [α-^{32}P]dNMP incorporated into SV40-origin DNA per hour at 37 °C. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP, and the incorporation of ^{32}P-dGTP was quantified as described in Materials and methods

replication and that of ara-C required to inhibit intact MDA MB-468 cell DNA synthesis. A similar correlation between the IC_{50} values for the inhibition of intact cell DNA synthesis and in vitro replication has been found in a previous study carried out in our laboratory using camptothecin and ara-C with HeLa cells [4, 28]. This result further supports the usefulness of the synthesome as a powerful model system for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

Ara-CTP affects the formation of the DNA synthesome replication intermediates

The synthesome-mediated in vitro SV40 DNA replication reactions were performed in the absence or presence of increasing concentrations of ara-CTP (Materials and methods) and the types of daughter DNA molecules produced in the in vitro reaction were analyzed using neutral and alkaline gel electrophoresis.

We have previously shown that the DNA synthesome isolated from HeLa cells supports the in vitro origin-specific T-antigen-dependent SV40 DNA replication reaction [31, 41]. The results of this study clearly demonstrate that the DNA synthesome isolated from human

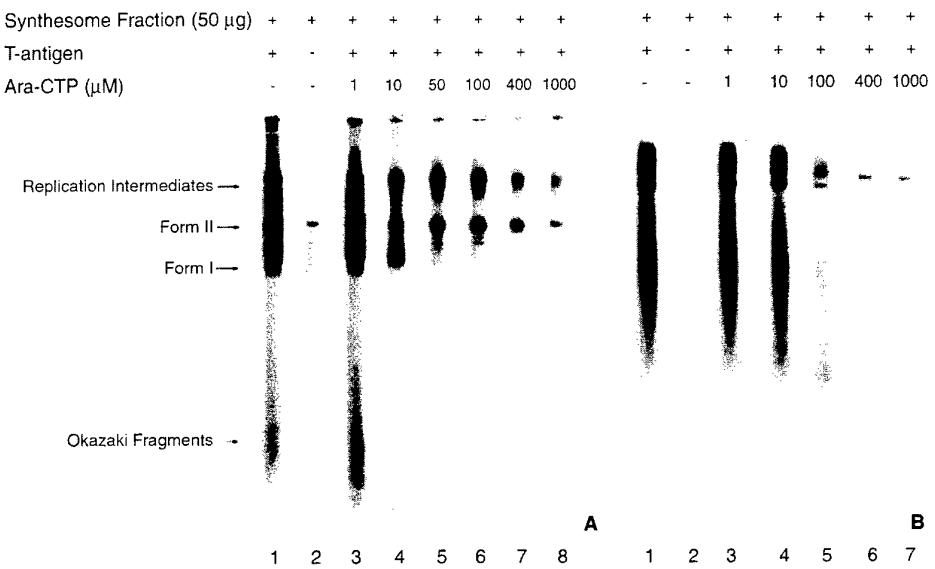

Fig. 4A,B Neutral (**A**) and alkaline (**B**) gel analysis of the synthesome-mediated in vitro SV40 DNA replication products formed in the absence or presence of increasing concentrations of ara-CTP. The DNA replication products formed in the in vitro DNA replication reaction were isolated by phenol/chloroform extraction, followed by precipitation with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA and electrophoresed through 1% agarose gels containing either TBE (**A**) (90 m*M* Tris/ 90 m*M* boric acid/2 m*M* EDTA) or alkaline (**B**) (50 m*M* NaOH/ 1 m*M* EDTA) buffers. Gels were dried and exposed to Kodak XAR-5 film at −80 °C for autoradiographic analysis of the resolved DNA replication products

breast cancer cells (MDA MB-468) was fully competent to produce newly replicated form I (superhelical) DNA, form II (nicked open circular) DNA, as well as higher order topological intermediates. The production of these DNA replication products was dependent on the presence of T-antigen (Fig. 4A,B; compare lanes 1 and 2).

Full-length daughter DNA molecules were obtained in the presence of low concentrations of ara-CTP (1 and 10 µ*M*; Fig. 4A,B, lanes 3 and 4) while at higher concentrations, there was an inhibition of full-length daughter DNA synthesis (Fig. 4A, lanes 5–8; Fig. 4B, lanes 5–7). These results are in accordance with our previous results using the DNA synthesome isolated from HeLa cells [41]. Our results also showed that specifically the initiation phase of DNA synthesis was inhibited by ara-CTP since the production of the short Okazaki fragments was suppressed at concentrations of the drug exceeding 10 µ*M* (Fig. 4A,B).

The synthesome-associated DNA polymerase α is sensitive to Ara-CTP while DNA polymerase δ is resistant to the inhibitory effect of that drug

Studies were initiated to determine the effect of ara-CTP on the activities of synthesome-associated DNA polymerases α and δ. Enzyme assays were carried out in the absence or presence of increasing concentrations of ara-CTP (Materials and methods). The concentration of ara-CTP required to inhibit 50% of the activity of the synthesome-associated DNA polymerase α was approximately 40 µ*M* (Fig. 5). This concentration was comparable to that required to effectively inhibit the in vitro SV40 DNA replication reaction (65 µ*M*; Fig. 3). Our results also clearly demonstrated that the synthesome-associated DNA polymerase δ seems to be resis-

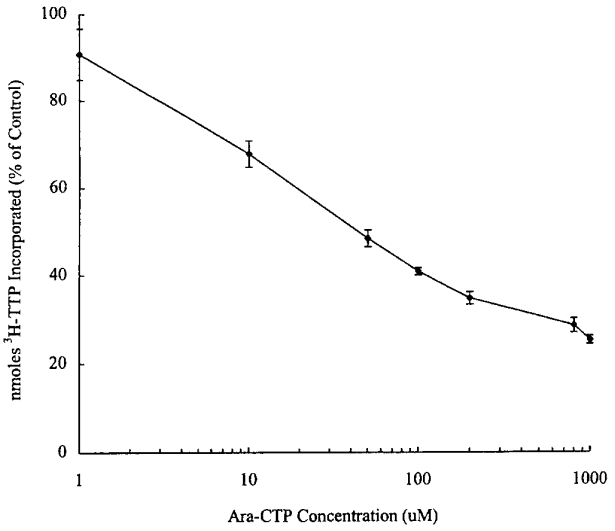

Fig. 5 Effect of different concentrations of ara-CTP on the synthesome-associated DNA polymerase α activity. The reaction was started by incubating the reaction mixture (20 µl) in the absence or presence of increasing concentrations of ara-CTP (1, 10, 50, 100, 200, 800, and 1000 µM) for 1 h at 37 °C. The reaction mixture was spotted onto DE81 filters, and the amount of ^{3}H-TTP retained on the filters was determined as described by Sambrook et al. [37]. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP

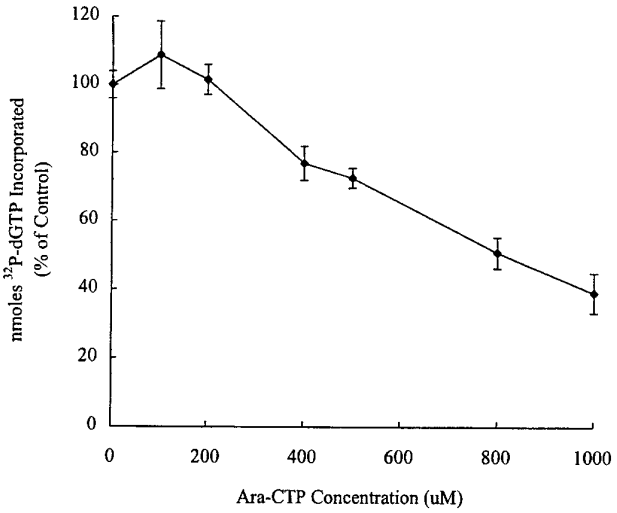

Fig. 6 Effect of ara-CTP on the synthesome-associated DNA polymerase δ activity. The assay was performed as described in the text and the amount of ^{32}P-dGTP incorporated into the DNA template was determined by binding to Whatman DE81 filters [37]. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP

tant to the inhibitory action of that drug (Fig. 6). These results are in agreement with those obtained by Han et al. [16] using the DNA synthesome isolated from HeLa and MCF-7 cells. The close correlation between the IC_{50} for ara-CTP-mediated inhibition of the synthesome-driven in vitro SV40 DNA replication reaction and that required to inhibit the synthesome-associated DNA polymerase α activity indicates that ara-C's action on DNA replication is mediated primarily through its inhibition of DNA polymerase α and suggests that this enzyme plays a key role during the initiation of DNA synthesis and Okazaki fragment formation.

Discussion

Ara-C is one of the most effective nucleoside analogues used in the treatment of acute myelogenous leukemia [8, 22, 32]. Ara-C, a deoxycytidine analogue, must be activated in the cell by the phosphorylation of the nucleoside to the monophosphate form (ara-CMP). This phosphorylation is catalyzed by the enzyme deoxycytidine kinase. Ara-CMP is then activated by conversion to the triphosphate form (i.e. ara-CTP) which is the biologically active form of the drug [6]. Ara-C inhibits cell proliferation and DNA replication in mammalian cells [13]. Yet despite a series of extensive studies, the mechanisms by which ara-C exerts its cytotoxic effect remain to be fully elucidated. Several hypotheses have been proposed to explain the inhibitory effect of ara-C on DNA replication. Previous studies have indicated that ara-CTP competes with dCTP for the binding sites on DNA polymerase α, inhibiting its enzymatic activity [11, 14]. However, it has been found that incorporation of ara-C into the DNA is the major mechanism by which the drug exerts its lethal effect [24, 27, 44] and that there is a strong correlation between the incorporation of ara-C into DNA and the loss of clonogenic potential [27]. Incorporation of ara-C into DNA has several effects. Fridland [9, 10] and Bell and Fridland [3] have demonstrated that incorporation of ara-C into DNA inhibits the initiation of new DNA synthesis. Other studies [33–35], however, have shown that the presence of ara-CMP at the 3′ terminus of DNA interferes with the elongation of a replicating DNA strand and not the initiation of synthesis.

