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We have recently described a multiprotein complex for DNA replication (DNA synthesome) isolated from MDA MB 468 human breast cancer cells, human breast tumor tissue and human breast tumor cell xenografts (Coll et al., 1996). In the presence of the viral large T-antigen and simian virus 40 (SV40) origin sequences, the DNA synthesome executes all of the steps required for the *in vitro* replication of the SV40 genome. Furthermore, the DNA synthesome isolated from human breast cancer cells possesses a lower fidelity for DNA synthesis *in vitro* than the synthesome purified from a non-malignant breast cell line. Our studies indicate that the following DNA replication proteins constitute the DNA synthesome: DNA polymerase α-primase, DNA polymerase δ, DNA polymerase ε, proliferating cell nuclear antigen (PCNA), RF-C, RP-A and DNA topoisomerases I, II.

The DNA synthesome represents the intact breast cell's DNA replication machinery; as such, the synthesome may serve as a powerful model to study the mechanisms of action of anti-breast cancer agents that target the DNA synthetic process. Our initial studies correlating the effects of camptothecin 11 (CPT-11) and etoposide (VP-16), on MDA MB 468 breast cancer cell survival, intact cell DNA synthesis and synthesome-mediated DNA replication, support the use of the synthesome as a drug model. Further studies may contribute important information about the cytotoxic effects of these agents, particularly the cellular consequences of drug-induced cleavable complex formation.

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b. Use HRE analysis to determine the induction of DNA single and double strand breaks by CPT-11 and VP-16 in replication intermediates.

INTRODUCTION

Breast cancer is one of the most commonly diagnosed female cancers and the second leading cause of cancer death among women (American Cancer Society, 1996). Recently, numerous reports have underscored the important role of cell proliferation rate as a prognostic factor for breast carcinoma. Studies using flow cytometry to measure the DNA content of breast tumor cells show a strong association between a high S-phase fraction and poor prognosis for relapse-free survival in patients with lymph node negative breast cancer (Keshgegian and Cnaan, 1995). In addition to a high rate of DNA synthesis, mammary cancer cells exhibit extensive DNA damage (Cropp, 1995; Witzig et al., 1994), as compared to non-malignant breast cells. The increased mutation frequency that accompanies the cellular transformation process is postulated to arise from molecular alterations of specific DNA replication and/or repair proteins (Popanda et al., 1995). For example, DNA polymerases α and ε purified from Novikoff hepatoma cells have altered physicochemical and catalytic properties compared to the respective polymerases isolated from normal liver cells (Popanda et al., 1995). Despite the knowledge that a high proliferation activity and increased mutation frequency correlate with breast cancer progression, there is a paucity of information regarding the regulation and precise molecular mechanisms of human breast cell DNA replication.

Over the last several years, a considerable effort has been mounted by a number of laboratories to identify the proteins required for mammalian cell DNA replication. As a result of these efforts, several proteins necessary for simian virus 40 (SV40) based cell-free DNA synthesis have been identified in human (HeLa and 293) cells; these polypeptides include: DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A), replication factor C (RF-C), and DNA topoisomerases I and II (Waga et al., 1994; Yang et al., 1987). Moreover, the functions that each of these individual proteins performs during DNA replication have been determined by utilizing the *in vitro* SV40 DNA synthesis system. Recent studies suggest that DNA polymerase α-primase synthesizes RNA-DNA primers required for the initiation of leading strand and Okazaki fragment synthesis (Denis et al., 1993; Waga et al., 1994). On the other hand, DNA polymerase δ is believed to conduct

the replication of the leading strand during DNA chain elongation (Denis et al., 1993; Prelich et al., 1988). According to a current model for eukaryotic DNA replication, the activities of both DNA polymerases α and δ are coordinated in part by RF-C, which serves as a connector or hinge between the proteins (Tsurimoto and Stillman, 1991). Also, PCNA--an accessory factor for polymerase δ--may participate in the coordination of leading and lagging strand synthesis by functioning as part of a molecular switch from the initiation to the elongation phase of DNA replication (Waga et al., 1994; Prelich et al., 1988).

Studies demonstrate that RP-A functions during the initiation stage of DNA synthesis to stabilize single-stranded regions created in replicating DNA by the helicase activity of the large T-antigen (Yang et al., 1987). Topoisomerase I, which also functions during the initiation stage of DNA synthesis, relaxes positive DNA supercoils as they accumulate ahead of the replication fork (Snapka, 1986). Such an action is necessary for translocation of the replication machinery along the parental template DNA during DNA synthesis. Finally, it has been shown previously that topoisomerase II mediates the segregation of newly replicated daughter DNA molecules (Snapka, 1986) following DNA synthesis. **To date, the manner in which these proteins associate with one another as well as their ability to act in concert to efficiently replicate DNA has not been adequately defined. As mammalian cell DNA replication represents an intricate yet highly coordinated and efficient process, it follows that all of the proteins that mediate DNA synthesis may be organized into a multiprotein complex. In support of this hypothesis, several reports have described the isolation of large macromolecular complexes of replication essential proteins from extracts of eukaryotic cells (Malkas et al., 1990a; Matthews and Slabaugh, 1986; Reddy and Fager, 1993; Hickey and Malkas, 1996).**

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex from human (HeLa) and murine (FM3A) mammary carcinoma cells using a series of differential centrifugations, polyethylene glycol precipitation and ion-exchange chromatography (Applegren et al., 1995; Wu et al., 1994). The integrity of the multiprotein complex was maintained after treatment with salt, detergents, RNase,

DNase, chromatography on Q-Sepharose or DE52-cellulose and electrophoresis through native polyacrylamide gels (Applegren et al., 1995; Wu et al., 1994; Tom et al., 1996). These results suggest that the association of the proteins with one another is independent of non-specific interactions with other cellular macromolecules. Most importantly, we have demonstrated that this complex of proteins is fully capable of supporting origin-specific and large T-antigen dependent papovavirus DNA replication *in vitro*. Since papovavirus is extensively dependent upon the host cell's DNA replication machinery for its own DNA synthesis, our results indicate that the isolated multiprotein complex plays a role not only in papovavirus DNA synthesis but in mammalian cell DNA replication as well.

We have recently determined that MDA MB 468 human breast cancer cells also utilize a multiprotein complex to carry out cellular DNA synthesis, and we have designated this complex the DNA synthesome (Coll et al., 1996). We have identified the proteins comprising the breast cell DNA synthesome using Western blot analyses and enzyme assays; these polypeptides include: DNA polymerases α, δ, ε, DNA primase, PCNA, RF-C, RP-A, and DNA topoisomerases I, II. Based on the fractionation and column chromatographic profiles of these protein components, we have proposed a model to represent the breast cancer cell DNA synthesome (Figure 1). As DNA polymerases α, δ, ε, DNA primase and RF-C were observed to copurify primarily with those fractions containing the replication-competent DNA synthesome, we propose that these proteins form the core of the DNA synthesome. In addition, we have included DNA ligase I as a member of the tightly associated components of the complex as it was observed to exclusively copurify with the DNA synthesome from FM3A and HeLa cells (Applegren et al., 1995; Wu et al., 1994). Unlike the other components, PCNA, RP-A and topoisomerases I and II were observed to co-fractionate and co-elute, following column chromatography, with those fractions containing the breast cancer cell DNA synthesome as well as with fractions lacking DNA replication activity. These results suggest that only a fraction of the cellular pools of PCNA, RP-A, and topoisomerases I, II, copurify with the DNA synthesome. This is consistent with the recognition that these proteins have

additional roles in mediating cellular functions such as transcription, recombination and repair.

Importantly, we have successfully isolated and purified a fully functional DNA synthesome from biopsied human breast tumor tissue as well as from human breast tumor cell xenografts (Coll et al., 1996). These latter results strongly suggest that the DNA synthesome mediates breast cancer cell DNA replication *in vivo*. Furthermore, our results from a forward mutagenesis assay (Coll et al., 1996) demonstrate that the synthesome isolated from MDA MB 468 breast cancer cells and human breast tumor tissue has a lower fidelity for DNA replication than the synthesome isolated from normal Hs587Bst breast cells. This finding implies that transformation to the malignant phenotype alters the process by which the synthesome from normal cells replicates and/or participates in the repair of DNA. **Ultimately, we anticipate that further study of the breast cancer cell DNA synthesome will provide important new insight into understanding the molecular mechanisms of breast cancer cell DNA replication as well as help discern how DNA replication fidelity is reduced in breast cancer cells.**

In addition to contributing insight into the mechanisms and regulation of breast cancer cell DNA replication, studies with the DNA synthesome could help elucidate the mechanisms of action of anti-breast cancer agents that directly target DNA synthesis. One advantage conferred by using the synthesome as a drug model is that it would permit the study of the interactions of anti-breast cancer agents with the entire breast cell DNA replication apparatus and not just individual proteins involved in DNA synthesis. Also, this novel model would allow the examination of the mechanisms of action of anti-cancer agents in the absence of any cellular processes that may secondarily affect DNA synthesis.

Two DNA topoisomerase inhibitors that are currently being evaluated for efficacy against breast cancer are irinotecan (CPT-11) and etoposide (VP-16). CPT-11 is an anticancer compound that is undergoing phase II clinical trials in breast cancer patients (Abrams et al., 1994). Preliminary phase II results indicate that the agent demonstrates potent activity against some types of breast cancer, with diarrhea and myelosuppression as the most commonly observed toxicities (Abrams et al., 1994). CPT-11 possesses a

unique mechanism of action in that it traps nuclear topoisomerase I in a ternary (drug-enzyme-DNA) cleavable complex (Schneider et al., 1990). In this state, topoisomerase I cannot perform its DNA single strand nicking-resealing function required for the relaxation of DNA supercoils that accumulate during replication and transcription. Additionally, VP-16 possesses efficacy in treating advanced refractory breast cancer when combined with cisplatin or used in high-dose polychemotherapy regimens (Pfeiffer et al., 1992; Vahdat et al., 1995). VP-16 traps topoisomerase II in a cleavable complex which prevents the enzyme from introducing transient double-strand breaks into the DNA phosphodiester backbone, an action required for the termination of DNA synthesis during interphase and the segregation of newly formed daughter DNA molecules during mitosis. For both CPT-11 and VP-16, cleavable complex formation represents an early step in a cascade of events that eventually leads to cell death (Schneider et al, 1990). Little is known about the cell-killing mechanism beyond cleavable complex formation.

We have previously reported that the anticancer compound, camptothecin (CPT), interacts with the HeLa cell DNA synthesome as it does with the intact HeLa cell's DNA replication machinery (Coll et al., 1996). We based this finding on the close correlation of CPT's IC_{50}s (concentration of drug inhibiting process by 50%) for HeLa cell colony formation, intact HeLa cell DNA synthesis and DNA synthesome mediated *in vitro* DNA replication. Initial studies from our laboratory also demonstrate that CPT-11 and VP-16 interact with the MDA MB 468 breast cancer cell DNA synthesome in a manner similar to their interactions with the intact breast cancer cell's DNA replication machinery. We anticipate that further studies with the breast cancer cell DNA synthesome will help elucidate the downstream events of cleavable complex formation as well as facilitate the development of improved CPT-11 and VP-16 analogues.

MATERIALS AND METHODS

Materials. CPT and VP-16 were purchased from Sigma Chemical Co. CPT-11 and SN-38 were obtained from UpJohn Pharmaceuticals for use in intact cell and *in vitro* studies.

Methods

Cell Culture. Suspension cultures of MDA MB-468 human breast cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and fetal bovine serum. Exponentially growing cells ($5x10^5$ cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS): 20 mM Na_2HPO_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation.

Human Breast Tumor Tissue. Biopsied breast tumor specimens from female breast cancer patients were immediately frozen at -80°C after resection. In order to examine the tumor tissue for the presence of a functional DNA synthesome, the breast tumor tissue was thawed and subjected to the purification protocol described in a later section of these Methods.

Isolation and Purification of the DNA Synthesome from Breast Cancer Cells and Breast Tumor Tissue: Cell Fractionation. MDA MB-468 cells were homogenized and the breast cell DNA synthesome purified according to our previously published procedures and as outlined in Figure 2. Briefly, the respective cell pellet was resuspended in 2 volumes of buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM AAN (pH 7.5) and 1 mM DTT) and homogenized using a loose-fitting Dounce homogenizer. The homogenate was then fractionated into a nuclear pellet and cytosolic extract. The nuclei was then extracted with a low salt buffer (0.15 M KCl) while the cytosolic fraction was used to prepare a post-microsomal supernatant (S-3). The nuclear extract and the post-microsomal supernatant was combined and adjusted to 2M KCl and 5% (w/v) polyethylene glycol. The mixture was then rocked for 1 h at 4°C, and centrifuged at 16,000 rpm for 15 minutes (4°C). The resulting supernatant was then dialyzed against buffer A (Malkas et al., 1990b) containing 0.25 M sucrose. The dialyzed fraction will be clarified by centrifugation at 16,000 rpm for 15 minutes and the supernatant solution layered onto a 1 ml 2 M sucrose cushion containing buffer A. After centrifugation at 40,000 rpm for 16 h (4°C), the supernatant S-4 and sucrose interface P-4 fractions was collected and dialyzed against buffer B (Malkas et al., 1990b). The fractions

were then tested for DNA polymerase α and *in vitro* SV40 DNA replication activities. The DNA synthesome was purified from breast cancer tissue according to a modified version of this isolation scheme. All steps of the fractionation process were altered to facilitate the purification of the DNA synthesome from small quantities of breast tumor tissue. **Column Chromatography.** The dialyzed MDA MB-468 P-4 fraction was loaded onto a 1 ml Q-Sepharose (Pharmacia) column (1 cm^3 bed volume/25 mg protein) pre-equilibrated with buffer B. The protein not binding to the matrix was collected and designated the column flow-through. After washing the matrix with 8 column volumes of buffer B, the column was eluted with 10 volumes of a linear 50-500 mM gradient of KCl. Fractions of 0.5 ml were collected and assayed for protein and enzymatic activity. Fractions containing the peak of DNA polymerase α and *in vitro* SV40 DNA replication activities were pooled, dialyzed against TDEG buffer (Malkas et al., 1990b) and stored at -80°C.

Velocity Sedimentation Analysis of the DNA Synthesome Isolated from MDA MB-468 Breast Cancer Cells. Approximately 500 µl of the DNA synthesome contained in the Q-Sepharose peak fraction was layered over a 10 ml 10-30% sucrose gradient containing 0.5 M KCl. Velocity sedimentation analysis was performed as described in a previously published report from this laboratory (Wu et al., 1994). The sedimentation analysis of marker proteins (horse spleen apoferritin (17S) and yeast alcohol dehydrogenase (7S)) were performed on parallel gradients to verify that the gradient is isokinetic.

Purification of SV40 Large T-antigen. SV40 large T-antigen was purified from 293 cells infected with a recombinant adenovirus vector, Ad-SVR284, as detailed elsewhere (Simanis and Lane, 1985).

***In vitro* SV40 DNA Replication Assay.** Assay reaction mixtures (12.5 µl) contained 80 mM Tris-HCl (pH 7.5); 7 mM $MgCl_2$; 1 mM DTT; 3-20 µg protein fraction; 0.5-1.0 µg purified SV40 large T-antigen; 25 ng plasmid $pSVO^+$ (Stillman et al., 1985) possessing an insert of SV40 replication-origin DNA sequences; 100 µM each dTTP, dATP, dGTP; 200 µM each rCTP, rGTP, UTP; 4 mM ATP; 25 µM [^{32}P]dCTP; 40 mM creatine phosphate; 1µg creatine kinase. Reactions were incubated for 2h at 35°C in the presence

of various concentrations of either CPT-11 or VP-16. The replication assay reaction products were processed using DE81 (Whatman) filter binding to quantitate the amount of radiolabel incorporated into the replication products (Sambrook et al., 1989). One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol of dNMP into SV40 replication-origin containing plasmid DNA per 2h under these described assay conditions.

DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures (LaMothe et al., 1981). One unit of DNA polymerase α activity is equivalent to 1nmol of total ^{3}H-TMP incorporated into DNA per hour at 35°C.

Immunodetection of DNA Polymerases δ, ε, RP-A, RF-C, PCNA and DNA Primase. Denaturing polyacrylamide gel electrophoresis of the various protein fractions was performed as previously described (Laemmli, 1970). The resolved polypeptides were transferred (15 volts, 16h, 4°C) to nitrocellulose membranes, and immunodetection of the respective DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham). A monoclonal antibody prepared against the C-terminal portion of DNA polymerase δ was used at a 1:100 dilution to probe membranes for the 125 kDa polymerase δ polypeptide. The anti-polymerase ε antibody which recognizes the 140 and >200 kDa forms of polymerase ε, was used at a 1:1000 dilution. Both the anti-RF-C monoclonal antibody (mAb-11), which recognizes the 140 kDa subunit of the RF-C protein-complex, and the anti-RP-A antibody (p34-20), which recognizes the 34 kDa subunit of RP-A, were generous gifts from Dr. Bruce Stillman. Both antibodies were used at a 1:500 dilution. The anti-DNA primase antibody, a gift from Dr. William Copeland, was used at a 1:500 dilution. The anti-PCNA antibody was used at a dilution of 1:1000. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots at a dilution of 1:5000.

MDA MB 468 Cell Survival Assays. MDA MB 468 cell survival assays were performed in the presence of increasing concentrations of either CPT-11 or VP-16, according to the procedure described by Ryan et al., 1994.