Using the purified human DNA synthesome which supports in vitro SV40 origin-specific DNA replication in the presence of the viral large T-antigen, we have found that the concentration of ara-C required to inhibit intact human breast cancer cell DNA synthesis, for the cell line MDA MB-468, was comparable to that required for the inhibition of synthesome-mediated in vitro SV40 DNA replication. The reason the concentration of ara-C required to inhibit intact cell DNA synthesis (115 μ*M*) is larger than the concentration required to inhibit in vitro DNA synthesis (65 μ*M*) is most likely due to one or more of the following factors. First, a larger dose of ara-C may be required than is needed to directly inhibit the purified DNA synthesome because ara-C must be transported across the cell membrane. Second, a higher concentration of ara-C may be required to compensate for that portion of drug that is inactivated by degradative enzymes (such as deoxycytidine deaminase). Third, the effective cellular concentration may be lower than the extracellular concentration of ara-C since the drug is readily exported from within the cell. Fourth, a larger dose of ara-C may be required for the intact cell study because ara-C must be converted to its active metabolite (ara-CTP) in order to produce its cytotoxic effect. Therefore, the concentration of the active metabolite that reaches the target site (i.e. the DNA synthetic apparatus in the nucleus) is probably less than that of the drug placed in the medium. However, these factors are not of consequence to the in vitro studies since a direct effect of the active metabolite of the drug (ara-CTP) on DNA synthesis was measured. Our results are therefore in agreement with those of a previous study in our laboratory examining the DNA synthesome isolated from HeLa cells [28].

The IC_{50} value of ara-C for inhibition of MDA MB-468 cell proliferation was found to be much lower than that for inhibition of either intact cell DNA synthesis or in vitro DNA synthesis (1 μ*M* compared to 115 or 65 μ*M*, respectively). Ara-C's mechanism of action includes not only inhibition of DNA polymerase and incorporation into DNA but also the generation of reactive oxygen intermediates [19], endoreduplication [42], alteration of the concentration of intracellular lipid second messengers such as ceramide [40], which is a potent inducer of apoptosis, and damaging the genomic DNA [15, 21]. Therefore, it is clear that the effect of ara-C on cell growth involves several mechanisms that collectively lead to inhibition of cell proliferation.

In our current study, the synthesome-mediated in vitro SV40 DNA replication reaction was performed in the presence of increasing concentrations of ara-CTP, and the types of daughter DNA molecules were analyzed using neutral and alkaline agarose gel electrophoresis. Our results clearly demonstrated that full-length daughter DNA molecules were produced in the presence of low concentrations of ara-CTP; however, at higher concentrations, the drug suppressed the formation of daughter DNA synthesis. Moreover, our results show that the initiation phase of DNA synthesis was specifically inhibited by ara-CTP since the production of short Okazaki fragments was suppressed at concentrations of the drug exceeding 10 μM. These results are consistent with those reported by Wills et al. [41] who found that the DNA synthesome isolated from HeLa cells was able to incorporate ara-CMP into internucleotide linkages, and to add deoxynucleotides at the 3′ terminus of a DNA strand containing incorporated ara-CMP, leading to the production of full-length daughter DNA molecules. Recently, Gmeiner et al. [12] studied the effect of ara-C substitution on the structural and thermodynamic properties of a model Okazaki fragment and found that the drug inhibits lagging strand DNA synthesis by destabilization of the interaction between the nascent DNA and the DNA template being replicated on the lagging strand of the replication fork.

Using highly specific antibodies recognizing DNA polymerases α or δ, Han et al. [16] demonstrated that the assay conditions for the synthesome-associated DNA polymerases α and δ were very specific for each enzyme and permitted the activity of each DNA polymerase to be measured while it was in the presence of the other polymerase (i.e., in the DNA synthesome). Our results have shown that ara-CTP preferentially inhibited the activity of the synthesome-associated DNA polymerase α enzyme, while the DNA polymerase δ appeared to be resistant to the inhibitory action of that drug. This result is in agreement with that of Han et al. [16] using the human cell DNA synthesome isolated from HeLa and MCF-7 cells. Our results indicate that the action of ara-C on DNA replication is mediated primarily through DNA polymerase α, and suggests that this enzyme plays a key role during the initiation of DNA synthesis and Okazaki fragments formation.

Taken together, our results suggest that the DNA synthesome can be a highly effective, powerful, and unique in vitro model system for studying the mechanism of action of ara-C and other nucleoside analogues that directly inhibit the DNA replication process.

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Title: DNA Replication is Mediated by a Discrete Multiprotein Complex

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SUMMARY

A discrete high molecular weight multiprotein complex containing DNA polymerase α has been identified by a native Western blotting technique. An enrichment of this complex was seen at each step in its purification. Further purification of this complex by ion-exchange chromatography indicates that the peak of DNA polymerase α activity co-purifies with the peak of *in vitro* SV40 DNA replication activity eluting from the column. The complex has a sedimentation coefficient of 18S in sucrose density gradients. We have designated this complex the DNA synthesome. We further purified the DNA synthesome by electroeluting this complex from a native polyacrylamide gel. The eluted complex retains *in vitro* DNA synthetic activity and, by Western blot analysis, contains DNA polymerase δ, proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A). Enzymatic analysis of the electroeluted DNA synthesome indicates that the synthesome contains topoisomerse I and II activities, and SDS-PAGE analysis of the electroeluted DNA synthesome revealed the presence of at least 25 major polypeptides with molecular weights ranging from 20 kD to 240 kD. Taken together, our evidence suggests that the DNA synthesome represents the fundamental DNA replication unit of the human cell.

INTRODUCTION

Mammalian cell DNA replication is an intricate and highly efficient process requiring the coordinated activity of a variety of enzymes and accessory proteins. The mechanism by which these proteins carried out DNA replication defines an important regulatory process capable of modulating cell proliferation. However, despite considerable effort from a variety of laboratories examining the replication process, the precise mechanism involved in coordinating the activity of these proteins is not fully understood. The elucidation of the role played by the human DNA replication apparatus, and its components in regulating the DNA synthetic process, is crucial to our understanding of both normal and aberrant cell proliferation. What is clear is that a minimum number of proteins are needed to drive a mammalian DNA replication fork. Several of these proteins have been identified using the simian virus (SV40) based *in vitro* DNA replication assay system (1, 2). These proteins include DNA polymerases α and δ, DNA topoisomerases I and II, DNA primase, DNA ligase I, proliferating cell nuclear antigen (PCNA)[1], replication factor C (RF-C), replication protein A (RP-A), and the nucleases RNase H1 and FEN1/RTH1. Increasing evidence has supported the concept that many of the enzymes and factors involved in the replication of mammalian DNA function together as an organized multiprotein complex (1-5). We have previously reported that a DNA replication-competent multiprotein form of DNA polymerase can be isolated from a variety of mammalian cell lines and tissues (6-10). The multiprotein form of DNA polymerase was purified from cells using a series of purification steps, which included differential centrifugation, polyethylene glycol (PEG) precipitation, ion-exchange chromatography and sucrose density gradient sedimentation. The sedimentation coefficient of the human cell replication-competent multiprotein form of DNA polymerase is 18S, as determined by sucrose gradient analysis (8-10). The integrity of the multiprotein form of

DNA polymerase was maintained after treatment with detergents, salt, RNase, DNase, ion-exchange chromatography using DEAE-cellulose (Whatman) or Q-Sepharose (Pharmacia), and following sedimentation through sucrose and glycerol density gradients. Our observations suggest that the ready co-purification of the proteins with one another was independent of non-specific interaction with other cellular macromolecular components (6, 8-10). Using enzymatic assays to functionally define the presence of specific components of this multiprotein complex, along with denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses, we demonstrated that DNA polymerases α, δ and ε, DNA ligase I, topoisomerases I and II, DNA primase, PCNA, RF-C, RP-A and DNA helicase co-purify with the replication-competent multiprotein form of DNA polymerase (6-10). Most importantly, we demonstrated that this 18S form of the DNA polymerase was fully competent to support SV40 origin-specific large T-antigen-dependent DNA replication *in vitro* (6, 8-10). Since replication of the SV40 genome is dependent on its host's DNA replication apparatus, our observation suggests that the multiprotein form of DNA polymerase must also play a role in human cell DNA replication.

Our observations led us to propose that the replication-competent multiprotein form of DNA polymerase represented an isolated multiprotein DNA replication complex (MRC) (6, 8). A model was proposed for this complex that was based on the observed fractionation, chromatography and density gradient sedimentation profiles for the proteins that co-purify with the replication-competent multiprotein complex (8-10). Recent co-immunoprecipitation studies support the model, and suggest that DNA polymerases α and δ, DNA primase, and RF-C, as well as PCNA, tightly associate with one another (11). Several other synthesome components were found to interact via direct contact with only PCNA or DNA polymerase α (11).

Reports of studies utilizing native polyacrylamide gel electrophoresis (PAGE) to identify high molecular weight protein species with associated DNA polymerase activity (3-5) are consistent with the study reported here. In this report, we present data from our non-denaturing polyacrylamide gel electrophoretic (native PAGE) analysis of the multiprotein DNA replication complex, which we have termed the DNA synthesome (9, 10). Our data indicates that the highly purified form of the synthesome is a singular and discrete high molecular weight protein species that supports large T-antigen SV40 origin-dependent DNA replication *in vitro*. Our results demonstrate that the DNA synthesome is the first discrete, fully functional mammalian cell multiprotein complex that is fully competent to support all phases of the SV40 origin dependent DNA replication process *in vitro*. Our data further suggests that the DNA synthesome may be the intact DNA synthetic apparatus of human cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Suspension cultures of MCF7 cells were grown in Joklik's modified Eagle's medium supplemented with 10% fetal bovine serum. Exponentially growing ($5x10^5$ cells/ml) cells were harvested and washed three times with phosphate-buffered saline (PBS). The cells were then pelleted and stored at -80°C prior to initiating synthesome isolation.

Subcellular fractionation—MCF7 cells were fractionated to the level of the previously described sucrose interface protein fraction (P4) using our published procedures (6) and as outlined in Fig. 1.