Measurement of Intact MDA MB 468 Cell DNA Synthesis. 5 x 10^4 exponentially growing MDA MB 468 breast cancer cells were seeded onto 60 mm cell culture plates and incubated at 37°C for 24 hrs. Cells were next incubated with increasing concentrations of either CPT-11, VP-16 or vinblastine in the presence of ^{3}H-thymidine. After a one hour incubation, cells were lysed and the amount of radiolabel incorporated into DNA determined by the isolation and counting of acid-insoluble material.

DNA topoisomerase I activity was measured by incubating 0.3 μg supercoiled pSVO+ DNA with either 8 μg Q-Sepharose peak, in the absence or presence of increasing concentrations of CPT-11, or 2 units purified topoisomerase I. Incubations were performed for 20 minutes at 37°C. Each reaction (15 μl) was stopped with 1% SDS and the DNA products resolved on a 1.0 % agarose gel containing TAE buffer (40 mM Tris acetate, 2 mM EDTA). After ethidium bromide (1 μg/ml) staining of the gels, topoisomers were visualized with an ultraviolet light source.

DNA topoisomerase II activity of the Q-Sepharose peak fraction was assayed in the presence of increasing concentrations of VP-16, using an assay kit purchased from TopoGen, Inc.

Southern Blots. Southern blotting will be performed according to the following procedure. Briefly, the plasmid DNA (pBRSV40ori), used as the input DNA in the *in vitro* DNA replication reactions, will be digested with various combinations of restriction endonucleases in order to generate distinct DNA fragments. Each set of digested plasmid DNA molecules will be run in adjacent lanes of a neutral 1.6% DNA agarose gel along with a size marker. The DNA fragments will be electrophoresed for 6 hours at 100 volts. The gel will then be stained with ethidium bromide in order to visualize the restriction fragments. The restriction DNA fragments will be transferred to a nitrocellulose filter membrane using 20X SSC. The filter membrane will then be baked in a vacuum oven at 80°C for 2 hours and the radiolabeled DNA replication products isolated from the *in vitro* replication reactions will be hybridized to the blot using 6X SSC, 10X Denhardt's solution, 0.1% SDS and 200 μg/ml denatured salmon sperm DNA at 65°C for 16 hours. The filters will then be washed twice at room temperature with 2X

SSC, 0.1% SDS, then once at 65°C with 0.1X SSC, 0.1% SDS. The filter will then be exposed to Kodak X-ray film (XAR-5).

RNase Protection Analysis and Probe Generation. The probes required for this analysis are easily prepared by identifying the plasmid DNA fragment in which the DNA replication products accumulate; removing this DNA fragment from the input pBRSV40ori plasmid; ligating this fragment into the "multiple cloning site" of a commercially available plasmid (pGem1); and preparing the radiolabeled RNA probes with bacteriophage SP6 and T7 RNA polymerases. The RNA probes will be hybridized to non-radioactive newly replicated DNA prepared from *in vitro* reactions containing non-radioactive dNTPs. The DNA/RNA hybrid is digested with RNase A and RNase T1, and the duplex DNA surviving digestion is analyzed under denaturing conditions of polyacrylamide gel electrophoresis. The size of the radiolabeled RNA surviving digestion will be estimated by comparing its position in the gel to that of molecular size standards separated in an adjacent lane of this same gel.

High Resolution Electrophoresis. Viral replication intermediates will be separated in the first dimension by electrophoresis at neutral pH in 0.8% agarose. For the second dimension, the gel lane from the neutral agarose will be cut out, soaked in alkaline buffer, embedded in an alkaline gel and run at a right angle to the first dimension. Under these conditions, replication intermediates denature. Only the pulse-labeled nascent DNA strands will contribute to the fluorographic image.

RESULTS / DISCUSSION

Human Breast Cancer Cell DNA Replication Proteins Co-fractionate as a Readily Sedimentable Form.

The DNA replication proteins of the MDA MB 468 human breast cancer cell line can be isolated in a readily sedimentable form. We subjected MDA MB-468 cells to the fractionation scheme outlined in Figure 2. The PEG NE/S-3, S-4 and P-4 fractions were collected and assayed for DNA polymerase α activity. The majority of the enzyme's activity partitioned with the sedimentable P-4 fraction following polyethylene glycol

precipitation and discontinuous gradient centrifugation of the NE/S-3 fraction (Table 1). This result is consistent with our earlier work on the purification of the DNA synthesome from HeLa and FM3A cells (Malkas et al., 1990b; Applegren et al., 1995; Wu et al., 1994), in which the DNA polymerase α activity contained in the DNA synthesome partitioned to the P-4 fraction at the sucrose interface.

In addition to determining DNA polymerase α activity, we assayed the PEG NE/S-3, S-4 and P-4 fractions for *in vitro* SV40 DNA replication activity. DE81 filter binding analysis was used to quantitate the level of ^{32}P-dCMP incorporation into SV40 DNA replication products. Following subfractionation of the PEG NE/S-3 fraction into the S-4 and P-4 fractions, the ability to support SV40 DNA replication *in vitro* partitioned exclusively with the sedimentable P-4 fraction (Table 1). This pattern of partitioning of DNA replication activity, is also consistent with our earlier work on the purification of the synthesome from HeLa and mouse FM3A cells (Malkas et al., 1990b; Applegren et al., 1995; Wu et al., 1994). Only negligible amounts of radiolabel were incorporated into DNA replication products when reactions lacked SV40 large T-antigen. These data indicate that all of the activities required to execute large T-antigen dependent SV40 DNA replication reside in the human breast cancer cell sedimentable P-4 fraction.

Further Purification of the Human Breast Cancer Cell DNA Synthesome.

We further purified the breast cancer cell DNA synthesome from the sedimentable P-4 fraction by Q-Sepharose anion-exchange chromatography; a method successfully employed for the purification of the DNA synthesome from HeLa cells (Malkas et al., 1990b; Applegren et al., 1995). The P-4 fraction was applied to a 1 ml Q-Sepharose column and the DNA synthesome eluted by a linear gradient of KCl (50-500 mM). Figure 3 shows the profile of DNA polymerase α activity as it eluted from the Q-Sepharose column. The DNA polymerase α activity eluted from the column as an initial sharp peak at lower salt concentrations (fractions 6-10), with an additional small peak of activity at higher salt concentrations (fractions 21-23). Negligible amounts of enzyme activity were found in the column flow-through and wash fractions (data not shown).

The peak of DNA polymerase α activity that eluted from the Q-Sepharose column (fractions 6-10), was designated the Q-Sepharose peak. Both the peak and the column

flow-through fractions were assayed for *in vitro* SV40 DNA replication activity. The Q-Sepharose peak contained over 80% of the large T-antigen dependent *in vitro* DNA replication activity; while the column flow-through fraction supported significantly less DNA synthesis (Table 2).

Velocity Sedimentation Analysis of the Breast Cancer Cell DNA Synthesome.

We determined the sedimentation coefficient of the breast cancer cell DNA synthesome by subjecting the Q-Sepharose peak fraction to velocity sedimentation analysis in a 10-30% sucrose gradient containing 0.5M KCl (Wu et al., 1994). The sucrose gradient fractions were collected and assayed for DNA polymerase α and *in vitro* SV40 DNA replication activities. Both activities co-sedimented in the sucrose gradient with a sedimentation coefficient of 18S (Figure 4; Table 2). This 18S sedimentation coefficient for the breast cell DNA synthesome corresponds to the S-value obtained for the HeLa cell DNA synthesome. Presumably, the 18S value of the human breast cancer cell DNA synthesome accounts for its ready sedimentation to the sucrose interphase following the centrifugation of the PEG NE/S-3 fraction (Figure 2).

The DNA Replication Proteins that Copurify with the Breast Cancer Cell DNA Synthesome.

We performed Western blot analyses and enzyme assays to identify the DNA replication proteins that copurify with the breast cancer cell DNA synthesome during its various stages of purification. As numerous studies have shown that DNA polymerase δ plays an integral role in the *in vitro* synthesis of SV40 origin containing DNA (Hurwitz et al., 1990; Prelich et al., 1987; Tsurimoto and Stillman, 1991;), we probed the PEG NE/S-3, P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions for the presence of the protein. Utilizing a monoclonal antibody prepared against the C-terminal peptide of DNA polymerase δ (Lee et al., 1984), we found that the protein exclusively co-purified with the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 5). The enzyme was not detectable in the replication-deficient S-4 and Q-Sepharose flow-through fractions.

In addition to DNA polymerase δ, we examined the human breast cancer cell fractions for the presence of RF-C (Tsurimoto and Stillman, 1991; Lee et al., 1991) and DNA primase. Immunoblot analyses, using antibodies that recognize either the 140 kDa subunit of the RF-C protein complex or the 58 kDa subunit of DNA primase, revealed that RF-C and DNA primase resided only in the replication-competent protein fractions (Fig. 5).

Western blot analysis also shows that the DNA replication protein, PCNA, was present in the replication-competent breast cancer cell fractions, as well as the S-4 and Q-Sepharose flow-through fractions (Figure 5). This result suggests that PCNA may not be as tightly associated with the DNA synthesome as compared to DNA polymerases α, δ, RF-C and DNA primase. Furthermore, immunodetection of RP-A (Wobbe et al., 1985; Fairman and Stillman, 1988) with a monoclonal antibody to the 70 kDa subunit of the protein, reveals that the polypeptide fractionated with both the replication-competent and -deficient fractions (Figure 5). These results suggest that only a fraction of the cellular pools of PCNA and RP-A copurify with the breast cancer cell DNA synthesome.

Furthermore, we determined whether the breast cancer cell DNA synthesome possesses DNA topoisomerase I activity by assaying several breast cancer fractions for their respective enzymatic activity. In Figure 6, lanes 1-3 show the conversion of supercoiled form I DNA to relaxed, open circular form II DNA by the topoisomerase I activity present in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Importantly, the relaxation of supercoiled plasmid DNA by the Q-Sepharose peak fraction was inhibited by 200 μM CPT (lane 4), a specific inhibitor of DNA topoisomerase I (Hsiang and Liu, 1988). This indicates that the conversion of supercoiled plasmid DNA to form II DNA was mediated specifically by topoisomerase I.

We also assayed the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions for DNA topoisomerase II activity. The decatenation of interlocked aggregates of *Crithidia fasisculata* kinetoplast DNA to monomeric, open circular DNA by the topoisomerase II enzyme present in all three fractions is shown in Figure 7 (lanes 2-4). In addition, we determined that the Q-Sepharose peak, flow-through

and sucrose gradient peak fractions were devoid of nuclease contamination because they did not support the relaxation of kinetoplast DNA to the linear DNA fragments (Figure 7). Moreover, as DNA topoisomerase II requires ATP for catalytic activity, incubation of the Q-Sepharose peak with a reaction buffer lacking ATP did not support the relaxation of kinetoplast DNA (Figure 7, lane 1).

Finally, we have just recently determined by immunoblot analysis and enzyme assay that DNA methyltransferase copurifies with the breast cancer cell DNA synthesome (data not shown). This enzyme is responsible for methylating newly replicated DNA molecules at the 5-position of cytosines in the CpG dinucleotide (Vertino et al., 1996).

Isolation of the DNA synthesome from Breast Tumor Tissue.

In order to verify that the DNA synthesome could be isolated from breast cancer tissue as well as breast cancer cells, we subjected biopsied human breast tumor tissue to a modified version of the purification scheme depicted in Figure 2. The alterations to the purification protocol were made to facilitate the isolation of the DNA synthesome from small quantities of breast tumor tissue. We collected and assayed the NE/S-3, S-4 and P-4 fractions for DNA polymerase α and large T-antigen dependent SV40 DNA replication activities. Table 3 shows that the majority of both activities partitioned exclusively with the sedimentable P-4 fraction after discontinuous gradient centrifugation of the NE/S-3 fraction.

We further purified the DNA synthesome that was isolated from the human breast tumor tissue, using ion exchange chromatography. We collected and assayed the column fractions for both DNA polymerase α and *in vitro* SV40 DNA replication activities. A peak of DNA polymerase α activity (fractions 2,3) was found to elute from the column in the presence of 1M KCl (Table 4). In contrast, only a minor amount of DNA polymerase α activity was found in the column flow-through fraction (Table 4). We also tested the fractions containing the peak polymerase α activity (fractions 2,3) as well as the column flow-through for *in vitro* SV40 DNA replication activity. Only fractions 2 and 3 supported SV40 DNA replication; the column flow-through did not contain DNA replication activity (data not shown).

We also fractionated breast cancer tissue-derived from a xenograft nude mouse model (Yue et al., 1994). Homogenous breast tumors were surgically excised from nude mice subcutaneously injected with MCF-7 breast cancer cells. Using the modified purification protocol, it was found that most of the DNA polymerase α and DNA replication activities resided with the sedimentable P-4 fraction following discontinuous gradient centrifugation of the NE/S-3 fraction (Table 5). These results suggest that the DNA synthesome exists as a functional complex within human breast cancer cells *in vivo*.

DNA Polymerase ε Copurifies with the Breast Cancer Cell DNA Synthesome.

Several lines of evidence support a role for DNA polymerase ε in cellular DNA replication. First, DNA polymerase ε is more abundant in proliferating tissues than in non-proliferating tissues (Tuusa et al., 1995). Second, when quiescent human fibroblast cells are stimulated to proliferate, the mRNA levels of DNA polymerase ε, like those of polymerase α, dramatically increase just prior to S-phase (Tuusa et al., 1995). Third, when the gene encoding the yeast homologue of DNA polymerase ε is mutated, the yeast cells fail to proliferate; suggesting a critical role for this polymerase in cell proliferation (Morrison et al., 1990). In order to determine whether DNA polymerase ε copurifies with the breast cancer cell DNA synthesome, we probed the MDA MB-468 derived protein fractions with an antibody that recognizes the >200 kDa polypeptide. Immunoblot analysis reveals that DNA polymerase ε was present in the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 8). Only a minor amount of DNA polymerase ε was present in the replication-deficient S-4 fraction (Figure 8), while none was detected in the Q-Sepharose flow-through (data not shown).

DNA Replication Fidelity of the Breast Cancer Cell DNA Synthesome.

The fidelity of DNA synthesis is mediated in part by the proof-reading capacity of the intrinsic 3'-5' exonuclease activity of DNA polymerase δ (Roberts and Kunkel, 1988). We employed a forward mutagenesis assay to measure the fidelity of the *in vitro* DNA synthesis process carried out by the breast cancer cell DNA synthesome (Roberts et al., 1991). In this assay we utilized the DNA synthesome isolated from MDA MB-468 breast cancer cells and human breast tumor tissue to replicate plasmid DNA containing the SV40 origin of replication and the *lac-Zα* gene. The results of the fidelity assay were

quantitated using the blue/white selection protocol (Roberts et al., 1991). These results were compared to the replication fidelity of the DNA synthesome isolated from non-malignant Hs587Bst breast cells. We determined that the DNA synthesome from MDA MB-468 cells possessed a replication fidelity approximately 6 fold lower than that of the synthesome from Hs587Bst cells (Table 6). Similarly, the DNA synthesome purified from human breast tumor tissue possessed an approximately 5-fold lower DNA replication fidelity than the Hs587Bst synthesome (Table 6). These differences in replication fidelity between the malignant and normal breast cell DNA synthesome suggest that transformation to the malignant phenotype alters the process by which the synthesome from normal cells replicates DNA.

Inhibition of MDA MB 468 breast cancer cell survival, intact cell DNA synthesis, and DNA synthesome-mediated *in vitro* replication by CPT, CPT-11 and VP-16

• ***Breast Cancer Cell Survival Assays***

We performed MDA MB 468 breast cancer cell survival assays to verify that CPT affects the ability of mammalian cells to proliferate, as reported by others (Andoh et al., 1987). We exposed MDA MB 468 breast cancer cells to increasing concentrations of CPT for a one hour interval, then washed the cells and incubated them in fresh drug-free media for an interval of 8-10 days. A one hour exposure of breast cancer cells to increasing concentrations of CPT impaired cell survival (data not shown). At approximately 0.6 μM CPT, only 50% of the breast cancer cells remained viable. At higher concentrations of the drug, cell colony formation was inhibited almost completely. This result, which is consistent with a previously published report that describes the effects of CPT on HeLa cell survival (Hennequin et al., 1994), indicates that CPT's toxicity to MDA MB 468 breast cancer cells may not be limited to the S-phase cell population.

We also exposed MDA MB 468 breast cancer cells to increasing concentrations of CPT-11 in the cell survival assay. We found that approximately 0.5 μM of the drug inhibited cell colony formation by 50%; again, with higher concentrations of CPT-11 impairing cell survival profoundly (Figure 9A). Furthermore, VP-16 inhibited MDA MB

468 cell survival by approximately 50% at 50 μM (Figure 9B). This result is consistent with a previously published report in which the IC_{50} for the inhibition of human colon cancer cell survival by VP-16 was determined (Inaba et al., 1994).