Low pressure liquid chromatography (LPLC)—P4 fraction (5 ml) containing approximately 30 mg of protein were loaded onto a *Bio-Rad* Q5 column pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1% glycerol and 1 mM EDTA). The column was then washed with buffer A and eluted using a gradient of 50-500 mM KCl in buffer A. One-ml fractions were collected. The eluted fractions were then dialyzed into buffer B (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT), and aliquots of each fraction were stored at -80°C.

Sucrose gradient sedimentation—A 10 ml continuous 10-30% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM KCl and 1 mM EDTA was formed using a Sorvall Gradient Maker. Approximately 1 mg of the pooled LPLC eluted fractions (fractions 27 and 28) containing the peak of the DNA polymerase α and *in vitro* SV40 DNA replication activities was loaded onto the gradient. The gradient was then centrifuged at 100,000 xg for 16 hours at a temperature of 4°C. After centrifugation, 1 ml fractions of the gradient were collected. The collected fractions were then dialyzed for 2 hours against two changes of buffer B. The dialyzed fractions were then stored at -80°C. Marker proteins having defined sedimentation coefficient

values were centrifuged alongside the gradients containing the LPLC fraction. The proteins alcohol dehydrogenase (7S) and thyroglobulin (19S) served as marker proteins. As described previously in Malkas *et al.* (6), the positions of these proteins in the density gradients were used to estimate the sedimentation coefficient of the replication-competent form of DNA polymerase α.

Electroelution of the multiprotein form of human cell DNA polymerase from preparative native polyacrylamide gels—Four percent native polyacrylamide gels containing a 3.5% stacking gel were prepared using the *Bio-Rad* mini-Protean II gel apparatus, as previously described (5). One milligram of pooled LPLC protein fraction was loaded onto the gel. Electrophoresis was initially started at 50 volts until the dye front entered the 4% separating PAGE gel, at which time the voltage was increased to 90 volts. Electrophoresis was continued until the dye front reached the bottom of the gel. Following electrophoresis, the 4% gel was trimmed to fit onto the *Bio-Rad* Mini Whole Gel Eluter, as described by the manufacturer. The gel was then soaked in 20 mM HEPES, pH 7.5, for 10 minutes and layered onto the elution chamber core. The Whole Gel Eluter was assembled according to the manufacturer's instructions, and elution of the resolved synthesome protein fraction from the 4% PAGE gel was performed as described in the *Bio-Rad* protocol provided with the apparatus. HEPES (20 mM, pH 7.5) was used as the elution buffer in these procedures. The electroelution was initiated at 60 mA for one hour, and then continued at 30 mA for an additional 2 hours. The proteins bound to the cellophane membrane at the end of the elution were removed by reversing the polarity of the electroeluter cell, and continuing electrophoresis for 30 seconds at 100 volts. The eluted fractions were harvested, with each fraction containing approximately 500 µl of liquid. The fractions were stored at -80°C until they were analyzed.

Western blot analysis following denaturing or native polyacrylamide gel electrophoresis— Either 8% or 4-20% linear gradient denaturing SDS polyacrylamide gels (SDS-PAGE) were used to resolve the individual polypeptides associated with the discrete high molecular weight form of DNA polymerase α purified by native PAGE (8). Native PAGE was carried out essentially as described (5), with SDS being excluded from the gel, loading buffer, electrophoresis buffer and transfer buffer. The resolved polypeptides were visualized either by silver staining (using a Silver Stain Plus kit purchased from *Bio-Rad*, CA, USA), or electrophoretically transferred to a nitrocellulose filter membrane for 18 hours at a temperature of 4°C and a voltage setting of 12 volts. The filter membranes from the SDS-PAGE analysis were then incubated with antibodies recognizing DNA polymerase δ, RP-A, PCNA or other proteins. The filter membrane prepared following native PAGE analysis of the *Bio-Rad* Q5 column purified complex was incubated with murine monoclonal antibody, SJK 132-20 (12), in order to specifically identify DNA polymerase α as a component of the chromatographically purified DNA synthesome. The filter membranes were then incubated with anti-mouse secondary antibody conjugated to horseradish peroxide, and the position of the specific proteins reacting with the primary antibodies (or the protein complex), was detected using a light-enhanced chemiluminescence system (Amersham).

Enzyme assays—DNA polymerase α activity was assayed according to published procedures (6) using an activated calf thymus DNA template (Sigma Co.). *—DNA polymerase δ activity* was detected essentially as described by Han *et al.* (to be published elsewhere). The 50 µl assay mixture contained 50 ng poly(dA)/oligo(dT) (20:1); 0.25 µl [α-^{32}P]dTTP; 2.5 µl core buffer (containing 10 mM $MgCl_2$; 10 µM TTP; 25 mM HEPES, pH 5.9; 200 µg/ml bovine serum albumin; 5% glycerol) and 30 µl of protein fraction. The reaction was carried out at 37°C for 15 minutes, and the whole reaction mixture was then spotted onto Whatman DE81 filters. The

filters were processed to quantify the amount of radiolabeled nucleotide incorporated into DNA template (13). —*In vitro SV40 DNA replication assay* was performed essentially as described in Malkas *et al.* (6) using a 25 µl assay volume. —*Analysis of in vitro SV40 DNA replication products*: The *in vitro* DNA replication reaction was performed at 37°C for 4 hours, and stopped by adding EDTA and SDS to a final concentration of 25 mM and 10%, respectively. The reaction mixture was then added to tubes containing 100 µg of yeast RNA in 1% SDS, and the mixture was subjected to proteolytic digestion for 1 hour at 37°C with 2 µg of proteinase K. DNA replication products formed in the assay were then isolated by extracting the digestion mixture twice with phenol/chloroform/isoamyl alcohol and one time with chloroform/isoamyl alcohol. The extracted DNA was then precipitated in the presence of 2 M ammonium acetate with 2-propanol and the DNA pellet collected by centrifugation. The pellet was finally resuspended in 10 mM Tris/1mM EDTA, and the reaction products were analyzed using a 1% alkaline agarose gel containing 50 mM NaOH/1 mM EDTA. The gels were dried, and autoradiograms of the dried gels were produced by exposing the dried gels to Kodak film at -80°C. —*Topoisomerase I activity* was measured using a commercially available kit and the instructions supplied by the manufacturer (TopoGene, Ohio, USA). —*Topoisomerase II activity* was determined using a commercially available assay kit (TopoGene, Ohio, USA).

RESULTS

Enrichment of a discrete high molecular weight form of DNA polymerase α during subcellular fractionation and purification of the human cell DNA synthesome—We previously demonstrated that a readily sedimentable form of DNA polymerase α, having a uniform sedimentation coefficient of 18S in sucrose density gradients, could be isolated from human cells (6, 8-10). Only the 18S form of DNA polymerase α was observed to be able to fully support large T-antigen dependent *in vitro* replication of SV40 origin containing DNA (6, 8-10). It was proposed that this replication-competent 18S form of the DNA polymerase represented a multiprotein DNA replication complex (designated the DNA synthesome), that could potentially function as an organized DNA synthetic apparatus in intact cells (1, 2, 6, 8-10). To begin to evaluate whether the replication-competent synthesome was a discrete multiprotein species, a methodology relying on native PAGE was developed for this analysis (5).

In this current report, non-denaturing (native) PAGE was used to determine whether we were enriching the abundance of a discrete high molecular weight form of DNA polymerase α using the subcellular fractionation procedure developed to achieve the partial purification of the DNA synthesome. MCF7 cell homogenates were fractionated according to the protocol outlined in Fig. 1, and the DNA synthesome was initially purified to the level of the DNA replication-competent P4 protein fraction, as described previously (6, 8-10). In those reports we demonstrated that the replication-competent P4 protein fraction contained a form of the human cell DNA polymerase α that had a sedimentation coefficient of 18S, while the replication-deficient S4 fraction lacked this form of the DNA polymerase (8-10). To determine whether this 18S form of the DNA polymerase present in the P4 was a discrete complex, we resolved 40 micrograms of each of the fractions obtained during the purification of the homogenate (H) (i.e.,

the S1, S2, S3, NE, NE/S3 and P4 fractions), using native PAGE. The resolved protein species were electrophoretically transferred to a nitrocellulose filter membrane (*Experimental Procedures*), and the nitrocellulose filter membrane was incubated with the antibody SJK 132-20 (12), which specifically recognizes human cell DNA polymerase α. The results of these Western blot analyses are shown in Fig. 2. A discrete high molecular weight form of DNA polymerase α with a consistent relative mobility just below that of thyroglobulin was identified in each fraction. There was an enrichment of this form of DNA polymerase α as a function of synthesome purification from the H through the P4 fraction. Our observation of the increasing abundance of the high molecular weight form of DNA polymerase α with each purification step correlated with the previously observed increase in the specific activity for synthesome-mediated T-antigen dependent *in vitro* SV40 DNA replication associated with each of these purification steps (6, 8).

The integrity of the high molecular weight form of DNA polymerase α was maintained following low pressure liquid chromatograph—Prior to this current report we demonstrated that both the 18S form of DNA polymerase and *in vitro* SV40 origin specific DNA replication activity were present in the human cell derived P4 fraction, and that these activities co-purified with one another following chromatography of the P4 fraction on the anion exchange resin Q-Sepharose (Pharmacia) (6, 8-10). We now report that the integrity of the high molecular weight form of DNA polymerase α, revealed by native PAGE, was also maintained following ion exchange chromatography. To demonstrate this, the P4 protein fraction was subjected to low pressure liquid chromatography (LPLC) on a *Bio-Rad* Q5 column as described in the *Experimental Procedures*. The fractions eluted from the columns were then assayed for both DNA polymerase α and *in vitro* SV40 DNA replication activity (*Experimental Procedures*). As observed

previously, the major peak of *in vitro* SV40 DNA replication activity (fractions 27 and 28) eluted from the column (Fig. 3*A*) exactly coincided with the peak of DNA polymerase α activity eluting from the column (Fig. 3*B*). The column fractions containing the highest levels of the co-purifying *in vitro* DNA replication and DNA polymerase α activities were pooled, as were column fractions surrounding the peak containing these activities. The protein species present in 20 μl of each of the pooled fractions were resolved by native PAGE using a 4% polyacrylamide gel, and the resolved protein species were electrophoretically transferred to a nitrocellulose filter membrane (*Experimental Procedures*). The nitrocellulose filter membrane was then incubated with the anti-DNA polymerase α antibody, SJK 132-20. It was observed that, relative to the P4 fraction, the high molecular weight form of DNA polymerase α was significantly enriched in the pooled column fractions containing the peak of co-eluting *in vitro* DNA replication and DNA polymerase α activities (Fig. 3*C*). Our data indicate that the integrity of the discrete high molecular weight form of DNA polymerase α was maintained after low pressure liquid chromatography. Our results also suggested a clear correlation between the ability of the chromatographically purified P4 fraction to support *in vitro* replication of SV40 origin containing DNA and the presence of the discrete high molecular weight form of DNA polymerase α in the replication-competent fraction(s).