• ***Intact Breast Cancer Cell DNA Synthesis Assays***

Topoisomerase I plays a pivotal role in DNA replication, facilitating replication fork migration by unwinding positive supercoils as they accumulate ahead of the fork. The necessity of topoisomerase I to DNA synthesis is underscored by the sensitivity of intact MDA MB 468 breast cancer cell DNA synthesis to low concentrations of CPT and CPT-11. Figure 10A shows that ^{3}H-thymidine incorporation into DNA by exponentially growing breast cancer cells is inhibited by 50% at approximately 0.04 μM CPT--a result consistent with previously published results on CPT's inhibition of nucleic acid synthesis in human leukemia HL60 cells (Kauffman, 1991). Preliminary results from our laboratory indicate that CPT-11 inhibited intact MDA MB 468 breast cancer cell DNA synthesis by approximately 50% at 1.8 μM (Figure 10B). The occurrence of low levels of DNA replication at higher drug concentrations suggests that topoisomerase II may partially substitute for the function normally provided by topoisomerase I (Annunziato, 1989).

Topoisomerase II also plays an integral role in DNA replication by facilitating the termination of DNA synthesis during interphase. We determined that approximately 2.4 μM VP-16 inhibited intact breast cancer cell DNA synthesis by 50% (Figure 10C). Intact cell DNA synthesis was impaired significantly at higher concentrations of the drug.

• ***In vitro DNA Replication Assays***

In vitro SV40 DNA replication assays were incubated with increasing concentrations of CPT, CPT-11, VP-16 and vinblastine, then analyzed for total ^{32}P-dCTP incorporation into DNA. Vinblastine, which inhibits mitotic microtubule assembly, was used as a negative control in these experiments. At concentrations ranging from 0.05-50 μM, vinblastine did not impair the DNA replication activity of the DNA synthesome. On the other hand, DNA synthesome-mediated replication was inhibited by almost 50% when reactions contained 0.5 μM CPT (Table 7). Furthermore, approximately 0.2 μM

SN-38 (the active metabolite of CPT-11) and 0.5 μM VP-16 inhibited *in vitro* DNA replication by 50% (Table 7). The relatively close correlation between the concentrations of these agents inhibiting DNA synthesis in intact cells and in the *in vitro* reactions supports the potential role of the DNA synthesome as a meaningful model to examine in detail the mechanisms of action of CPT-11 and VP-16.

Effect of CPT on DNA synthesome-associated and purified topoisomerase I activity

We performed topoisomerase I assays to establish that the topoisomerase I activity present in the DNA synthesome is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA into a form II DNA (Figures 11A and 11B, lanes 1 and 7 respectively). The pattern of topoisomers produced by the DNA synthesome-associated topoisomerase I is indistinguishable from that generated by the purified enzyme (Figure 12, lane 7).

In order to examine the effects of CPT and SN-38 on complex associated-topoisomerase I activity, we performed topoisomerase I assays in the presence of increasing concentrations of these drugs. In these assays, inhibition of topoisomerase I activity by CPT and SN-38 results in the accumulation of form I DNA. We observed an extensive level of inhibition of the complex associated-topoisomerase I activity by CPT (Figure 11A, lanes 3-6); as little as 0.5 μM CPT caused a significant retention of form I DNA. From these experiments, we determined that 0.5 μM CPT inhibited complex associated-topoisomerase I activity approximately 50%. This concentration of CPT is comparable to that which inhibits both *in vitro* and intact cell DNA synthesis by 50%; supporting the premise that the inhibition of DNA replication by CPT results from the drug's inhibition of topoisomerase I activity. Furthermore, a comparison of Figures 11A and 12 reveals that the complex-associated topoisomerase I is more sensitive to CPT poisoning than the purified enzyme.

Similar to CPT, 0.5-5 μM SN-38 inhibited synthesome-associated topoisomerase I activity significantly (Figure 11B, lanes 1-5). This concentration range of SN-38 corresponds with those concentrations of the agent inhibiting intact and *in vitro* DNA replication by 50%. Again, supporting the premise that the inhibition of DNA replication results from the drug's inhibition of topoisomerase I activity. Experiments to determine

the effects of SN-38 on purified topoisomerase I activity are currently in progress; as are experiments to examine the effects of VP-16 on complex-associated and purified topoisomerase II activity.

CONCLUSION

In this report, we have described for the first time the purification of a multiprotein DNA replication complex isolated from human breast cancer cells and breast tumor tissues. The integrity of the breast cancer cell DNA synthesome was maintained after treatment with high salt, polyethylene glycol precipitation, anion-exchange chromatography and sucrose gradient sedimentation. These results suggest that the co-purification of the synthesome's proteins with one another is independent of non-specific interactions with other cellular macromolecules. In addition, upon velocity sedimentation analysis of the breast cancer cell DNA synthesome, both the DNA polymerase α and DNA replication activities co-migrated in the sucrose gradient with a coefficient of 18S. This 18S sedimentation coefficient is comparable to that obtained for the HeLa cell DNA synthesome (Malkas et al., 1990b; Applegren et al., 1995).

Our data show that the DNA polymerase α and DNA replication activities of the synthesome isolated from breast cancer cells and breast tumor tissues, was enriched by the successive steps of the purification process. Furthermore, the P-4 as well as the Q-Sepharose peak fractions from the breast cancer cells and tissues possessed comparable levels of *in vitro* SV40 DNA replication activity. Overall, the isolation of the DNA synthesome as a fully functional complex from human and nude mouse xenograft breast tumor tissues, strongly suggests that the synthesome mediates DNA replication *in vivo*.

We have identified several of the key DNA replication proteins comprising the breast cancer cell DNA synthesome utilizing immunoblot analyses and enzymatic assays; these proteins include: DNA polymerase δ, PCNA, DNA polymerase α, DNA primase, RF-C, RP-A, DNA topoisomerases I, II, DNA polymerase ε and DNA methyltransferase. All of these polypeptides, excluding DNA polymerase ε and DNA methyltransferase, have been shown to be required for the faithful replication of SV40 DNA *in vitro* (Hurwitz et al., 1990; Malkas et al., 1990a; Stillman et al., 1992). We are presently

characterizing the breast tumor tissue-derived synthesome with respect to its protein components. Presumably, all of the proteins comprising the breast cancer cell DNA synthesome copurify with the tumor tissue-derived synthesome, as it is fully capable of supporting SV40 DNA replication *in vitro.*

In order to preserve the integrity of the information contained in DNA, normal mammalian cells must replicate their DNA with an error frequency as low as 10^{-10} (Tuusa et al., 1995). Such a high fidelity for DNA replication must be maintained by the DNA synthesis and DNA repair systems functioning within the cell. We utilized a forward mutagenesis assay (Roberts et al., 1988) to examine the fidelity with which the breast cancer cell and human breast tumor tissue DNA synthesome replicates plasmid DNA containing the *lac-Zα* gene. This assay detects point mutations occurring within the *lac-Zα* gene as well as frame-shift mutations occurring in other positions on the plasmid. We found a 5-6 fold decrease in the replication fidelities of the DNA synthesome isolated from malignant breast cells and tissue compared to that of the normal breast cell DNA synthesome (Table 6). Our results are consistent with the observation that mammary cancer cells accumulate extensive genetic damage (Thorgeirrson et al., 1993; Nutter et al., 1994). The significant difference in the replication fidelities between the malignant and normal breast cell DNA synthesome suggests that transformation alters the process by which the latter replicates and/or participates in the repair of DNA. Indeed, it has been demonstrated that specific DNA replication proteins are targets for molecular modification during cellular transformation (Loeb, 1991). Importantly, we have determined by two-dimensional polyacrylamide gel electrophoresis that significant physical differences exist between the protein components of the DNA synthesome purified from malignant and normal breast cells (Applegren, Bechtel, Malkas; unpublished data). We are currently conducting experiments to determine the precise molecular changes that occur to the components of the breast cell DNA synthesome during the transformation process.

Most importantly, in this report we have presented data that establishes that the DNA synthesome represents a powerful model for examining in detail the mechanisms of action of CPT-11 and VP-16. We base this assertion on the finding that the DNA

synthesome is as sensitive to CPT, CPT-11 and VP-16 poisoning as intact breast cancer cell DNA synthesis. This result supports the premise that the DNA synthesome represents the intact breast cancer cell's DNA replication machinery. In addition, we found a close correlation between the concentrations of CPT and SN-38 inhibiting 50% of *in vitro* DNA replication and complex-associated topoisomerase I activities. This is consistent with the premise that CPT and SN-38 inhibit nucleic acid synthesis by selectively targeting DNA topoisomerase I.

Ultimately, the complete characterization of the DNA synthesome may lead to a better understanding of the molecular mechanisms of breast cancer cell DNA replication. Moreover, the employment of the DNA synthesome as a model to examine in detail the mechanisms of action of anti-breast cancer agents that directly target DNA synthesis could facilitate the development of improved anti-breast cancer agents. We are currently performing studies to determine precisely which stage of DNA synthesis is halted by CPT-11 and VP-16; these studies will also allow us to determine the specific DNA sequences with which these agents interact. Future studies with the DNA synthesome may help to uncover the lethal cellular consequences of cleavable complex formation.

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Appendix A:

Figure Legends and Figures

Figure 1. Model for the human breast cell DNA synthesome. A full description of the model is presented in the text.

Figure 2. Flow diagram of the isolation scheme used to purify the DNA synthesome from MDA MB-468 human breast cancer cells. A detailed description of the isolation scheme is presented in the text and Materials and Methods.

Figure 3. Q-Sepharose chromatographic profile of DNA polymerase α activity in the MDA MB-468 derived P-4 fraction. A description of the column preparation and elution conditions are provided in the text and Materials and Methods.

Figure 4. Velocity sedimentation analysis of the DNA synthesome present in the MDA MB-468 Q-Sepharose peak fraction. 0.8 ml of the Q-Sepharose peak fraction were layered onto a 9 ml 10-30% sucrose gradient containing 0.5 M KCl. Centrifugation was performed as described in the Materials and Methods. One unit of activity denotes 1 nmol of TMP incorporated into DNA per hour at 35°C.

Figure 5. Western blot analysis of the MDA MB-468 breast cancer cell derived fractions. Thirty micrograms of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak (QS), Q-Sepharose flow-through (FT) and sucrose gradient peak (SG)) were resolved on 8% polyacrylamide gels, then transferred to nitrocellulose membrane filters. The membranes were incubated with primary antibodies against (a) DNA polymerase δ, (b) RP-A, (c) RF-C, (d) PCNA and (e) DNA primase. Following incubation with the appropriate species-specific secondary antibody conjugated to horseradish peroxidase, the immobilized proteins were detected using a light-enhanced

chemiluminescence system (Amersham). The relative positions of the protein molecular weight markers are designated by the markers.

Figure 6. DNA topoisomerase I activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Reaction assays containing 8 μg of the Q-Sepharose peak (QS), 8 μg of the Q-Sepharose flow-through (FT) or 20 μg of the sucrose gradient peak (SG) were incubated for 30 minutes at 37°C with 0.3 μg of pSVO$^+$ plasmid DNA. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (0.5 μg/ml) staining of gels, topoisomers were visualized with an ultraviolet light source. Lanes 1-3 show the conversion of supercoiled, form I DNA to relaxed, open circle form II DNA by the topoisomerase I activity present in the SG, FT and QS fractions, respectively. Lane 4 shows the inhibition of QS topoisomerase I activity by 200 μM of camptothecin. Lane 5 shows the position of supercoiled plasmid pSVO$^+$.

Figure 7. DNA topoisomerase II activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Decatenation reactions were performed in topoisomerase II buffer (TopoGen) with 0.15 μg of KDNA and 10μg of the respective protein fraction. Lane 1 shows the position of KDNA networks after incubation with Q-Sepharose peak (QS) in a buffer lacking ATP. Lanes 2-4 show the relaxation of KDNA to nicked, open circular DNA by the topoisomerase II activity present in the QS, flow-through (FT) and sucrose gradient peak (SG) fractions. Lane 5 shows the positions of the decatenated KDNA markers: nicked, open circular (top), linear (bottom). All reactions were stopped by the addition of a stop buffer containing 1% SDS. Reactions were loaded directly onto a 1% agarose gel containing 0.5 μg/ml ethidium bromide. After electrophoresis, DNA products were visualized with an ultraviolet light source.

Figure 8. Immunoblot analysis for the presence of DNA polymerase ε in the MDA MB-468 breast cancer cell derived fractions. 50 μg of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak (QS) and sucrose gradient peak (SG)) were resolved on a 8% polyacrylamide gel then transferred to a nitrocellulose membrane. The membranes were incubated with a primary antibody against human DNA polymerase ε. Following incubation with an anti-mouse secondary antibody conjugated to horseradish peroxidase, the immobilized protein was detected using a light-enhanced chemiluminescent system (Amersham).

Figures 9A, 9B

Effect of CPT-11 and VP-16 on MDA MB 468 cell survival

500 MDA MB 468 breast cancer cells were seeded onto 60-mm cell culture plates and incubated for 24 hours at 37°C. Cells were then exposed for one hour to increasing concentrations of CPT-11 (A) or VP-16 (B) (0, 0.05, 0.5, 5, 50 500 μM) dissolved in DMSO. Control plates contained DMSO alone. After removal of drug from the cell cultures, the cells were rinsed twice with warm phosphate-buffered saline and then incubated in fresh drug-free media for an additional 8-10 days. Colonies were visualized by staining with Giemsa stain. These graphs represent the average of 2 independent experiments; bars represent deviations from the average. For those symbols apparently lacking error bars, the deviation from the average was contained within the symbol.

Figures 10A, 10B, 10C

Effect of CPT, CPT-11 and VP-16 on intact MDA MB 468 cell DNA synthesis

5 x 10^4 MDA MB 468 breast cancer cells were seeded onto 60-mm cell culture plates and incubated for 24 hours at 37°C. The cells were then labeled with ^{3}H-thymidine (1 μCi/ml of medium) and exposed to increasing concentrations of CPT (A), CPT-11 (B) and VP-16 (C) (0, 0.05, .5, 5, 50, 500 μM) dissolved in DMSO. Plates containing DMSO alone served as controls. After a one hour incubation, cells were lysed and the level of

DNA synthesis was measured by the isolation and counting of ^{3}H in acid-insoluble material. These graphs represent the average of 2 separate experiments; error bars represent standard error of the mean.

Figures 11A, 11B

Inhibition of DNA synthesome-associated and purified topoisomerase I activity by CPT

(A) Reaction assays containing 8 μg of the Q-Sepharose peak were incubated for 30 minutes at 37°C with 0.3μg supercoiled pSVO+, in the presence of increasing concentrations of CPT (0, 0.05, 0.5, 5, 50 μM) dissolved in DMSO. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (1μg/ml) staining of the gels, topoisomers were visualized by illuminating gels with an ultraviolet light source. Lane 2 shows the position of supercoiled $pSVO^{+}$ DNA in the presence of DMSO. Lane 1 shows the conversion of supercoiled DNA to relaxed, open circle form II DNA by the synthesome-associated topoisomerase I present in the Q-Sepharose peak. Lanes 3-6 show the inhibition of complex-topoisomerase I activity by increasing concentrations of CPT. (B) Experiment was performed as above using 2 units of purified topoisomerase per assay. Lane 7 shows the conversion of supercoiled DNA to relaxed form II DNA by 2 units of purified topoisomerase I. Lane 1 shows the position of supercoiled $pSVO^{+}$ DNA in the presence of DMSO. Lanes 2-6 show the inhibition of topoisomerase I activity by increasing concentrations of CPT.

Figure 12

Inhibition of DNA synthesome associated topoisomerase I activity by SN-38

Reaction assays containing 8 μg of the Q-Sepharose peak were incubated for 30 minutes at 37°C with 0.3μg supercoiled pSVO+, in the presence of increasing

concentrations of SN-38 (0, 0.05, 0.5, 5, 50, 500 μM) dissolved in DMSO. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (1μg/ml) staining of the gels, topoisomers were visualized by illuminating gels with an ultraviolet light source. Lane 6 shows the position of supercoiled $pSVO^+$ DNA in the presence of DMSO. Lane 7 shows the conversion of supercoiled DNA to relaxed, open circle form II DNA by the synthesome-associated topoisomerase I present in the Q-Sepharose peak. Lanes 2-6 show the inhibition of complex-topoisomerase I activity by increasing concentrations of SN-38

.

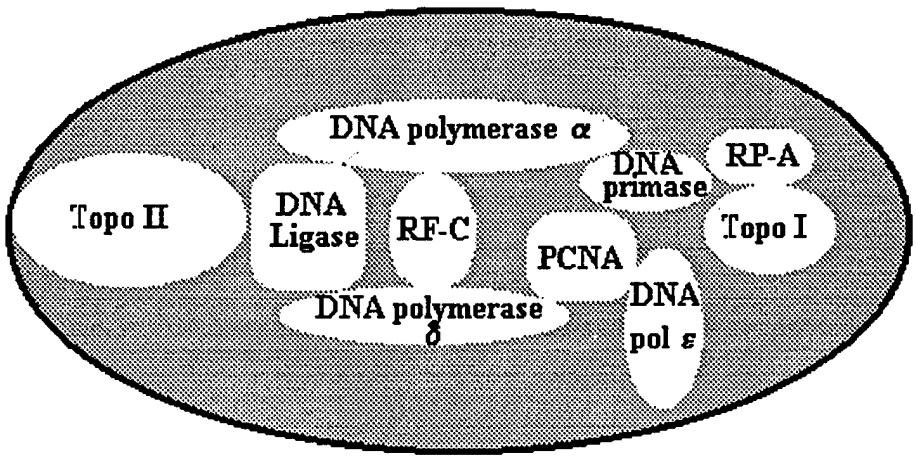

DNA polymerase α
DNA primase
RP-A
Topo II
DNA Ligase
RF-C
Topo I
PCNA
DNA polymerase δ
DNA pol ε

Figure 1

Fractionation Scheme for the Breast Cancer Cell DNA Synthesome

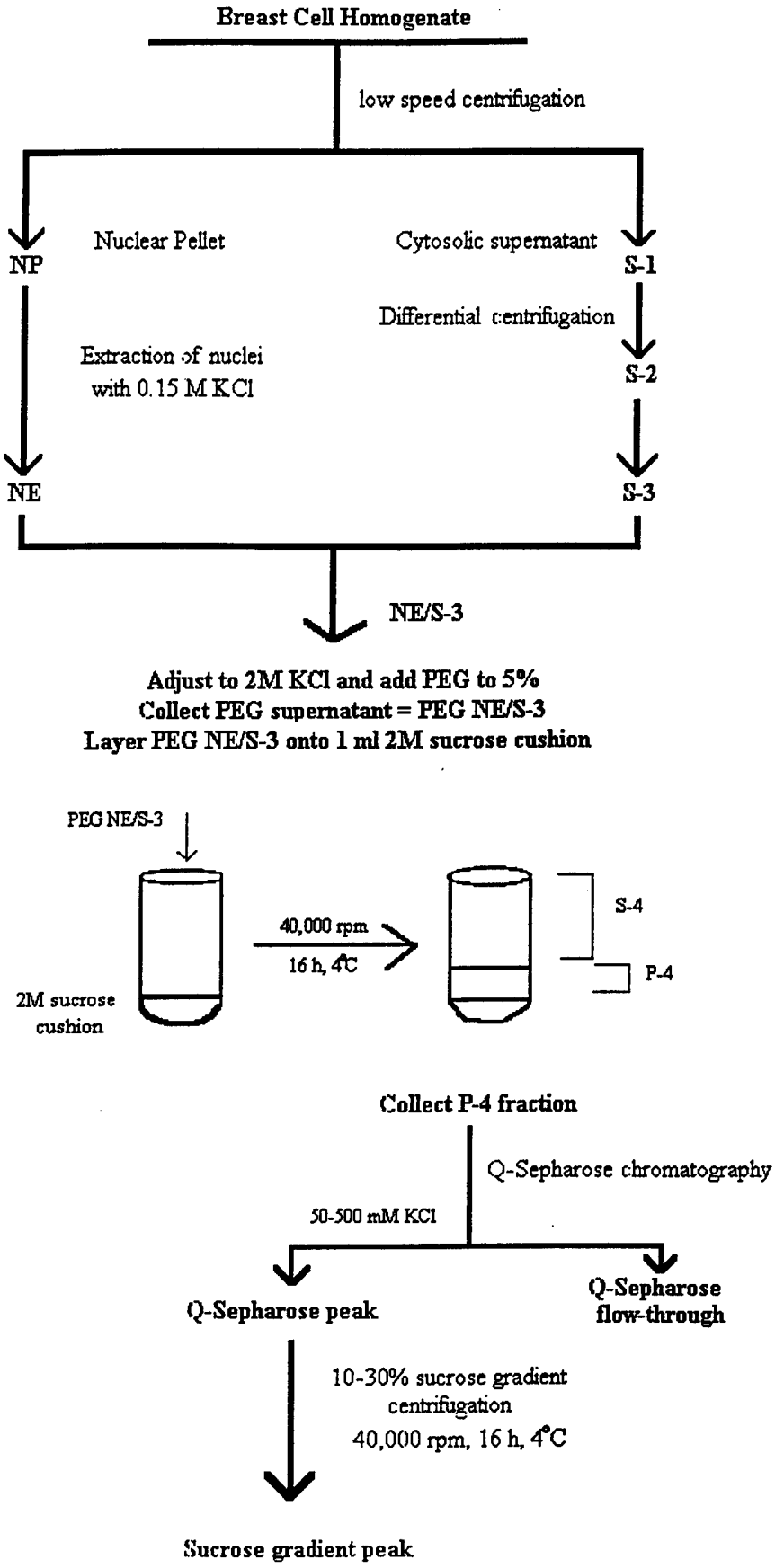


Figure 2

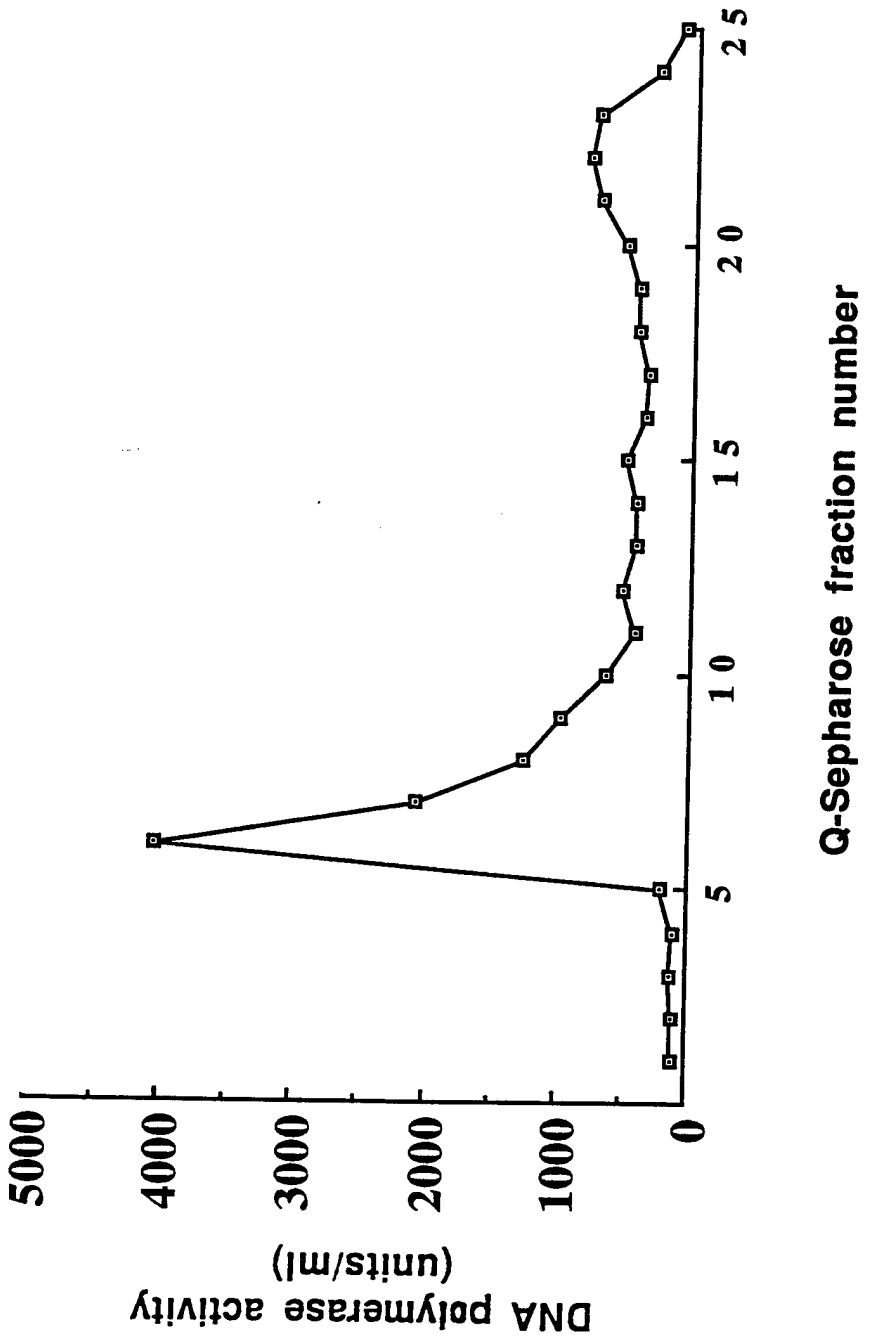

DNA polymerase activity
(units/ml)
5000
4000
3000
2000
1000
0
5
10
15
20
25
Q-Sepharose fraction number

Figure 3

Profile of DNA Polymerase α Activity in the Sucrose Gradient Fractions

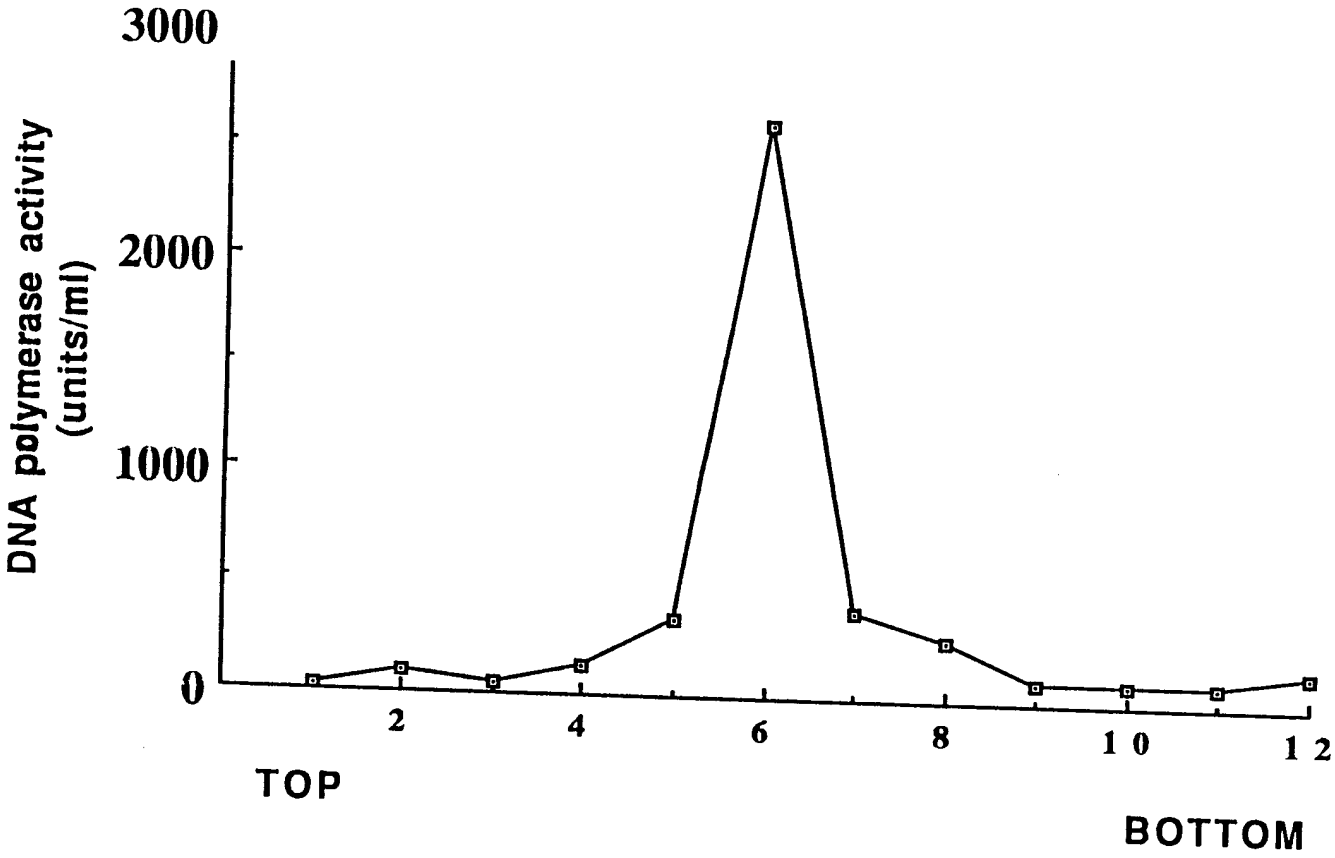


Figure 4

Western Blot Analysis of DNA Replication Proteins Present in the Breast Cancer Cell MRC