The discrete high molecular weight form of DNA polymerase α has a sedimentation coefficient of 18S—The replication-competent multiprotein form of the human DNA polymerase α has been previously shown to have a sedimentation coefficient of 18S in sucrose density gradients (8, 10). We now attempted to determine the sedimentation coefficient of the discrete high molecular weight form of the DNA polymerase α eluted from LPLC. The pooled *Bio-Rad* Q5 column eluted fractions (fractions 27 and 28) containing the peak of the DNA polymerase α and *in vitro*

SV40 origin-specific DNA replication activities (Fig. 3*A* and 3*B*), as well as the high molecular weight form of the DNA polymerase α (Fig. 3*C*), were subjected to sucrose density gradient analysis as described in the *Experimental Procedures*. It was observed that the discrete high molecular weight form of DNA polymerase α present in chromatographic fractions 27 and 28 had a sedimentation coefficient of 18S (Fig. 4). This sedimentation coefficient correlated exactly with that determined previously for the replication-competent multiprotein form of this DNA polymerase (8-10). Our result suggested that the discrete high molecular weight form of DNA polymerase α identified by native PAGE analysis could, in fact, be the previously described 18S replication-competent DNA polymerase termed the DNA synthesome (8-10).

The discrete high molecular weight form of DNA polymerase α was stable to electroelution procedures following native PAGE resolution—In order to attempt to establish a relationship between the previously described 18S replication-competent DNA synthesome (8-10) and the discrete high molecular weight form of the DNA polymerase α identified using native PAGE (Fig. 2, 3*C* and 4), it was necessary to extract the high molecular weight DNA polymerase α protein species from the native gel. This was accomplished by the use of an electroelution method devised to extract the replication competent form of the DNA polymerase α from the 4% non-denaturing polyacrylamide gel (*Experimental Procedures*). For this study the pooled protein fraction derived from LPLC (Fig. 3*A* and 3*B*; fractions 27 and 28) of an MCF7 cell P4 fraction containing both the major peak of DNA polymerase α and *in vitro* SV40 DNA replication activities was subjected to native PAGE (*Experimental Procedures*). The native 4% polyacrylamide gel containing the resolved protein fraction was eluted using a *Bio-Rad* Whole Gel Eluter, and individual electroelution fractions were collected as described in the *Experimental Procedures*. The electroelution fractions found to contain significant levels of

protein (fractions 5, 9, 11 and 13) were resolved again by native PAGE followed by silver staining the gel. Fig. 5*A* shows the different protein species resolved by the native gel. The protein species observed in lane 1 of the gel had a relative mobility consistent with that obtained for the discrete, replication-competent, high molecular weight form of DNA polymerase α (compare *lane 1* with *lane 5*).

To verify that the protein species eluted in fraction 5 from the native polyacrylamide gel (Fig. 5*A*, *lane 1*), was in fact the discrete high molecular weight form of DNA polymerase α, this electroeluted fraction was analyzed by native PAGE along with a P4 fraction, and the resolved protein species were transferred electrophoretically to a nitrocellulose filter membrane (*Experimental Procedures*). The nitrocellulose filter membrane was then subjected to Western blot analysis using anti-DNA polymerase α. It was observed that the electroeluted fraction (fraction 5), contained the discrete high molecular weight form of DNA polymerase α (Fig. 5*B*) found to be present in the P4 fraction (Fig. 2).

The native PAGE purified high molecular weight form of DNA polymerase α contains in vitro DNA synthetic activity—The high molecular weight form of DNA polymerase α that was present in fraction 5 of the electroelution step (described above) was evaluated for both its ability to support T-antigen dependent DNA replication *in vitro* and for the presence of specific replication essential proteins. Each of the above-described electroeluted fractions from the native polyacrylamide gel were assayed for *in vitro* SV40 DNA replication activity. It was observed that the major peak of *in vitro* replication activity was present in electroelution fraction 5 (Fig. 6*A*). Our analysis of fraction 5 indicated that the level of *in vitro* DNA synthetic activity supported by this fraction was significantly enriched over the levels supported by the various replication-competent fractions obtained at earlier steps in the purification. Fraction 5 had a

specific activity for T-antigen-dependent *in vitro* DNA replication that was approximately 3696-fold higher than that observed in MCF7 cell homogenates (Table I). The ability of the high molecular weight form of DNA polymerase α to produce full-length daughter DNA molecules is shown in Fig. 6*B*. Fig. 6*B* also demonstrates that the *in vitro* replication reaction supported by the electroeluted synthesome (fraction 5) was T-antigen dependent.

The assay component requirements for *in vitro* SV40 DNA replication activity supported by the highly purified high molecular weight form of DNA polymerase (i.e., the DNA synthesome) present in electroelution fraction 5 are shown in Table II. It was observed that synthesome-driven *in vitro* SV40 DNA replication activity required the large T-antigen protein as well as an SV40 replication origin containing DNA. The replication reaction was dependent on the presence of both ribonucleotides and deoxyribonucleotides, Mg^{2+}, and an ATP regenerating system. The inhibition of DNA synthetic activity by antibodies directed against either the DNA polymerase α polypeptide or the DNA polymerase δ accessory protein PCNA indicates that both DNA polymerase α and δ mediate synthesome-driven *in vitro* SV40 DNA replication.

The discrete high molecular weight form of DNA polymerase (i.e., the DNA synthesome) contains DNA replication essential proteins—SDS-PAGE analyses of the electroeluted DNA synthesome revealed the presence of approximately 25 polypeptides staining with silver and spanning a wide range of molecular weights (Fig. 7). The highly purified DNA synthesome was also examined for the presence of DNA replication essential proteins using Western blot analyses and specific enzymatic activities. It was observed that the replication-competent synthesome (electroelution fraction 5) contained enzymatic activities for DNA polymerase α and δ as well as topoisomerases I and II (Fig. 8*A-D*). Our data indicate that these protein activities

co-purify with one another and with *in vitro* DNA synthetic activity. Western blot analyses of the electroeluate fraction 5 also revealed the presence of PCNA, RP-A and the 125kDa polypeptide of DNA polymerase δ in this fraction (Fig. 9). Thus our results indicate that the PCNA and RP-A proteins also co-purify with the enzymatic activities reported in Fig. 8.

DISCUSSION

Since the development of the *in vitro* SV40 DNA replication model system by Li and Kelly (14), progress has been rapid in delineating the minimal number of enzymes and factors required for the synthesis of mammalian DNA. The majority of studies have utilized partially purified enzymes and factors to reconstitute DNA synthetic activity (15, 16). Although some data exist on the functional association of several enzymes and factors (1, 2), the precise molecular interactions that occur between the proteins required to support DNA replication remain largely undefined. The ability to reconstitute DNA replication activity utilizing individual components does not exclude the existence of large multiprotein complexes containing many or all of the proteins that function *in vivo* to replicate DNA.

Although previous studies have reported the existence of multiprotein complexes that can participate in mammalian cell DNA replication (reviewed in 1, 2), we report the purification of a functional and discrete multiprotein complex, which we have termed the DNA synthesome. Our results demonstrate that the DNA synthesome is a functional multiprotein complex that is fully competent to participate in all of the reactions required for large T-antigen-dependent *in vitro* replication of SV40 origin containing DNA, and potentially the replication of genomic DNA in mammalian cells.

In addition to MCF7 cells, we have also identified this high molecular weight complex in the HeLa cell line (5), the human leukemia cell line HL-60 (10), human esophageal cells (our unpublished data), and a variety of other types of human cells and tissues. Therefore, our observations indicate that the presence of this complex is not specific to MCF7 cells. Since antibodies specific to all of the individual replication proteins assumed to be present in the DNA synthesome were not available for immunoblot analyses of non-denaturing polyacrylamide gels,

we are unable to confirm the identity of all of the proteins associated with this complex. However, experiments in our laboratory are underway to identify those individual proteins shown on the SDS-silver stain gel using a combination of mass spectroscopy and protein micro-sequencing.

The actual molecular mass of this complex cannot be estimated by native PAGE, because migration of the complex is not simply dependent on its molecular weight, but also on its shape, and net charge of the complex, and on the pH of the electrophoresis buffer system. The identification and stoichiometry of each individual protein component of the synthesome will assist in defining the actual mass of this high molecular weight complex. Taken together, the correlation between enrichment in the abundance and activity of individual components of the DNA synthesome throughout its purification (as well as the enhancement in the inherent *in vitro* SV40 DNA replication activity of the complex), suggests that the DNA synthesome exists as a discrete entity within the cell, and that use of the synthesome driven assay system may enable us to accurately model, *in vitro*, many of the events that occur during intact mammalian cell DNA synthesis. We therefore propose that this high molecular weight DNA polymerase α containing complex, the DNA synthesome, represents a discrete functional unit of the cellular DNA replication machinery. With respect to the regulation of the activity of the DNA synthesome, we have shown that this high molecular weight DNA polymerase α containing complex appeared to disassemble in terminally differentiated HL-60 cells (10), while in actively cycling cells this complex stays together throughout the cell cycle. The data of Lin *et al.* (10) suggest that the DNA synthesome is not assembled just prior to or during S phase and then disassembled just after S phase, but rather is disassembled only when the cells permanently leave the cell cycle. Our demonstration that a discrete complex can be isolated and purified from asynchronous

cultures of proliferating cells is consistent with this suggestion. Furthermore, the ability of the complex to support all of the reactions needed to replicate SV40 origin containing DNA *in vitro*, throughout each step of its purification, suggests that the interactions between the components of the DNA synthesome are specific and of sufficiently high affinity so as to maintain the replicative function of the complex.