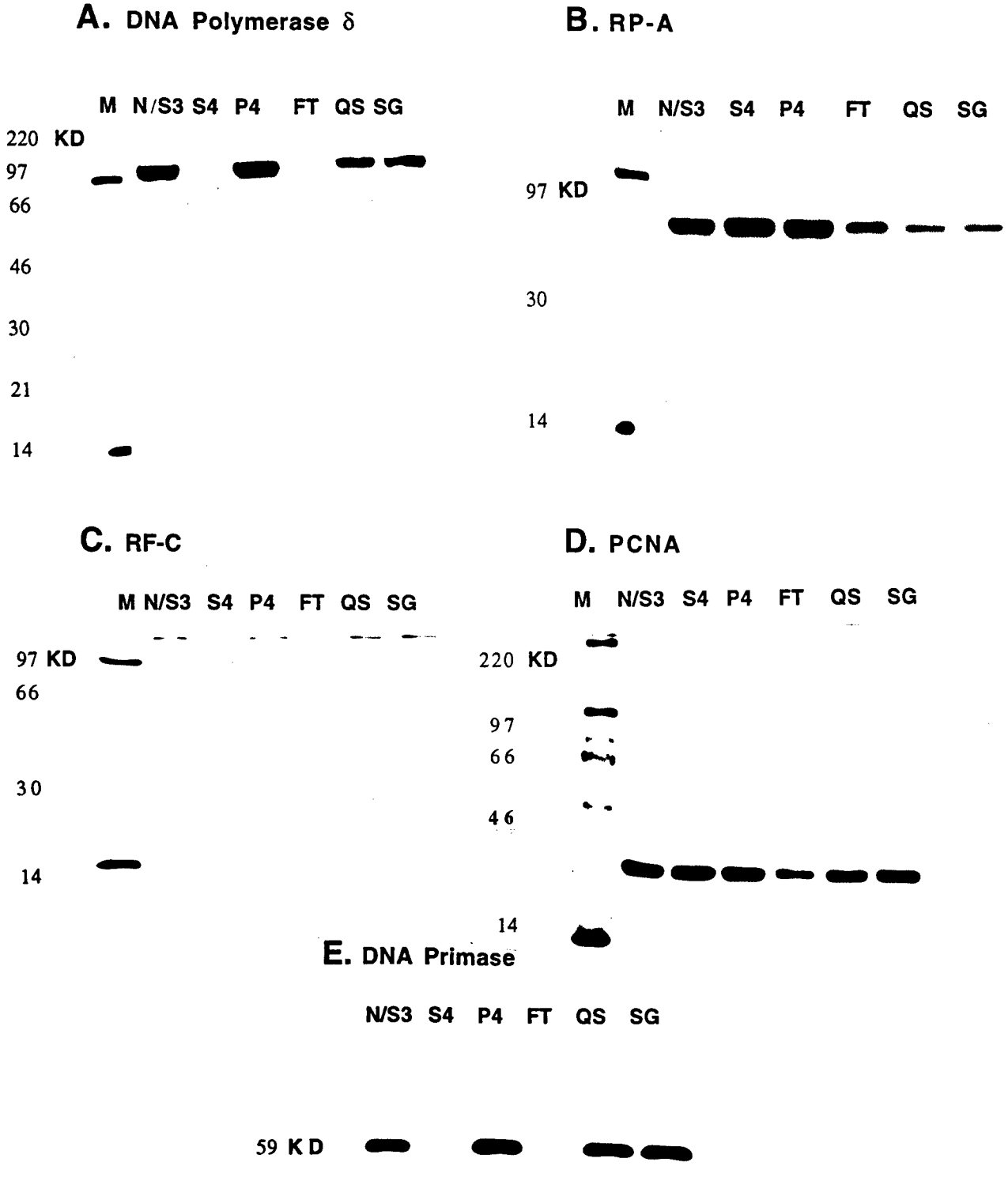


Figure 5

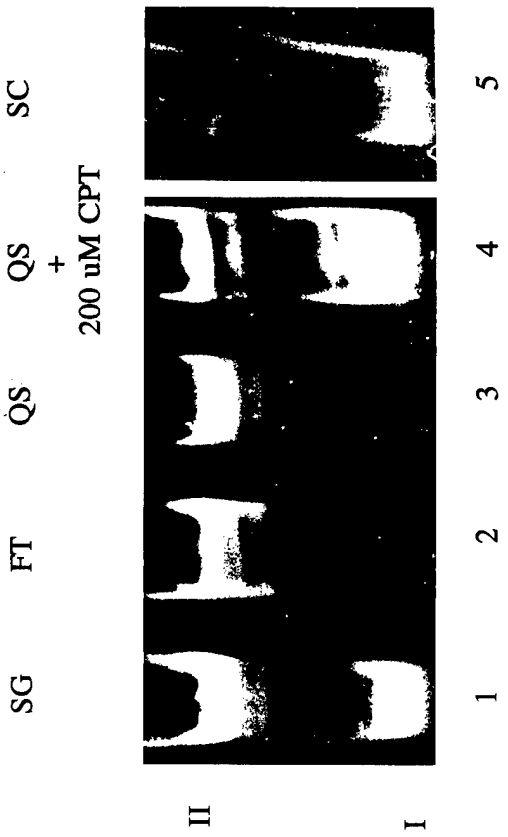


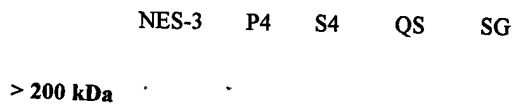
Figure 6

KDNA networks

Decatenated DNA
Linear DNA

1 2 3 4 5

Figure 7



> 200 kDa

Figure 8

Effect of CPT-11 on MDA MB 468 Breast Cancer Cell Survival

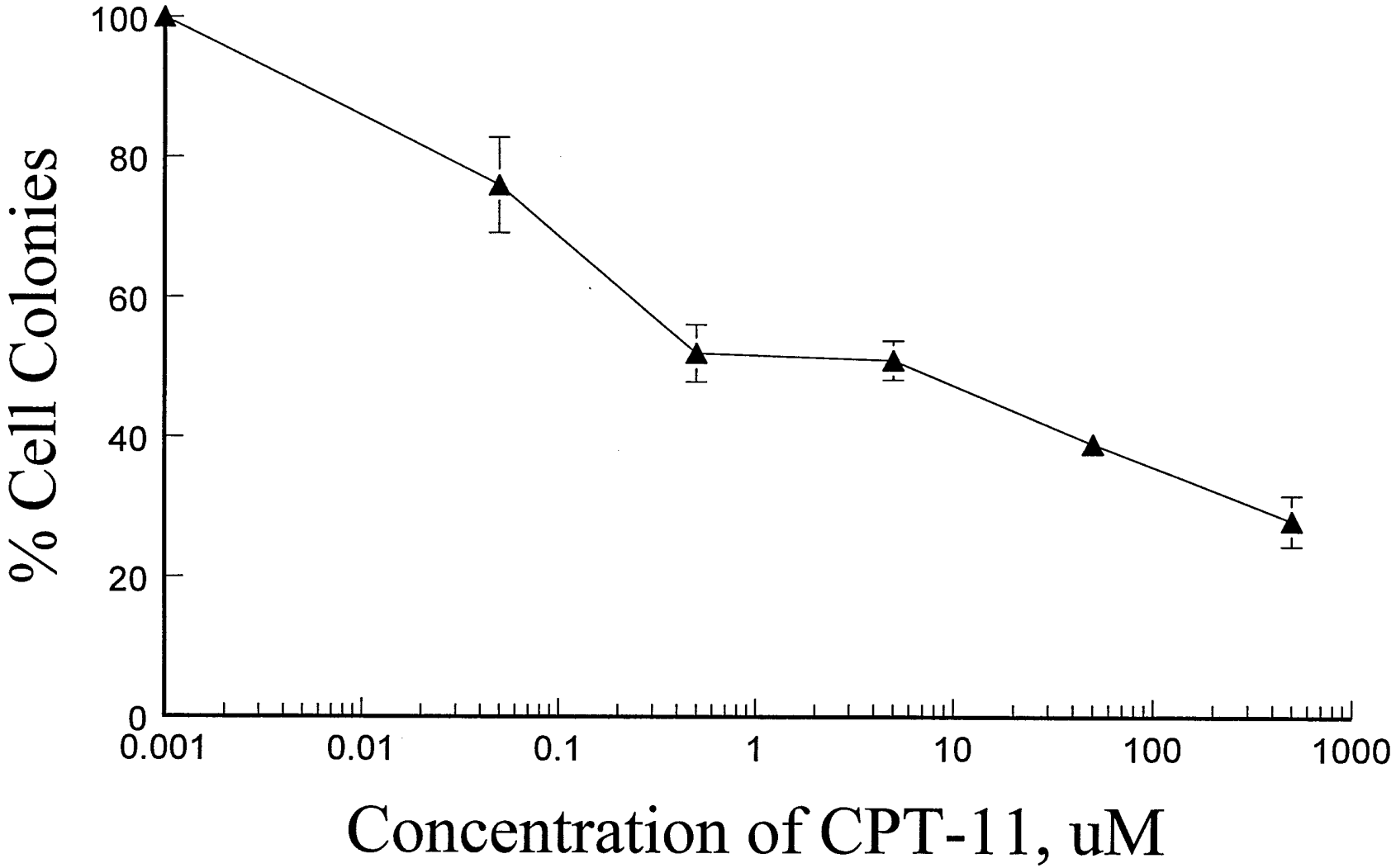


Figure 9a

Effect of Etoposide on MDA MB 468 Breast Cancer Cell Survival

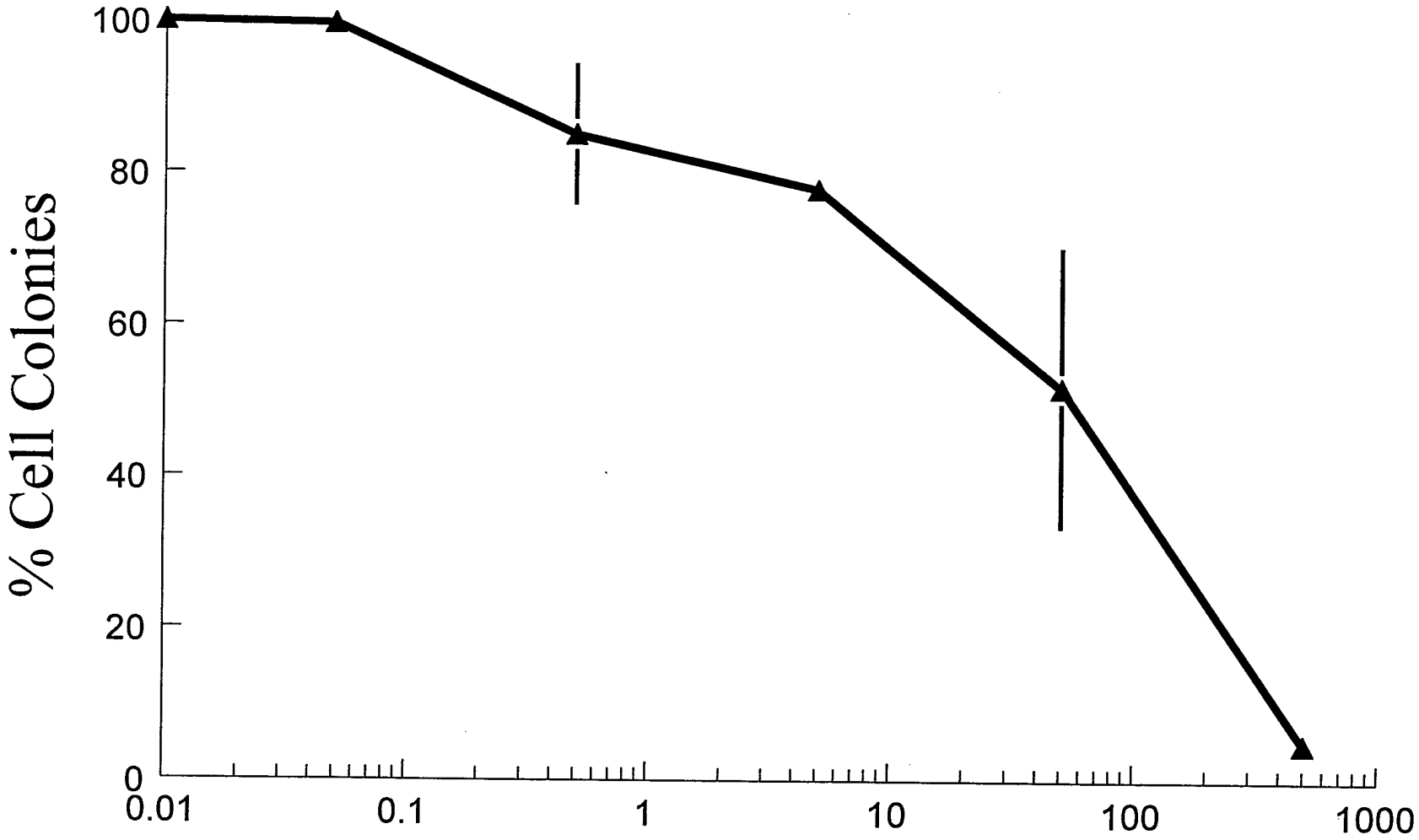


Figure 9b

Effect of Camptothecin on MDA MB 468
Intact Cell DNA Synthesis

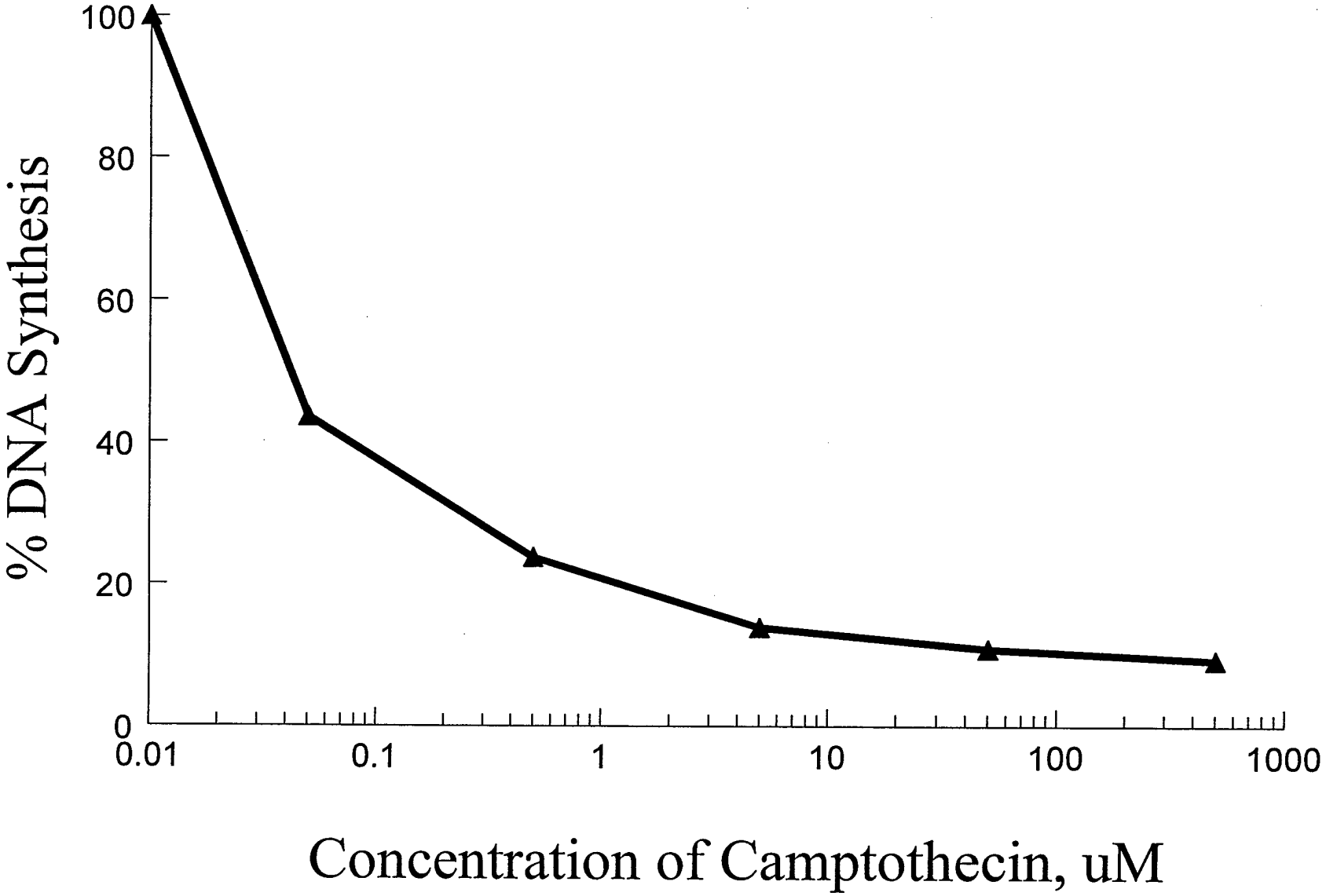


Figure 10a

Effect of CPT-11 on Intact MDA MB 468 Breast Cancer Cell DNA Synthesis

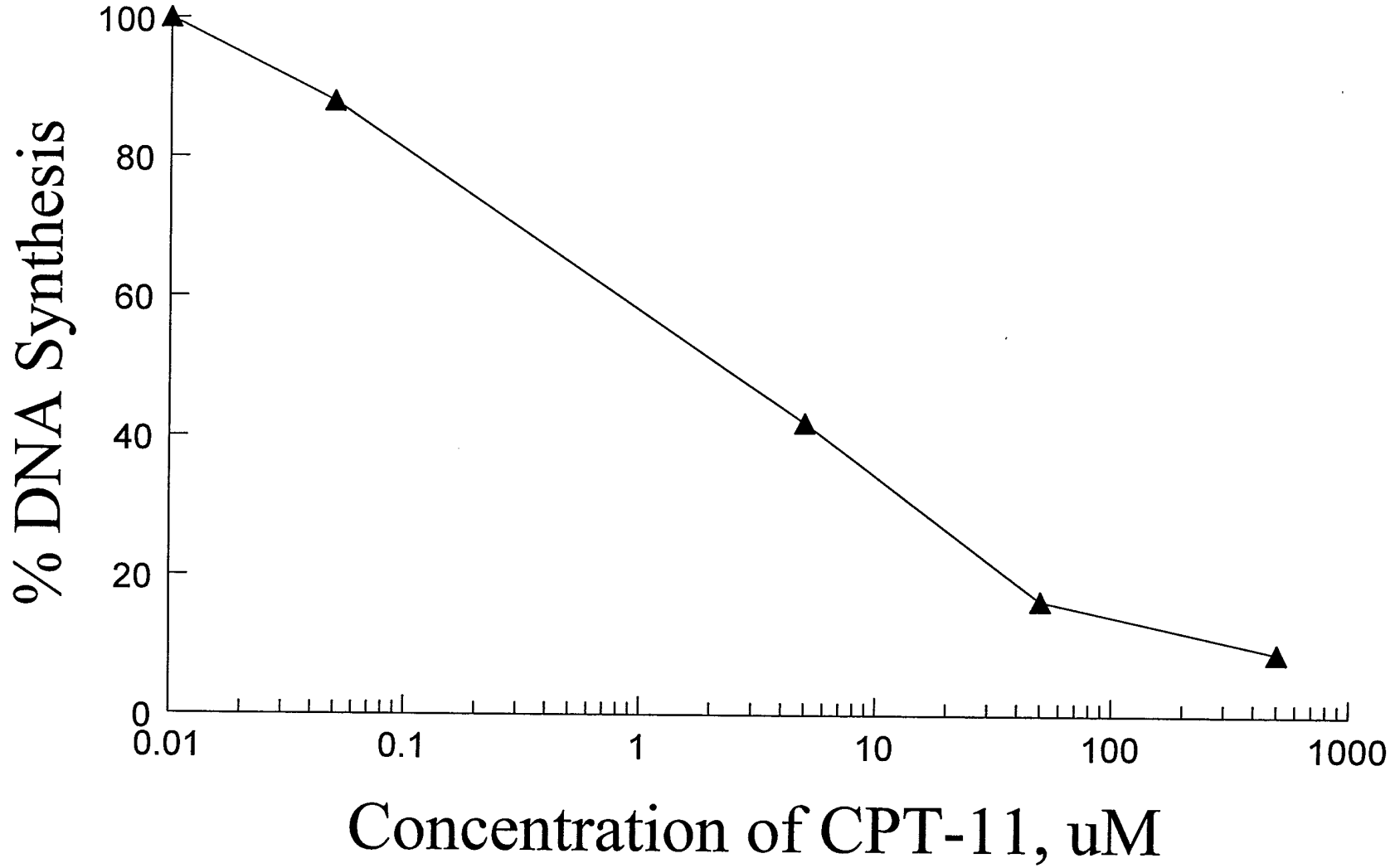


Figure 10b

Effect of Etoposide on MDA MB 468
Intact Cell DNA Synthesis

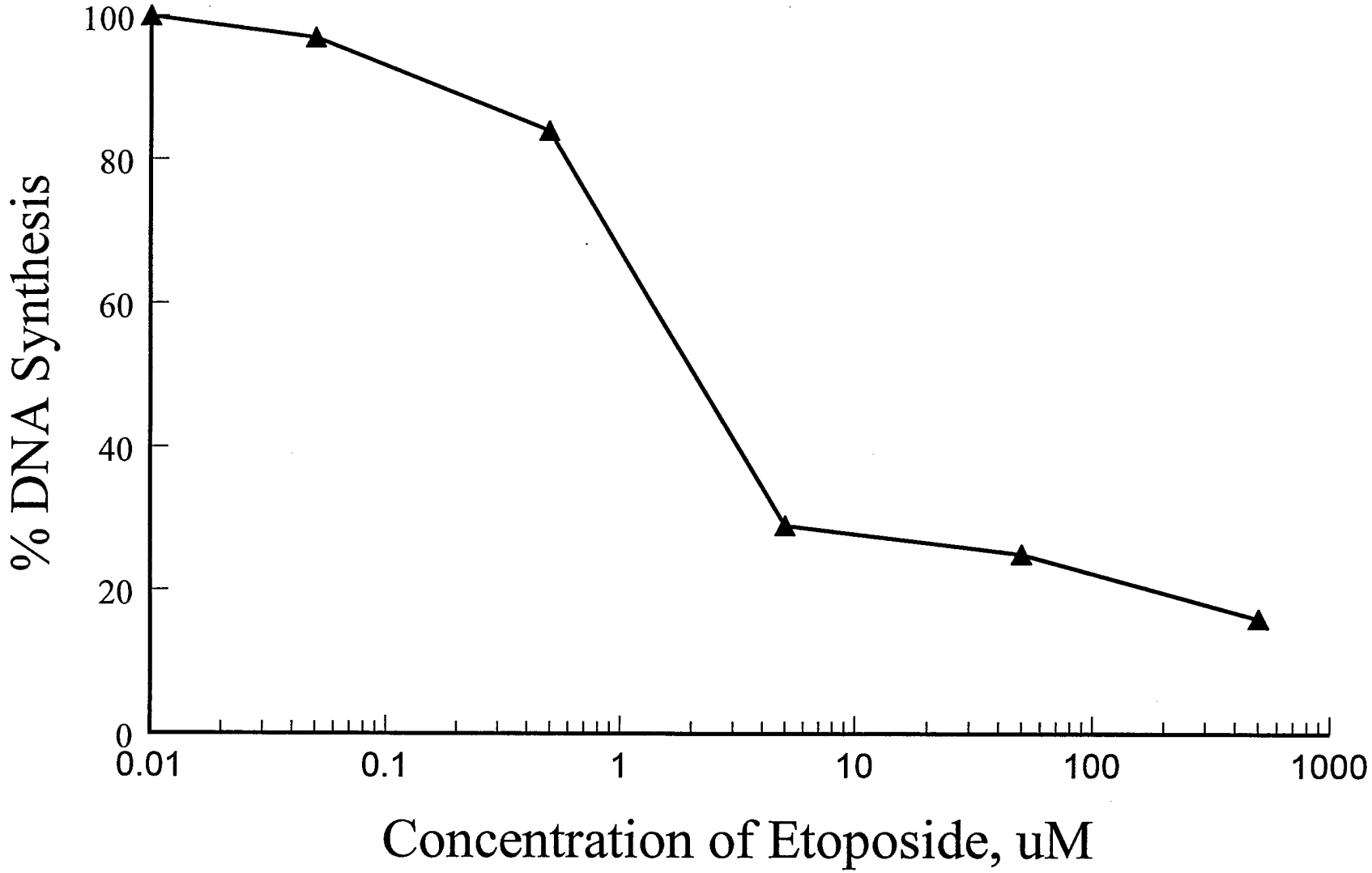


Figure 10c

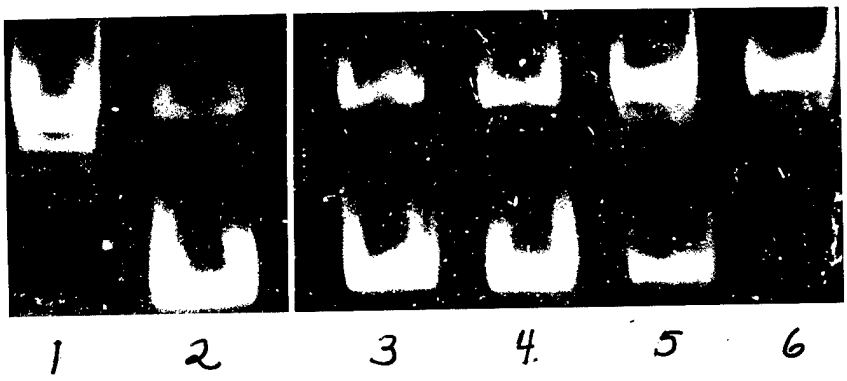


Figure 11a

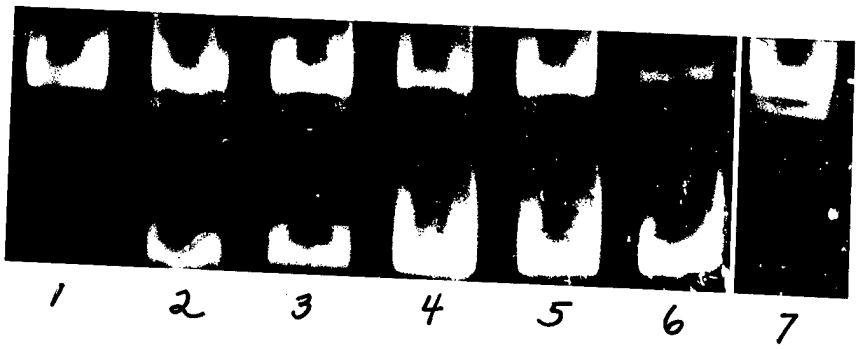


Figure 11b

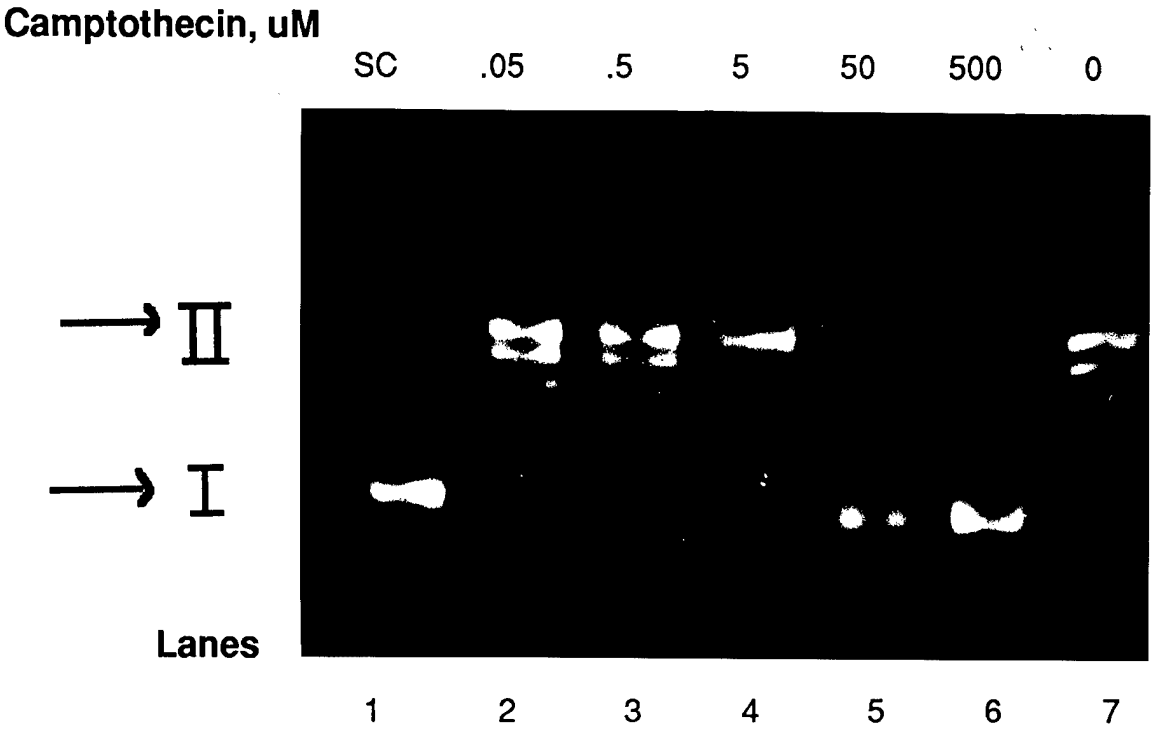


Figure 12

Table 1. DNA polymerase α and *in vitro* DNA replication activities fractionate with the P-4 fraction.

Fraction	PEG NE/S-3	S-4	P-4
DNA polymerase α*	132.5	0.3	188.3
DNA replication +T+	103.8	8.8	110.6
DNA replication -T+	3.1	0.0	0.2

*DNA polymerase activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity equals 1 nmole of ^{3}H-TMP incorporated into DNA per hour at 35°C. These values represent the average of three experiments.

+*In vitro* SV40 DNA replication assays were performed as described in our previously published procedures. One unit of activity is equal to the incorporation of 1 pmole of ^{32}P-dCMP into SV40 origin containing DNA per 2 hours at 35°C. These values represent the average of three experiments.

Table 2. *In vitro* DNA replication activities fractionate with the Q-Sepharose and sucrose gradient peak fractions.

Fraction	Q-Sepharose Peak	Flow Through (FT)	Sucrose Gradient
DNA replication $+T^{+}$	136.6	3.4	141.2
DNA replication $-T^{+}$	8.5	1.8	10.2

$^{+}$*In vitro* SV40 DNA replication assays were performed as described in our previously published procedures. One unit of activity is equal to the incorporation of 1 pmole of ^{32}P-dCMP into SV40 origin containing DNA per 2 hours at 35°C. These values represent the average of three experiments.

Table 3. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from human breast tumor tissue.

Fraction	NE/S-3	S-4	P-4
DNA polymerase α*	27.8	1.7	37.5
DNA Replication +T#	29.5	1.8	122.9
DNA Replication -T#	5.74	0.5	12.1

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}H-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

In vitro SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol of ^{32}P-dCMP into SV40 origin containing DNA. These values represent the average of two independent experiments.

Table 4. DNA polymerase α activity of the column purified DNA synthesome from human breast cancer tissue.

Fraction	Peak	Flow-through
DNA polymerase α*	77.3	1.3

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}H-TMP incorporated into DNA per hour at 35°C. Fractions 2 and 3 constitute the peak of DNA polymerase α activity. These values represent the average of two independent experiments.

Table 5. DNA polymerase α and *in vitro* SV40 DNA replication activities of the DNA synthesome from nude mouse tumor tissue.

Fraction	NE/S-3	S-4	P-4
DNA polymerase α*	40.6	2.0	123.2
DNA Replication +T#	57.2	11.2	158.7
DNA Replication -T#	5.5	4.1	9.6

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}H-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

In vitro SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol of ^{32}P-dCMP into SV40 origin containing DNA. These values represent the average of two independent experiments.

Table 6. DNA replication fidelity of the breast cell DNA synthesome: Blue/White selection assay.

Origin of Synthesome	% Mutants (average per 10^4 colonies)
MDA MB-468 breast cancer cell line	1.20% +/- 0.2%
Human breast tumor tissue	0.93% +/- 0.3%
Hs578Bst non-malignant breast cell line	0.19% +/- 0.08%

An *in vitro* DNA replication fidelity assay was used to measure the fidelity with which the synthesome from MDA MB 468 breast cancer cells, human breast tumor tissue and Hs578Bst breast cells replicates plasmid DNA. The replicated plasmid DNA, containing the bacterial lac-Z gene, was Dpn I digested and electroporated into E. coli, which were then plated onto LB agar containing the chromogenic substrates X-gal and IPTG. Transformed bacteria expressing a non-mutated lac-Z gene (encodes the B-galactosidase enzyme) formed blue colonies on the plate, while bacteria containing DNA with mutations in the lac-Z gene formed white colonies. Mutations occurring in locations other the lac-Z gene on the pBK-CMV plasmid are not detected by this forward mutagenesis assay. Consequently, the reported percentage of white colonies provides a minimum estimate of the actual number of mutations occurring in DNA synthesome mediated DNA replication. The percentage of mutant colonies expressed for each breast cell DNA synthesome is the average number taken from 3 separate assays of 10^4 transformed colonies each.

Table 7. IC_{50}s for the inhibition of *in vitro* DNA synthesis by CPT, SN-38 and VP-16.

Drug	IC_{50}s for in vitro DNA synthesis*
CPT	**0.5 μM**
SN-38	**0.2 μM**
VP-16	**0.5 μM**
Vinblastine	**No inhibitory effect at concentrations 0.05-50 μM.**

**In vitro* SV40 DNA replication assays were performed as described in the Material and Methods. The reported IC_{50} concentrations are representative of those values typically obtained in these reactions.

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BIOGRAPHICAL SKETCH

EDUCATION

Institution and Location	Degree	Year
University of Maryland at Baltimore Pharmacology and Experimental Therapeutics Baltimore, Maryland	PhD candidate	1993-present
University of Notre Dame Notre Dame, Indiana	B.S., Biological sciences	May, 1992

PREVIOUS RESEARCH EXPERIENCE

- Undergraduate research assistant
1/92-9/92 *Molecular parasitology* Research goal focused on the development of a malaria vaccine (Plasmodium *falciparum*). The experimental design utilized immunological and molecular biology techniques to study the malarial sporozoite proteins involved in red blood cell invasion.

AWARDS AND RECOGNITION

- United States Army Medical Research and Development Command Breast Cancer Predoctoral Fellow (7/94 - 7/98)
- Sigma Xi Grant-in-Aid of Research (7/94 - 7/95)
- University of Maryland Graduate Student Association Travel Award (1/95)
- Invited Participant of the American Association for Cancer Research sponsored *Histopathobiology of Cancer Workshop*, Keystone, Colorado, July 9-16, 1995
- Who's Who among U.S. Graduate Students (1996)
- The National Dean's List (1996)

EXTRACURRICULAR ACTIVITIES

- Associate member of the American Association for Cancer Research (7/95-present)
- Member of the Association for Women in Science (AWIS; 1994-present)
- Department of Pharmacology and Experimental Therapeutics representative to the Student Government Association (1993-'94)
- Associate Member of Sigma Xi, the Scientific Research Society (1996)
- Women in Cancer Research (1996)

ABSTRACTS

Coll, J., Wei, Y. and Malkas, L. A unique model system to investigate the mechanisms of action of camptothecin on DNA synthesis. Presented at the ASBMB national meeting in Washington, D.C., May 1994.

Malkas, L., Wu, Y., Applegren, N., Li, N., *Coll, J.*, Tuteja, N. and Hickey, R.J. The mammalian cell multiprotein DNA

replication complex (MRC). Presented at the ASBMB national meeting in Washington, D.C., May 1994.

Coll, J., Glazer, J., Hickey, R.J. and Malkas, L.H. Multiprotein replication complex protein-protein interactions. Presented at the Third McGill University Conference on Regulation of Eukaryotic DNA Replication in Montreal, Quebec, Canada, October, 1994.

Coll, J., Hickey, R.J., Zhou, Q. and Malkas, L.H. A multiprotein DNA replication complex (MRC) isolated from human breast cancer cells. Presented at the AACR Histopathobiology of Cancer Workshop in Keystone, Colorado, July 1995.

Coll, J., Hickey, R.J., Weeks, J., Schnaper, L., Yue, W., Brodie, A. and Malkas, L.H. Human breast cancer cells mediate DNA replication via an organized DNA synthesome. Presented at the American Association for Cancer Research Meeting in Washington, D.C., April, 1996.

Bechtel, P., *Coll, J.*, Malkas, L. and Hickey, R. The identification of structural alterations in the DNA synthetic apparatus of human breast cancer cells. Presented at the American Association for Cancer Research Meeting in Washington, D.C., April, 1996.

MANUSCRIPTS

Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., *Coll, J.*, Wills, P., Swaby, R., Wei, Y., Quan, J.Y., Lee, M. and Malkas, L.H. (1995) Further characterization of the human cell multiprotein DNA replication complex (MRC). Journal of Cellular Biochemistry 59:1-16.

Coll, J., Hickey, R.J., Wei, Y. and Malkas, L.H. (1996) The human cell multiprotein DNA replication complex (MRC): The effect of camptothecin on its ability to support *in vitro* DNA synthesis. Cancer Chemotherapy and Pharmacology (in press).

Coll, J.M., Weeks, J.G., Hickey, R.J., Schnaper, L., Wue, Y., Brodie, A.H.M., Svaoja, J. and Malkas, L.H. (1996) The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture. Oncology Research (submitted).

Cancer Chemother Pharmacol (1996) 00: 000–000

ORIGINAL ARTICLE

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The human cell multiprotein DNA replication complex (MRC): the effect of camptothecin on its ability to support in vitro DNA synthesis

Received: 24 March 1995/Accepted: 22 March 1996

Abstract *Purpose*: We have previously reported on the isolation and characterization of a multiprotein DNA replication complex (MRC) from HeLa cells that fully supports in vitro DNA replication. Based upon its ability to replicate DNA in a cell-free environment (devoid of other cellular processes) the MRC may serve as a unique model system for investigating the mechanisms of action of anticancer drugs that directly affect DNA synthesis. The experiments described in this report were performed to establish whether the MRC could serve as a model system to examine in detail the mechanism of action of camptothecin, a DNA topoisomerase I inhibitor. *Methods*: We examined the effects of increasing concentrations of camptothecin on HeLa cell survival, intact HeLa cell DNA synthesis and MRC-mediated in vitro DNA replication. We also performed topoisomerase I assays in the presence of increasing concentrations of camptothecin to study the direct effects of the agent on MRC-associated topoisomerase I activity. Furthermore, we employed an SDS precipitation assay to measure the formation of MRC-associated topoisomerase I-cleavable complexes in the presence of increasing concentrations of camptothecin. *Results*: We found a close correlation between the IC_{50} values for intact HeLa cell DNA synthesis (0.15 μM) and MRC-mediated in vitro DNA synthesis (0.05 μM). Similarly, we found that 0.05 μM camptothecin inhibited MRC-associated topoisomerase I activity by approximately 50%. In addition, we found that the formation of MRC-associated topoisomerase I-cleavable complexes increased linearly with increasing concentrations of camptothecin. *Conclusions*: The data presented in this report support the use of the MRC as a model system to study the mechanism of action of camptothecin. We anticipate that future studies with the MRC will help elucidate the cellular consequences of camptothecin-cleavable complex formation.

Key words Camptothecin · Topoisomerase I · DNA replication complexes · Anticancer agents · In vitro model system

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Introduction

Camptothecin is a plant alkaloid that demonstrates potent antitumor activity against a wide range of human cancers [17]. It inhibits both RNA and DNA synthesis in mammalian cells during S phase and causes subsequent G2 cell cycle arrest [18]. Camptothecin possesses a unique mechanism of action in that it traps nuclear topoisomerase I in a reversible enzyme-drug-DNA cleavable complex [9, 11]. In this state, topoisomerase I cannot perform its DNA single strand nicking resealing function required for the relaxation of supercoiled DNA. However, it is unclear whether or not this mode of action is responsible for camptothecin's cytotoxicity in vivo. Researchers speculate that the interaction of drug-stabilized cleavable complexes with advancing replication forks culminates

change To [5, 9, 11]

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in replication fork breakage and the accumulation of irreversible topoisomerase I-linked DNA single-strand breaks [6, 18]. These drug-induced recombinational events appear as smears and late-Cairns arcs when the aberrant DNA replication products are resolved on two-dimensional gels [16, 20].