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FOOTNOTES

The abbreviations used are: PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; MRC, multiprotein replication complex; LPLC, low pressure liquid chromatograph; PBS, phosphate buffered saline; DTT, dithiothreitol; SV40, simian virus 40; BSA, bovine serum albumin; ROC, relaxed open circular; SC, super coiled; CCC, covalently closed circular; Tag, SV40 large T antigen.

FIGURE LEGENDS

FIG. 1. Flow diagram of the subcellular fractionation scheme used to partially purify the DNA synthesome from MCF7 cells.

FIG. 2. Native Western blot analysis of various protein fractions obtained at different stages in the purification of the human cell DNA synthesome. Forty micrograms of protein from each purification step of the subcellular fractionation used to purify the DNA synthesome (Fig. 1), were resolved by native-PAGE using a 4% polyacrylamide gel. The resolved proteins were transferred to a nitrocellulose filter membrane, and the membrane was incubated with the murine monoclonal antibody SJK 132-20 (*Experimental Procedures*). The relative positions of specific molecular markers (thyroglobulin, 669 kDa), bovine serum albumin (BSA) trimer (198 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) resolved in the native gel system are indicated on the Fig.

FIG. 3. Enzymatic and Western blot analyses of the *Bio-Rad* Q5 chromatographic fractions for DNA polymerase α and *in vitro* SV40 replication activities, and DNA polymerase α polypeptide. The *Bio-Rad* Q5 column LPLC elution conditions were described in *Experimental Procedures*. Three microliters of each pooled fraction were assayed for ***A. in vitro* SV40 DNA replication** and ***B.* DNA polymerase α activities**, as described in the *Experimental Procedures*. One unit of DNA polymerase α activity was defined as 1 nM of total [α-^{32}P]dNMP incorporated per hour at 37°C into an activated calf thymus DNA template. One unit of *in vitro* SV40 DNA synthesis activity was defined as the incorporation of 1 nM of total [α-^{32}P]dNMP into an SV40 origin-containing DNA per hour at 37°C. ***C.* Native Western blot analysis of *Bio-Rad* Q5**

eluted fractions. *Bio-Rad* Q5 column eluted fractions were pooled and 20 μl of each of the pooled fractions were resolved using 4% native gel PAGE and the resolved proteins transferred to a nitrocellulose filter membrane. The membrane was incubated with the murine monoclonal antibody SJK 132-20, which specifically recognizes DNA polymerase α (*Experimental Procedures*). The relative positions of specific molecular standards are indicated alongside the scan of the gel.

FIG. 4. Native Western blot analysis of various protein fractions obtained following sucrose gradient velocity sedimentation of the *Bio-Rad* Q5 column eluate. Forty microliters of each of the sucrose gradient fractions and 40 μg of Q5 column elute (pooled fractions 27 and 28) were resolved using native PAGE (4% gel), and the resolved proteins were transferred to a nitrocellulose filter membrane. The membrane was incubated with the murine monoclonal antibody SJK 132-20, (*Experimental Procedures*). Numbers at the top of the figure represent sucrose gradient fractions. The relative positions of specific molecular standards are listed alongside the figure.

FIG. 5. Silver stain and Western blot analyses following non-denaturing PAGE of the electroeluted fractions. *A.* Silver stain of resolved protein species following non-denaturing PAGE of electroeluted fraction 5. Fifty microliters of each of the collected electroeluted fractions were resolved by native PAGE using 4% gels, and the resolved proteins were stained with silver stain (*Experimental Procedures*). *Lanes 1-4* contain electroeluted fractions 5, 9, 11, and 13. One microgram of pooled Q5 column eluted fractions 27 and 28 (Fig. 3) was used for comparison of the relative mobility of the high molecular mass complexes. ***B.***

Western blot analysis following non-denaturing PAGE of electroeluted fraction 5. One microgram of electroeluted fraction 5 (denoted as E in the Fig.) and 40 μg of P4 (Fig. 2) were resolved by 4% native PAGE and transferred to a nitrocellulose filter membrane. The membrane was then incubated with antibody SJK 132-20 to identify the relative position of DNA polymerase α on the filter membrane (*Experimental Procedures*). The relative positions of specific molecular standards are listed alongside the scan of the blot.

FIG. 6. T-antigen dependent *in vitro* SV40 DNA replication activity of the electroeluted fractions. A. *In vitro* SV40 DNA replication activity. The *in vitro* SV40 DNA replication assay was performed on individual electroeluted fractions as described (*Experimental Procedures*). One unit of *in vitro* SV40 DNA synthesis activity was defined as 1 picomole of total [α-^{32}P]dNMP incorporated into SV40 origin-containing DNA per hour at 37°C. **B. Replication products formed by electroeluted fraction 5.** The replication products formed in the *in vitro* replication assay by electroeluted fraction 5 (E) and the P4 fraction were extracted and analyzed by 1% alkaline agarose gel electrophoresis (*Experimental Procedures*). CCC: covalently closed circular; Linear: single-stranded linear DNA.

FIG. 7. SDS-PAGE analysis of the replication-competent DNA synthesome in electroeluted fraction 5. The components of the highly purified DNA synthesome were resolved using a 4-20% linear gradient of polyacrylamide (SDS-PAGE) under denaturing conditions. The resolved polypeptide bands were visualized by silver staining. The relative positions of specific molecular mass standards are listed alongside the scan of the gel.

FIG. 8. Analysis of the DNA polymerases activities and replication proteins contained in the electroeluted DNA synthesome. Electroeluted fractions (15 and 30 µl) were assayed for **A. DNA polymerase α activity** and **B. DNA polymerase δ activity**, respectively, as described in the *Experimental Procedures*. One unit of DNA polymerase α activity equals one picomole of total [α-^{32}P]dNMP incorporated into an activated calf thymus DNA template per hour at 37°C. One unit of DNA polymerase δ activity equals one picomole of [α-^{32}P]dTMP incorporated into the DNA template per 15 minutes at 37°C. **C. Topoisomerase I activity assay of the purified DNA synthesome.** Agarose gel electrophoretic analysis showing the conversion of 0.2 µg of supercoiled plasmid DNA to the relaxed form by electroeluted fraction 5 (E). The assays were performed as described in the *Experimental Procedures*. Reactions containing two units of purified topoisomerase I were used as a positive control. Reactions containing the topoisomerase I specific inhibitor, camptothecin (200 µM), were used in the assay as a negative control. ROC: Relaxed Open Circular, SC: Supercoil. **D. Topoisomerase II activity assay of the purified DNA synthesome.** The presence of topoisomerase II in electroeluted fraction 5 (E) was assayed by decatenation of kinetoplast (KDNA) as described in the *Experimental Procedures*. Linear and decatenated KDNA served as reference markers, and topoisomerase II activity was inhibited in one reaction by the inclusion of 100 µM VP-16. This reaction served as a negative control.

FIG. 9. Western blot analysis of DNA synthesome-associated replication essential proteins. The proteins contained in one microgram of electroeluted fraction 5 were resolved using 8% polyacrylamide gel under denaturing conditions (*Experimental Procedures*). The resolved polypeptides were transferred onto a nitrocellulose filter membrane, and the membrane was

incubated with antibodies recognizing either DNA polymerase δ (Transduction Laboratories), RP-A, or PCNA (Calbiochem) (*Experimental Procedures*).

Figure 1

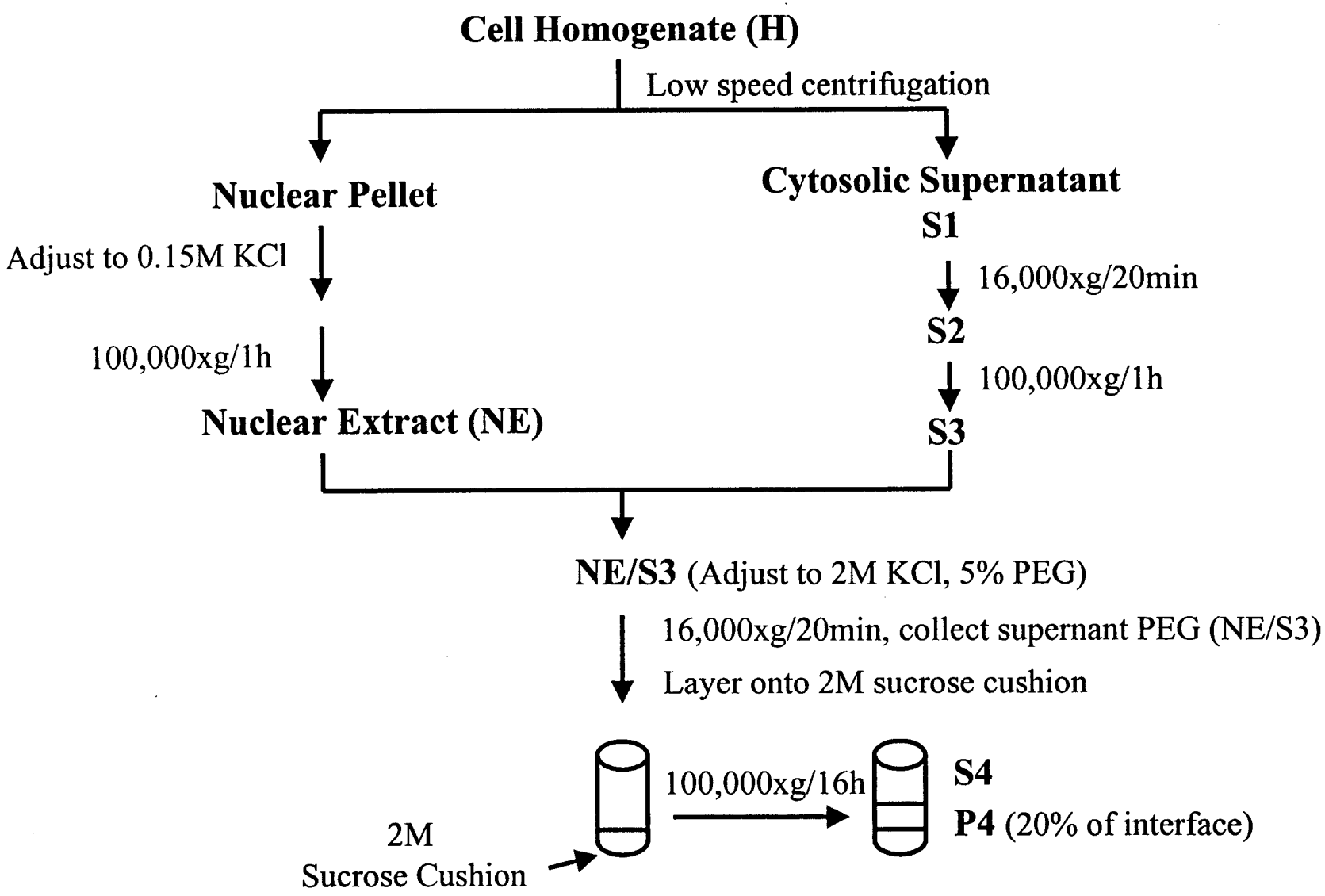
Cell Homogenate (H)
Low speed centrifugation
Nuclear Pellet
Adjust to 0.15M KCl
100,000xg/1h
Nuclear Extract (NE)
Cytosolic Supernatant
S1
16,000xg/20min
S2
100,000xg/1h
S3
NE/S3 (Adjust to 2M KCl, 5% PEG)
16,000xg/20min, collect supernant PEG (NE/S3)
Layer onto 2M sucrose cushion
2M
Sucrose Cushion
100,000xg/16h
S4
P4 (20% of interface)

Figure 2

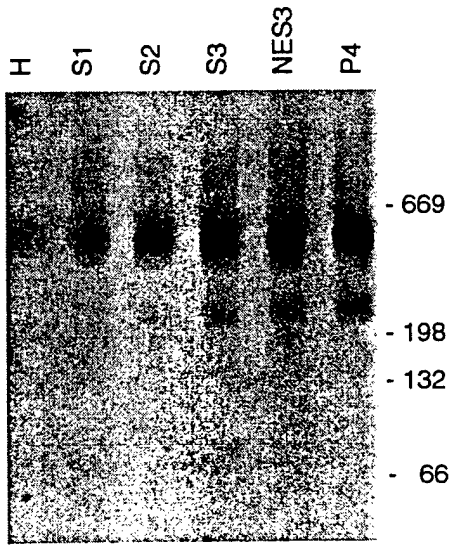

Figure 3A

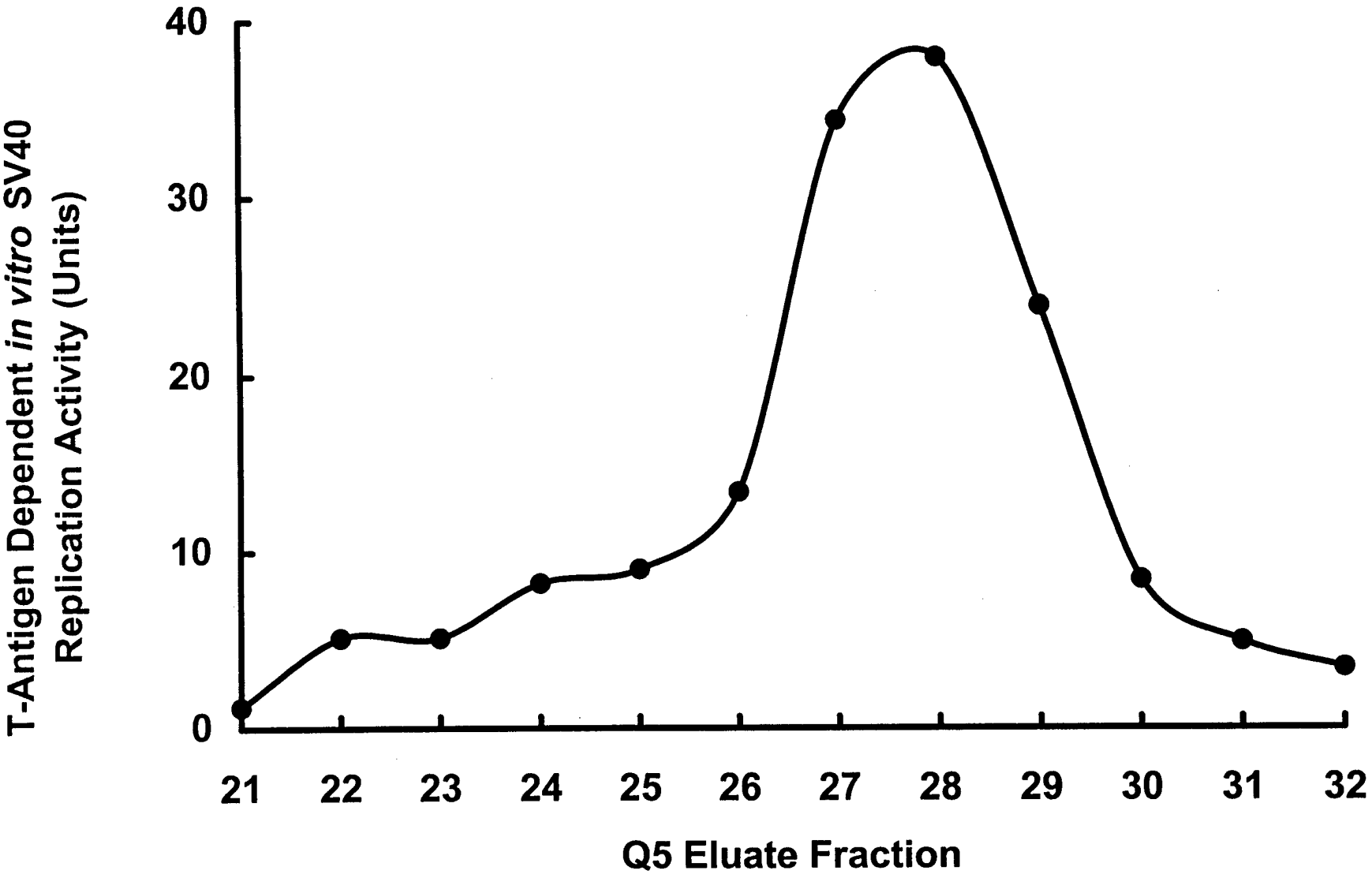
T-Antigen Dependent *in vitro* SV40 Replication Activity (Units)
40
30
20
10
0
21
22
23
24
25
26
27
28
29
30
31
32
Q5 Eluate Fraction

Figure 3B

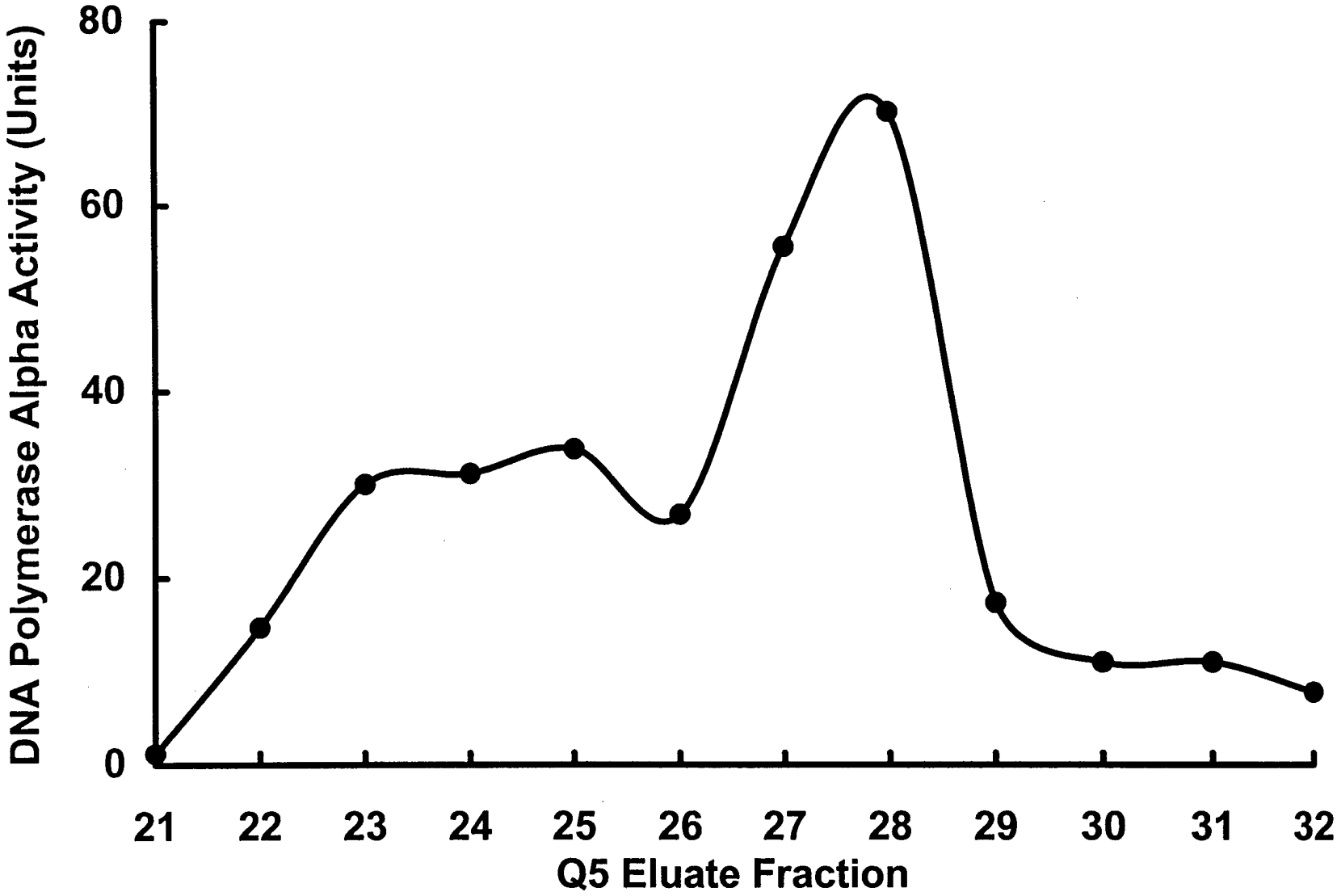
DNA Polymerase Alpha Activity (Units)
80
60
40
20
0
21
22
23
24
25
26
27
28
29
30
31
32
Q5 Eluate Fraction