We have utilized a novel in vitro system to examine the inhibition of DNA synthesis and DNA topoisomerase I activity by camptothecin. Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex (MRC) from human cells that supports origin-specific and large T-antigen-dependent simian virus 40 (SV40) DNA replication in vitro [4, 7, 14, 15]. The integrity of the MRC is maintained following treatment with detergents, salt, RNase and DNase, suggesting that the association of the proteins with one another is independent of nonspecific interactions with other cellular macromolecules [24]. We have shown that the DNA replication activity of the human cell MRC is comparable to that observed in intact cells [3]. The proteins observed to copurify with the MRC include the key DNA synthetic enzymes DNA polymerase α, DNA polymerase δ, DNA primase, PCNA, DNA ligase I, DNA helicase, RF-C, RPA, RNase H, DNA topoisomerase II and a fully functional topoisomerase I [3, 15, 24].

In this report, we describe experiments that establish that the MRC could serve as a valuable, in vitro model to examine in detail the mechanism of action of camptothecin. Such work would help elucidate the cellular consequences of cleavable complex formation as well as facilitate the development of improved camptothecin analogues.

Materials and methods

Materials

Camptothecin was purchased from TopoGen, Ohio. The drug was dissolved in dimethyl sulfoxide and stored in aliquots at − 20 °C.

Purified topoisomerase I was also purchased from TopoGen. One unit of enzyme relaxes 0.5 μg DNA in 15 min at 37 °C. The enzyme was supplied at a concentration of 2 units/μl, 4 ng/μl.

α^{32}P-dCTP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) and ^{3}H-thymidine (90 Ci/mmol; 37 MBq; 2.5 mCi/ml) were obtained from DuPont New England Nuclear.

Cell culture and harvest

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

Fractionation and chromatographic scheme for isolation of HeLa cell MRC

The HeLa cell MRC was isolated as described by Malkas et al. [15]. The protein fraction designated Q-Sepharose peak (1 μg/μl), which contains the DNA replication-competent MRC, was used in the experiments described in this report.

HeLa cell survival assays

HeLa cell survival assays were performed in the presence of increasing concentrations of camptothecin, according to the procedure described by Ryan et al. [18].

Measurement of intact HeLa cell DNA synthesis

Exponentially growing HeLa cells were incubated at 37 °C with increasing concentrations of camptothecin in the presence of ^{3}H-thymidine. After a 1-h incubation, cells were lysed [8] and the amount of radiolabel incorporated into DNA was determined by the isolation and counting of acid-insoluble material [19].

In vitro SV40 DNA replication assay

Assay reaction mixtures (25 μl) contained 80 m*M* Tris-HCl, pH 7.5; 7 m*M* $MgCl_2$; 1 m*M* DTT; 1–5 μg Q-Sepharose peak; 0.5–1.0 μg purified SV40 large T-antigen; 25 ng plasmid pSVO + containing an insert of SV40 replication-origin DNA sequences [21]; 100 μ*M* each dTTP, dATP, dGTP; 200 μ*M* each rCTP, rGTP, rUTP; 4 m*M* ATP; 50 μ*M* α^{32}P-dCTP; 40 m*M* creatine phosphate; and 1 μg creatine kinase. The standard reaction, conducted in the absence or presence of camptothecin, was incubated for 2 h at 37 °C. Replication assay products were processed using DE81 filter binding to determine the amount of radiolabel incorporated into acid-insoluble material [19].

Topoisomerase I assay

Topoisomerase I activity was measured by incubating 0.3 μg supercoiled pSVO + DNA with either 8 μg Q-Sepharose peak, in the absence or presence of increasing concentrations of camptothecin, or 2 units purified topoisomerase I. Incubations were performed for 20 min at 37 °C. Each reaction (15 μl) was stopped with 1% SDS and the DNA products resolved on a 1.0% agarose gel containing TAE buffer (40 m*M* Tris acetate, 2 m*M* EDTA). After ethidium bromide (1 μg/ml) staining of the gels [19], topoisomers were visualized with an ultraviolet light source.

Quantitation of the amount of topoisomerase I present in the Q-Sepharose peak

The densities of fully converted form II DNAs produced by purified topoisomerase I (5 units) and Q-Sepharose peak (3 μl) were scanned by a laser densitometer. The form II DNA relaxed by the purified enzyme possessed a twofold greater density than that relaxed by the MRC-associated enzyme. Based upon this comparison, a mathematical relationship was used to estimate the amount of topoisomerase I present in the Q-Sepharose peak:

$$\text{topo I in Q-Seph peak (ng/μl)} = \frac{0.5\ (10\ \text{ng purified topo I})}{3\ \mu\text{l Q-Seph peak}}$$

$$= 1.66\ \text{ng/μl}$$

5′ end-labeling of linear plasmid DNA

Plasmid pSVO + DNA (20 μg) was digested with 40 units Hind III for 60 min at 35 °C. The digested DNA (1 μg) was incubated with 1 unit of Klenow fragment (5 units/μl) for 30 min at room temperature in a reaction mixture containing: 2.5 μl end-labeling buffer (0.5 *M* Tris-HCl (pH 7.6), 0.1 *M* $MgSO_4$, 1 m*M* dithiothreitol, 500 μg/ml BSA); 0.75 μl 100 m*M* $MgCl_2$; 2.5 μl 10X dGTP; 2.5 μl 10X dATP; 2.5 μl 10X dTTP; 4 μl ^{32}P-dCTP (3000 Ci/mmol, 10 mCi/ml); 2.25 μl dH_2O. Reactions were stopped by the addition of 1 μl 0.5 *M* EDTA and heating to 65 °C for 5 min; the reaction mixtures were then diluted to 100 μl with a buffer containing 0.1 *M* NaCl, 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA. Unincorporated deoxynucleotide triphosphates were removed by chromatography through a p60 gel filtration column [19].

SDS precipitation of topoisomerase I-cleavable complexes

Cleavage of DNA by human topoisomerase I was performed as follows. Briefly, 5 units purified topoisomerase I or 8 μg Q-Sepharose peak, 1 μl end-labeled pSVO + DNA and increasing concentrations of camptothecin were added to a reaction buffer containing 10 m*M* Tris-HCl (pH 9.0), 0.5 m*M* EDTA and 10 μg/ml BSA. After a 5-min incubation at 37 °C, SDS precipitation of double-stranded DNA topoisomerase I complexes was performed as described by Liu et al. [13].

Results

Inhibition of HeLa cell survival, intact HeLa cell DNA synthesis, and MRC-mediated DNA synthesis by camptothecin

We performed HeLa cell survival assays (Materials and methods) to verify that camptothecin affects the ability of mammalian cells to proliferate, as previously reported [1]. We exposed HeLa cells to increasing concentrations of camptothecin for a 1h, then washed the cells and incubated them in fresh drug-free medium for of 8–10 days. Figure 1 shows that exposing HeLa cells to increasing concentrations of camptothecin for 1 h, impaired cell survival. At the highest concentration of camptothecin used in this experiment (5 μ*M*), approximately 66% of the HeLa cells remained viable. This result is indicative of the S-phase specificity of the drug and is consistent with previous reports on camptothecin cytotoxicity [22].

Topoisomerase I plays a pivotal role in DNA replication, facilitating replication fork migration by unwinding positive supercoils as they accumulate ahead of the fork. The necessity for topoisomerase I in DNA synthesis is underscored by the sensitivity of intact HeLa cell DNA synthesis to low concentrations of camptothecin. Figure 2 shows that ^{3}H-thymidine incorporation into DNA by exponentially growing HeLa cells (Materials and methods) is inhibited by 50% at approximately 0.15 μ*M* camptothecin–a result consistent with previously published results on camptothecin's inhibition of nucleic acid synthesis in HL60

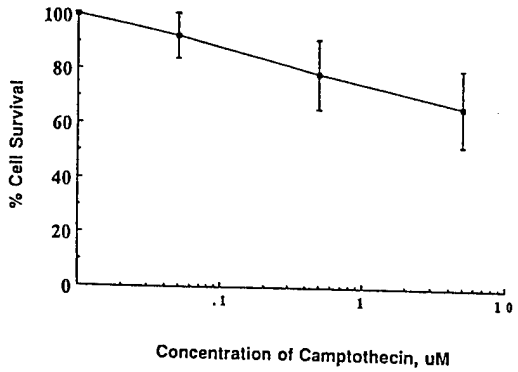


Fig. 1 Effect of camptothecin on HeLa cell survival. HeLa cells (500) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. Cells were then exposed for 1 h to increasing concentrations of camptothecin (0, 0.05, 0.5, 5 μ*M*) dissolved in DMSO. Control plates contained DMSO alone. The number of colonies formed in the presence of DMSO were within 5% of those formed in the absence of DMSO. After removal of drug from the cell cultures, the cells were rinsed twice with warm phosphate-buffered saline and then incubated in fresh drug-free medium for an additional 8–10 days. Colonies were visualized by staining with trypan blue dye. This graph represents the average of three independent experiments; bars represent standard deviations from the average. For the point apparently lacking error bars, the deviation from the average was contained within the symbol.

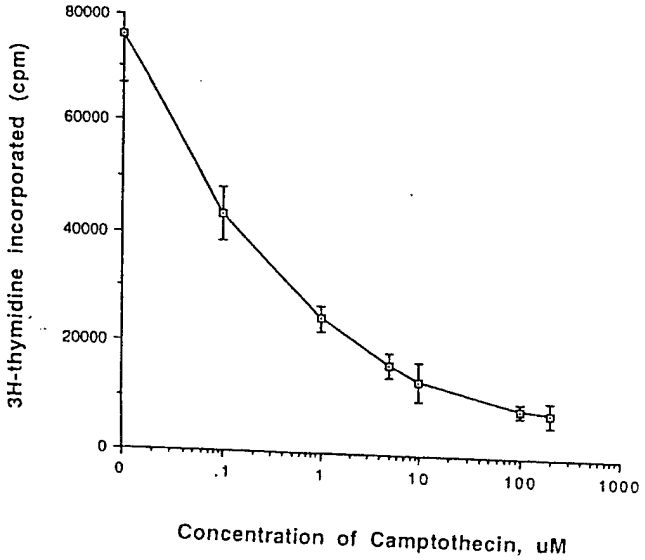


Fig. 2 Effect of camptothecin on intact HeLa cell DNA synthesis. HeLa cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then labeled with ^{3}H-thymidine (1 μCi/ml of medium) and exposed to increasing concentrations of camptothecin (0, 0.1, 1, 5, 10, 20, 100 μ*M*) dissolved in DMSO. Plates containing DMSO alone served as controls. After a 1-h incubation, cells were lysed and the level of DNA synthesis was measured by the isolation and counting of ^{3}H in acid-insoluble material. This graph represents the average of four separate experiments; error bars represent standard error of the means.

cells [12]. The occurrence of low levels of DNA replication at higher drug concentrations suggests that topoisomerase II may partially substitute for the function normally provided by topoisomerase I [2].

Table 1 Effect of camptothecin on MRC-meditated SV40 DNA replication in vitro. MRC-driven in vitro SV40 DNA replication assays were performed in the presence of increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50 μM) dissolved in DMSO. Reactions containing DMSO alone served as a control. The amount of ^{32}P-dCTP incorporated into DNA replication assay products was determined using DE81 filter binding

Camptothecin concentration (μM)	^{32}P-dCTP incorporation into DNA (open)	Inhibition (%)
0	36387	0
0.05	15049	59
0.5	14315	61
5	11343	69
50	3822	89

Similarly, a low concentration of camptothecin was found to inhibit the ability of the MRC to support in vitro DNA replication. In vitro SV40 DNA replication assays (Materials and methods) incubated with increasing concentrations of camptothecin were analyzed for total ^{32}P-dCTP incorporation into DNA. MRC-mediated DNA replication was inhibited by almost 60% when reactions contained 0.05 μM camptothecin (Table 1). The results presented in Table 1 are typical of those obtained in this type of experiment. The relatively close correlation between the concentrations of camptothecin inhibiting DNA synthesis in intact cells and in the MRC-driven in vitro reactions supports the potential role of the MRC as a meaningful model to study camptothecin and other anticancer agents that directly affect DNA synthesis.

Effect of camptothecin on MRC-associated topoisomerase I activity

We performed topoisomerase I assays to establish that the topoisomerase I activity present in the MRC is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA into a form II DNA (Fig. 3, lane 6). The pattern of topoisomers produced by the MRC-associated topoisomerase I was indistinguishable from that generated by the purified enzyme (Fig. 3, lanes 6 and 8). Furthermore, we assessed the level of topoisomerase I activity present in the Q-Sepharose peak by laser densitometry to quantitate the densities of fully converted form II DNAs produced by the MRC-associated and purified enzymes (data not shown). We found that the form II DNA generated by the purified topoisomerase I enzyme possessed a twofold greater density than that generated by the MRC-associated enzyme. Therefore, we estimated that the level of topoisomerase I present in the Q-Sepharose peak was approximately 1.66 ng/µl (Materials and methods).

In order to examine the effect of camptothecin on MRC-topoisomerase I activity, we performed topoisomerase I assays in the presence of increasing concentrations of the drug (Materials and methods). In these assays, inhibition of topoisomerase I activity by camptothecin resulted in the accumulation of form I DNA. We observed an extensive level of inhibition of the MRC-topoisomerase I activity by camptothecin (Fig. 3, lanes 1–5); as little as 0.05 μM camptothecin caused a significant retention of form I DNA. From these experiments, we determined that 0.05 μM camptothecin inhibited MRC-topoisomerase I activity by over 50%. This concentration of camptothecin is comparable to that which inhibits both in vitro and intact cell DNA synthesis by 50%, supporting the premise that the inhibition of DNA replication by camptothecin results from the drug's inhibition of topoisomerase I activity.

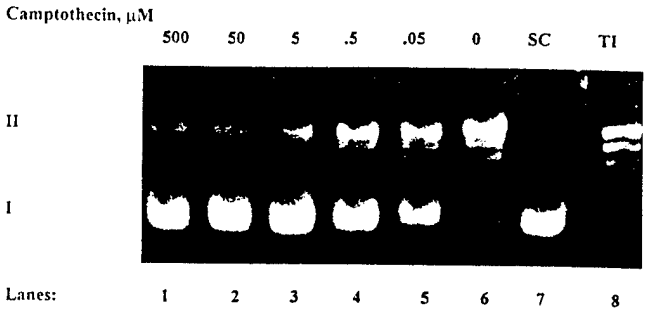
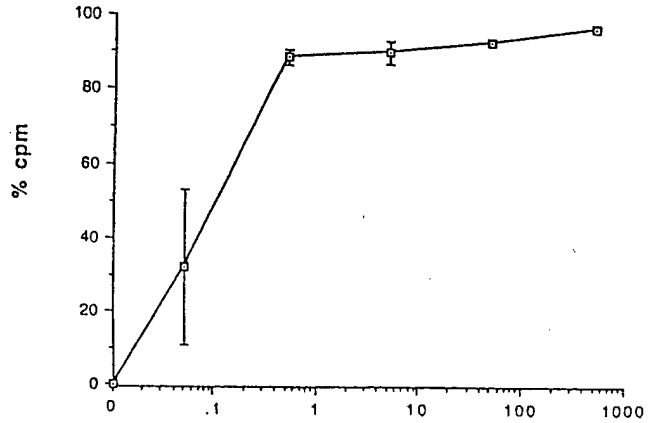


Fig. 3 Inhibition of MRC-associated topoisomerase I activity by camptothecin. Reaction assays containing 8 µg of the Q-Sepharose peak were incubated for 15 min at 37 °C with 0.3 µg supercoiled pSVO+, in the presence of increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50, 500 μM) dissolved in DMSO. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (1 µg/ml) staining of the gels, topoisomers were visualized by illuminating gels with an ultraviolet light. Lane 7 shows the position of supercoiled pSVO+ DNA in the presence of DMSO (*lane 6* conversion of supercoiled DNA to relaxed open circle form II DNA by the MRC-associated topoisomerase I present in the Q-Sepharose peak, *lanes 1–5* inhibition of MRC-topoisomerase I activity by increasing concentrations of camptothecin, *lane 8* conversion of supercoiled DNA to relaxed form II DNA by 2 units of purified topoisomerase I)

SDS precipitation of camptothecin-stabilized cleavable complexes

Camptothecin inhibits topoisomerase I activity by trapping the enzyme in a reversible enzyme-drug-DNA cleavable complex, the formation of which leads to replication fork arrest and DNA fragmentation [22]. Studies have revealed that precipitation of the cleavable complex by protein denaturants yields DNA single-strand breaks and results in the covalent linkage of topoisomerase I to the 3′ end of the breaks via a phosphotyrosine bond [10]. Utilizing 5′ end-labeled substrate DNA, the relative amount of cleavable complexes stabilized by camptothecin can be quantified via

the isolation and counting of radioactive topoisomerase I-linked DNA breaks [13]. Figure 4 shows the precipitation of purified and MRC-associated topoisomerase I DNA cleavable complexes in the presence of increasing concentrations of camptothecin (Materials and methods). The formation of drug-stabilized purified topoisomerase I-cleavable complexes leveled off at 0.5 μ*M* camptothecin (Fig. 4A), while the formation of drug-stabilized MRC-associated topoisomerase I-cleavable complexes increased linearly with increasing amounts of drug (Fig. 4B). This is a consequence of the greater amount of MRC-associated topoisomerase I used in these assays compared to the purified enzyme. Overall, the increase in MRC-topoisomerase I cleavable complex formation at higher concentrations of camptothecin correlates with our observation regarding the loss of MRC-associated topoisomerase I activity at higher concentrations of camptothecin.