Figure 3C

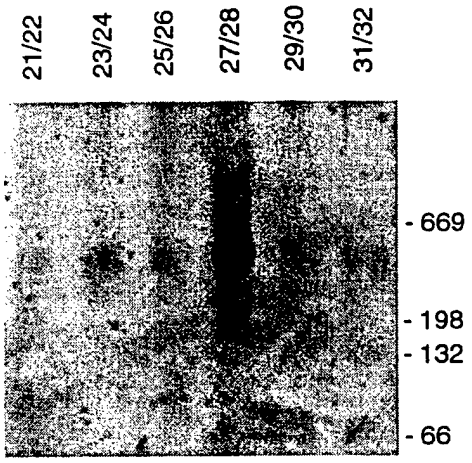

Figure 4

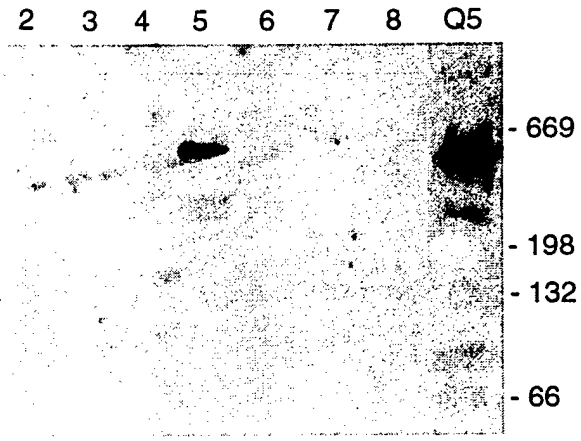

Figure 5A

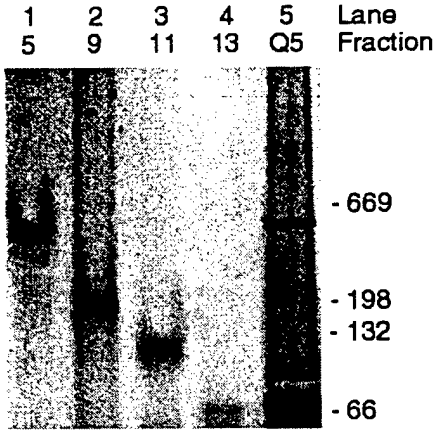

Figure 5B

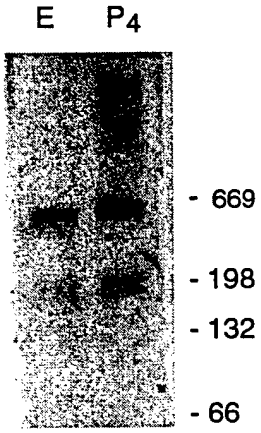

Figure 6A

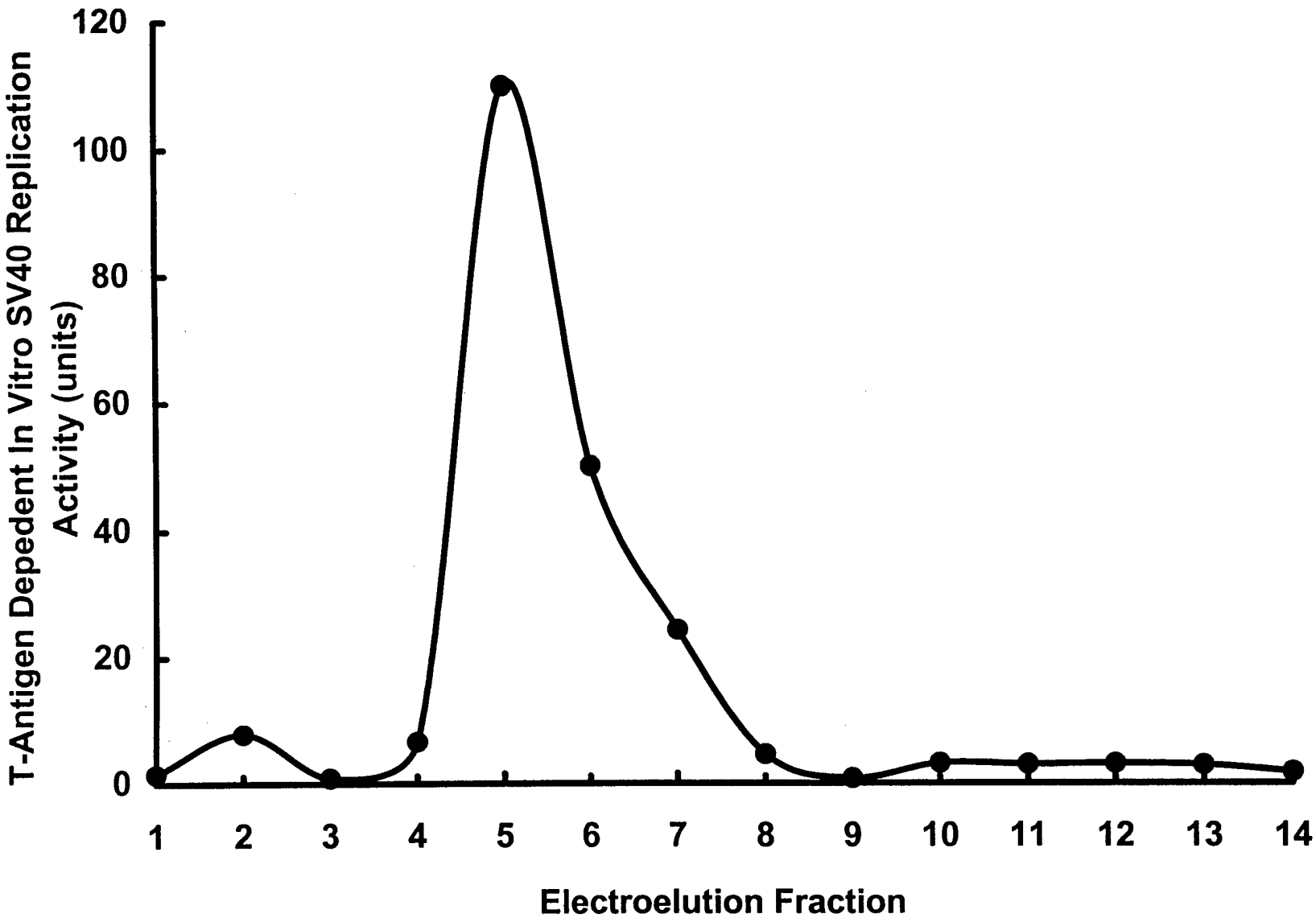
T-Antigen Depedent In Vitro SV40 Replication Activity (units)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Electroelution Fraction

Figure 6B

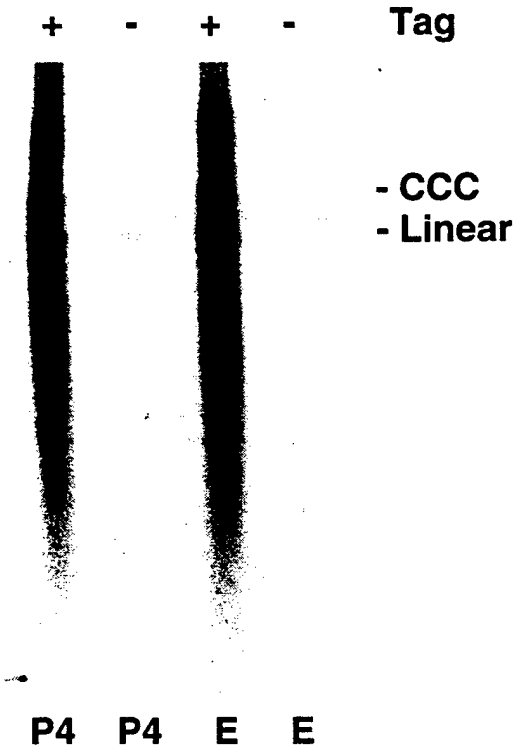

Figure 7

Figure 8A

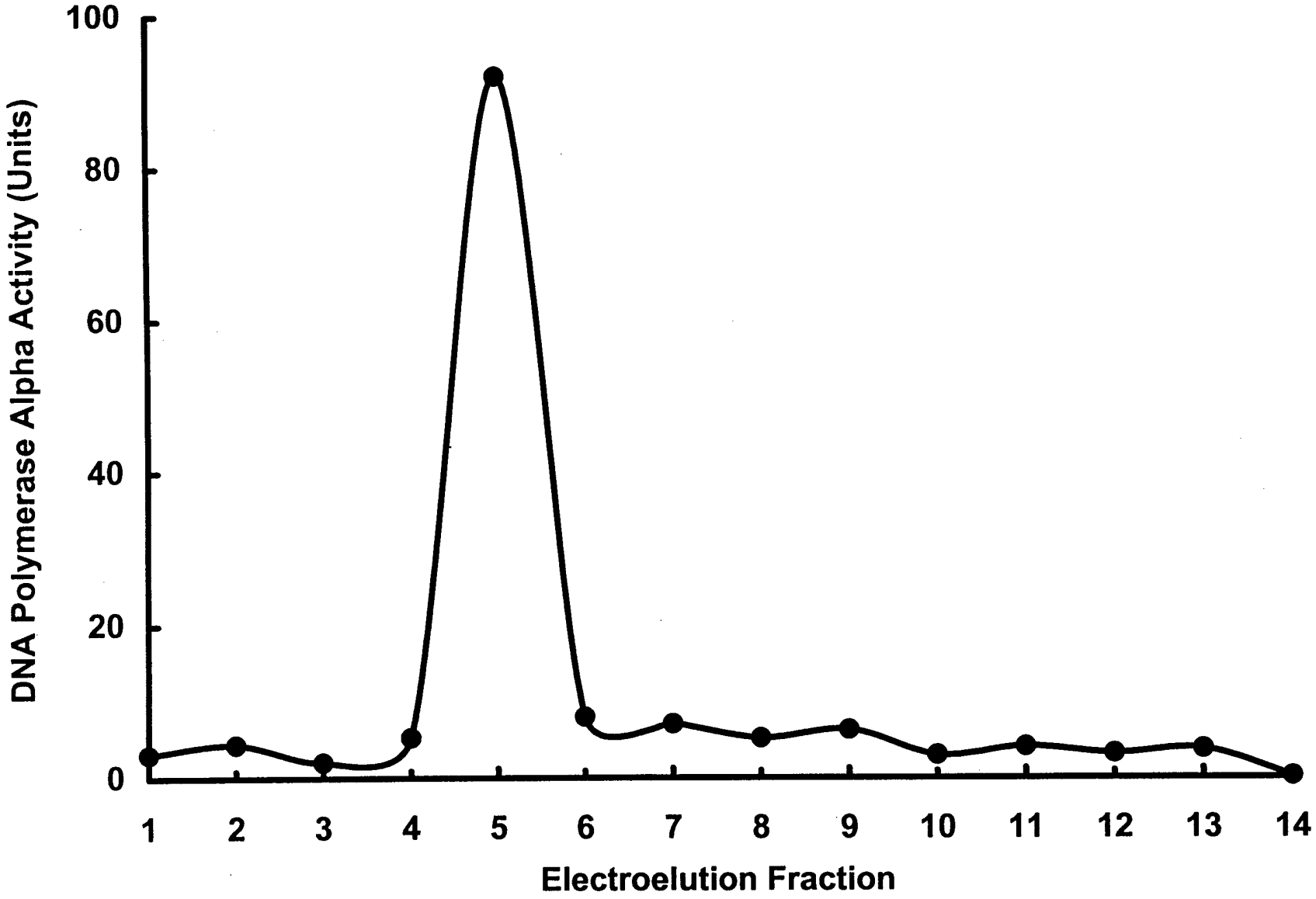
100
80
60
40
20
0
DNA Polymerase Alpha Activity (Units)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Electroelution Fraction

Figure 8B

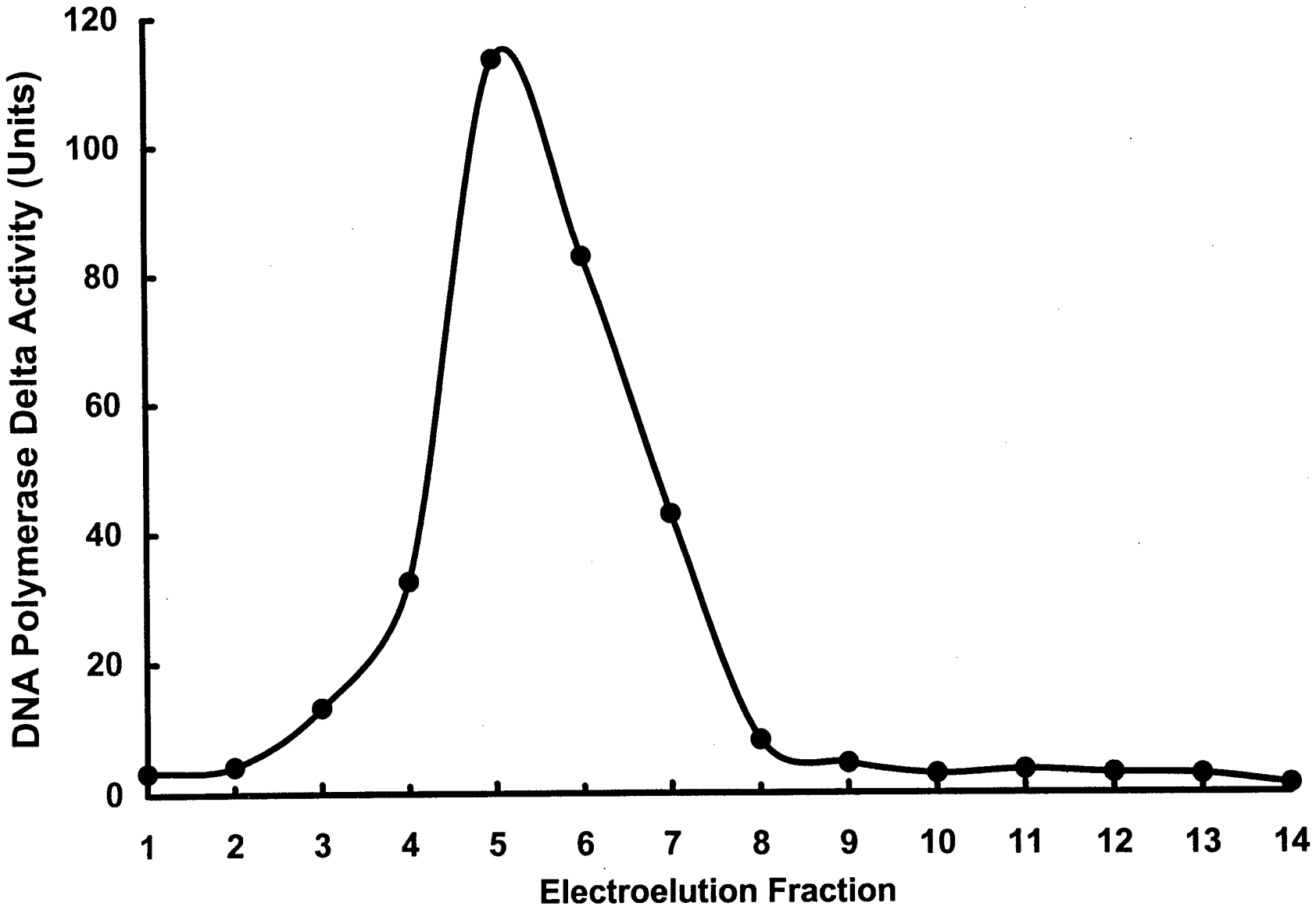
DNA Polymerase Delta Activity (Units)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Electroelution Fraction

Figure 8C

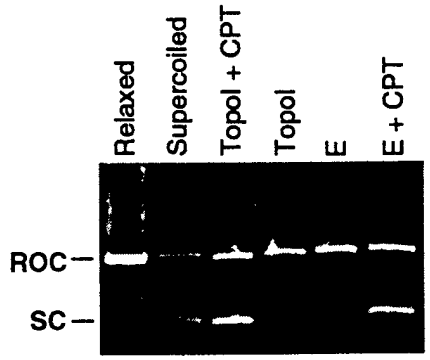

Figure 8D

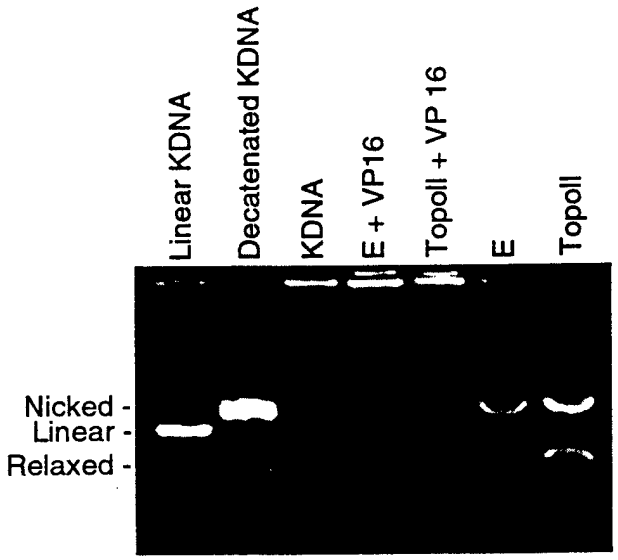
Linear KDNA
Decatenated KDNA
KDNA
E + VP16
TopoII + VP 16
E
TopoII
Nicked -
Linear -
Relaxed -

Figure 9

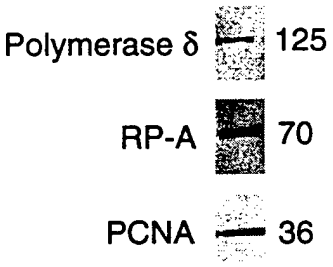

Table I

Specific activity analysis of *in vitro* SV40 DNA replication activity as a function of synthesome purification. The *in vitro* DNA replication reactions were performed as described in the *Experimental Procedures*. One unit of activity was defined as [α-^{32}P]NMP incorporated into SV40 origin containing DNA per mg protein/hr at 37°C.

Fractions	Specific Activity (units/mg)	Purification Fold
H	0.14	1.0
S1	0.22	1.6
S2	0.66	4.7
S3	0.71	5.1
NE/S3	1.03	7.3
P4	2.55	18.2
Q5	5.25	37.5
Sucrose Peak	67.68	483.2
E	517.53	3696.1

Table II

Assay component requirements for DNA synthesome driven *in vitro* DNA replication: The reactions were performed as described in the *Experimental Procedures*. Individual assay components were omitted from specific reactions as indicated. Electroeluted fraction 5 was preincubated with specific antibodies recognizing either PCNA or DNA polymerase α for 1 h at 4°C prior to the addition of the other assay components, and the reactions were performed at 37°C for 1 hr (*Experimental Procedures*). Relative DNA synthesis was calculated as the percentage of [α-^{32}P]dNMP incorporated into DNA relative to a control reaction containing all of the reaction components but lacking any neutralizing antibody.

Assay components omitted or added	Relative DNA synthesis
(+) T-Antigen	100
(-) T-Antigen	0
(-) CPK/PC	9
(-) Mg^{2+}	0
(-) DNA	0
(-) dATP, dGTP, dTTP	0
(-) ATP, CTP, GTP, UTP	8
(+) polymerase α antibody (1μg)	11
(+) PCNA antibody (1.5μg)	31