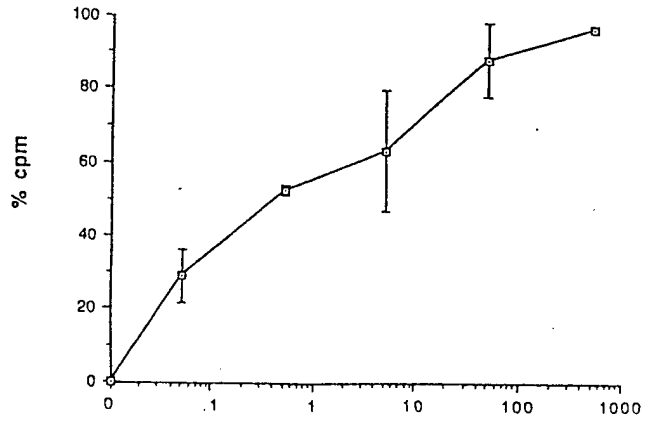


Fig. 4 A, B SDS precipitation of camptothecin-topoisomerase I cleavable complexes. **A** Reaction assays containing 5 units of purified topoisomerase I and 1 ng of 5′ end-labeled pSVO+ DNA were incubated for 5 min at 37 °C with increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50, 500 μ*M*) dissolved in DMSO. Reactions were stopped by the addition of a solution containing 2% SDS, 2 m*M* EDTA and 0.5 mg/ml sheared salmon sperm DNA [12]. After SDS precipitation of topoisomerase I-DNA cleavable complexes, pellets were transferred to vials containing scintillation cocktail and counted. The data presented are the average of two independent experiments; error bars represent the standard deviations from the average. **B** SDS precipitation assays were performed as described for A using 8 μg MRC. The data are the average of three independent experiments; error bars represent the standard error of the mean

Discussion

Although human cell-derived DNA polymerases α and δ, RP-A, RF-C, topoisomerases I and II, and PCNA have been identified as playing roles in SV40 DNA replication in vitro, their functional organization allowing for the efficient replication of DNA in vivo has not been well defined. Over the past several years, a number of researchers have reported on the roles that multiprotein complexes play in eukaryotic cellular DNA replication [14]. Our laboratory was the first to describe an MRC isolated from human cells that faithfully duplicates DNA in the presence of large T-antigen and SV40 viral origin sequences [3, 7, 15]. In addition to identifying many of the key DNA replication enzymes that comprise the MRC, we have performed experiments that suggest that the MRC replicates DNA in vitro in a manner similar to that observed in intact cells [3, 15, 24].

We believe that the MRC will prove to be a powerful in vitro system to study the mechanisms of action of anticancer agents that directly affect DNA synthesis. First, as discussed earlier in this report, the DNA replication activity of the MRC is as sensitive to camptothecin poisoning as intact cell DNA synthesis. In another study, in which investigators examined the effect of camptothecin on SV40 DNA replication mediated by HeLa cell extracts, it was found that much higher concentrations of camptothecin are required to inhibit DNA synthesis as compared to its effect with intact cells [23]. This result suggests that the DNA replication activity of the crude HeLa cell extracts is not especially sensitive to the action of camptothecin. It also provides support for the use of the MRC as an appropriate system to study camptothecin's mechanism of action. Secondly, MRC-intact topoisomerase I activity was inhibited by 50% at 0.05 μ*M* camptothecin. This concentration correlates with those required to inhibit in vitro and intact cell DNA replication by 50%, supporting the premise that camptothecin inhibits nucleic acid synthesis by selectively targeting topoisomerase I. Finally, the increased formation of MRC-intact topoisomerase I-DNA cleavable complexes at higher concentrations of camptothecin is consistent with the more pronounced inhibition of MRC-intact topoisomerase I activity by higher concentrations of the drug. Our results suggest that camptothecin interacts with the intact cell topoisomerase

I and the MRC-associated topoisomerase I in a similar manner.

Several aspects of camptothecin cytotoxicity remain to be investigated; for example, uncovering the lethal cellular events that occur beyond cleavable complex formation. We believe that some of these questions can be answered by employing the MRC as a novel system to study the effects of camptothecin on DNA replication. We anticipate that future studies with the MRC will facilitate a greater understanding of camptothecin's mechanism of action and contribute pertinent information required for the development of analogues with improved anticancer activity.

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The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture*

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Key Words: breast cancer; DNA replication; multiprotein complex; fidelity; DNA polymerase

ABSTRACT

In this report, we describe the isolation and purification of an organized multiprotein complex for DNA replication from MDA MB-468 human breast cancer cells. This complex, which we designate the DNA synthesome, possesses a sedimentation coefficient of 18S as determined by sucrose density gradient analysis. Importantly, the DNA synthesome fully supports the *in vitro* replication of simian virus 40 (SV40) origin-containing DNA in the presence of the viral large T-antigen. Since the SV40 virus utilizes the host's cellular proteins for its own DNA replication, our results indicate that the DNA synthesome may play a role not only in viral DNA synthesis but in human breast cell DNA replication as well. Our studies demonstrate that the following DNA replication proteins constitute the DNA synthesome: DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A), replication protein C (RF-C), DNA topoisomerases I, II, and DNA polymerase ε. In addition, we successfully isolated the DNA synthesome from human breast tumor tissue as well as xenografts from nude mice injected with the human breast cancer cell line MCF-7. The DNA synthesome purified from the breast cancer tissues fully supports SV40 DNA replication *in vitro*. Furthermore, our results obtained from a forward mutagenesis assay suggest that the DNA synthesome isolated from a non-malignant breast cell line, mediates SV40 DNA replication by an error-resistant mechanism. In contrast, the DNA synthesome derived from malignant breast cells and tissue, exhibited a lower fidelity for DNA synthesis *in vitro*. Overall, our data support the role of the DNA synthesome as mediating breast cell DNA replication *in vitro* and *in vivo*. We expect that the continued characterization of the DNA synthesome will provide important insight into the molecular mechanisms regulating breast cancer cell DNA replication.

Title Footnote:

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INTRODUCTION

Breast cancer is one of the most commonly diagnosed female cancers and the second leading cause of cancer death among women [1]. Recently, numerous reports have underscored the important role of cell proliferation rate as a prognostic factor for breast carcinoma. Studies using flow cytometry to measure the DNA content of breast tumor cells show a strong association between a high S-phase fraction and poor prognosis for relapse-free survival in patients with lymph node negative breast cancer [2]. In addition to a high rate of DNA synthesis, mammary cancer cells exhibit extensive DNA damage [3], as compared to non-malignant breast cells. The increased mutation frequency that accompanies the cellular transformation process is postulated to arise from molecular alterations of specific DNA replication and/or repair proteins [4]. Despite the knowledge that a high proliferation activity and increased mutation frequency correlate with breast cancer progression, there is a paucity of information regarding the regulation and precise molecular mechanisms of human breast cell DNA replication.

To date, several mammalian enzymes and proteins have been shown to be required for DNA replication *in vitro* [5-10]. In particular, the proteins necessary to support SV40 based cell-free DNA synthesis include: DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A), replication factor C (RF-C), and DNA topoisomerases I and II [11]. As mammalian cell DNA replication represents an intricate yet highly coordinated and efficient process, it follows that the proteins mediating DNA synthesis may be organized into a multiprotein complex. In support of this hypothesis, several reports have described the isolation of large macromolecular complexes of replication essential proteins from extracts of eukaryotic cells [9,11,12].

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex from human (HeLa) cells and murine (FM3A) mammary carcinoma cells that fully supports origin specific and large T-antigen dependent papovavirus DNA replication *in vitro* [13-15]. The multiprotein complex was observed to retain its ability to

replicate papovavirus DNA after additional purification by anion-exchange chromatography and sucrose or glycerol gradient sedimentation [13-15]. In addition, the integrity of the multiprotein complex was maintained after treatment with salt, detergents, RNase, DNase and electrophoresis through native polyacrylamide gels [15,16]. These results suggest that the association of the proteins with one another is independent of non-specific interactions with other cellular macromolecules.

We report here, for the first time, that breast cancer cells also utilize a multiprotein complex to carry out cellular DNA synthesis, and we now designate this complex the DNA synthesome. We describe the isolation and purification of the DNA synthesome from MDA MB-468 human breast cancer cells and most importantly from human breast tumor cell xenografts, as well as from biopsied human breast tumor tissue. Furthermore, we discuss the results of a forward mutagenesis assay which establish that the DNA synthesome isolated from breast cancer cells and tissue has a lower fidelity for DNA replication than the DNA synthesome isolated from a normal breast cell line. Ultimately, we anticipate that the complete characterization of this DNA synthesome will lead to important new insights into understanding the molecular mechanisms of breast cancer cell DNA replication.

Materials and Methods

Materials. [α-^{32}P]dCTP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) and [^{3}H]thymidine (90 Ci/mmol; 37 MBq; 2.5 mCi/ml) were obtained from DuPont New England Nuclear (Boston, MA). Camptothecin was purchased from TopoGen, Inc. (Columbus, Ohio). The drug was dissolved in dimethyl sulfoxide and stored in aliquots at -20°C. Purified topoisomerase I enzyme (4 ng/ml) and a topoisomerase II assay kit were purchased from TopoGen, Inc. (Columbus, Ohio).

Cell Culture. Suspension cultures of MDA MB-468 human breast cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and fetal bovine serum. Exponentially growing cells ($5x10^5$ cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS): 20 mM Na_2HPO_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation. Hs587Bst cells were cultured in monolayer in Dulbecco's Modified medium supplemented with 30 ng/ml of epidermal growth factor and 10% fetal bovine serum. Sub-confluent cells were harvested and washed three times with PBS. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 minutes, 4°C) and the pellets stored at -80°C until fractionation. MCF-7 cells were cultured in Eagle's minimum essential medium containing 5% fetal bovine serum and 600 μg/ml of neomycin sulfate, as described in Yue et al. [17]. Subconfluent MCF-7 cells were scraped into Hank's solution and centrifuged at 1000 rpm for 2 minutes at 4°C. The cells were then prepared for inoculation into intact nude mice according to published procedures [17].

Human Breast Tumor Tissue. A biopsy from an infiltrating ductal type carcinoma of the female mammary gland was immediately frozen at -80°C after resection. In order to examine the tumor tissue for the presence of a functional DNA synthesome, the breast

tumor tissue was thawed and subjected to the purification protocol described in a later section of these Materials and Methods.

Isolation and Purification of the DNA Synthesome from Breast Cancer Cells: Cell Fractionation. MDA MB-468 (20 g) and Hs587Bst (2 g) cells were homogenized and the breast cell DNA synthesome was purified according to our previously published procedures [13-15] and as outlined in Figure 1. Briefly, the respective cell pellet was resuspended in 2 volumes of buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM AAN (pH 7.5) and 1 mM DTT) and homogenized using a loose-fitting Dounce homogenizer. The homogenate was then fractionated into a nuclear pellet and cytosolic extract. The nuclei were extracted with a low salt buffer (0.15 M KCl) while the cytosolic fraction was used to prepare a post-microsomal supernatant (S-3). The nuclear extract and the post-microsomal supernatant were combined and adjusted to 2M KCl and 5% (w/v) polyethylene glycol. The mixture was rocked for 1 h at 4°C, then centrifuged at 16,000 rpm for 15 minutes (4°C). The resulting supernatant was then dialyzed against buffer A [13] containing 0.25 M sucrose. The dialyzed fraction was clarified by centrifugation at 16,000 rpm for 15 minutes and the supernatant solution was layered onto a 1 ml 2 M sucrose cushion containing buffer A. After centrifugation at 40,000 rpm for 16 h (4°C), the supernatant S-4 and sucrose interface P-4 fractions were collected and dialyzed against buffer B [13]. The fractions were then immediately tested for DNA polymerase α and *in vitro* SV40 DNA replication activities. **Column Chromatography**. 5 mls of the dialyzed MDA MB-468 P-4 fraction were loaded onto a 1 ml Q-Sepharose (Pharmacia) column (1 cm^3 bed volume/25 mg protein) pre-equilibrated with buffer B. The protein not binding to the matrix was collected and designated the column flow-through. After washing the matrix with 8 column volumes of buffer B, the column was eluted with 10 volumes of a linear 50-500 mM gradient of KCl. Fractions of 0.5 ml were collected and assayed for protein and enzymatic activity. Fractions containing the peak of DNA polymerase α and *in vitro* SV40 DNA replication activities were pooled, dialyzed against TDEG buffer [13] and stored at -80°C.

Velocity Sedimentation Analysis of the DNA Synthesome Isolated from MDA MB-468 Breast Cancer Cells. 0.6 ml (600 μg of protein) of the DNA synthesome present in the Q-Sepharose peak fraction was layered over a 10 ml 10-30% sucrose gradient containing 0.5 M KCl. Velocity sedimentation analysis was performed as described in a previously published report from this laboratory [15]. The sedimentation analysis of marker proteins (horse spleen apoferritin (17S) and yeast alcohol dehydrogenase (7S)) was performed on parallel gradients to verify that the gradient was isokinetic.

Micro-isolation and Purification of the DNA Synthesome from Breast Tumor Tissue: Cell Fractionation. The DNA synthesome was purified from breast cancer tissue according to a modified version of the isolation scheme depicted in Figure 1 and as described in a previous section of these methods. All steps of the fractionation process were altered to facilitate the purification of the DNA synthesome from small quantities of breast tumor tissue. The human breast tumor (8.55 g) was dissected and finely chopped with a tissue chopper at 4°C. The minced breast tissue was then suspended in 1 volume of buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM AAN (pH 7.5) and 1 mM DTT) and homogenized using a 1 ml Dounce homogenizer. The homogenate was centrifuged at 2,000 rpm for 10 minutes (4°C), and the crude nuclear pellet (NP) and cytosolic fraction (S-1) were collected separately. The nuclear pellet was resuspended in 1 volume of nuclei extraction buffer [13] containing 0.15 M KCl. After rocking the nuclear pellet for 2 h at 4°C, the extracted nuclei were centrifuged at 22,300 rpm for 2.1 minutes (4°C) using a TLA 100.3 rotor and the supernatant (NE) retained. In order to remove mitochondria and microsomes, the S-1 fraction was subjected to differential centrifugation using a TLA 100.3 rotor: 17,800 rpm, 3.2 minutes and 59,700 rpm, 22.2 minutes, respectively. The final post-microsomal supernatant (S-3) was collected. The NE was combined with the S-3 fraction; 4.5 mls of the NE/S-3 fraction were then layered over a 0.5 ml 2 M sucrose cushion. After centrifugation at 40,000 rpm for 16h (4°C) using a Beckman SW55.Ti swinging bucket rotor, the S-4 and P-4 fractions were collected, dialyzed against a low-salt TDEG buffer,

and assayed for their respective enzymatic activities. These same steps were followed to purify the DNA synthesome from the xenografts grown in nude mice. **Column Chromatography.** 0.7 mls of the dialyzed human breast tumor P-4 fraction (3.3 mg protein) were loaded onto a 0.15 ml DE52 cellulose column, pre-equilibrated in TDEG buffer containing 5 mM KCl. The protein not binding to the matrix was collected, and designated the column flow-through. The column was washed with 8 column volumes of pre-equilibration buffer. Matrix-bound protein was then eluted with 8 volumes of TDEG buffer containing 1M KCl. Fractions of approximately 0.1 ml were collected, dialyzed against TDEG buffer, and assayed for their DNA polymerase α and *in vitro* DNA replication activities.

Purification of SV40 Large T-antigen. SV40 large T-antigen was purified from 293 cells infected with a recombinant adenovirus vector, Ad-SVR284, as detailed elsewhere [18].

***In vitro* SV40 DNA Replication Assay.** Assay reaction mixtures (12.5 μl) contained 80 mM Tris-HCl (pH 7.5); 7 mM $MgCl_2$; 1 mM DTT; 3-20 μg protein fraction; 0.5-1.0 μg purified SV40 large T-antigen; 25 ng plasmid $pSVO^+$ [19] containing and insert of SV40 replication-origin DNA sequences; 100 μM each dTTP, dATP, dGTP; 200 μM each rCTP, rGTP, UTP; 4 mM ATP; 25 μM [α-^{32}P]dCTP; 40 mM creatine phosphate; 1μg creatine kinase. Each reaction was incubated for 2h at 35°C. The replication assay reaction products were processed using DE81 (Whatman) filter binding to quantitate the amount of radiolabel incorporated into the replication products [20]. One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol of dNMP into SV40 replication-origin containing plasmid DNA per 2h under these described assay conditions.

Enzyme Assays. DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [21,22]. One unit of DNA polymerase α

activity is equivalent to 1nmol of total [^{3}H]TMP incorporated into DNA per hour at 35°C. The assay for **DNA topoisomerase I** activity is a modification of published methods [19] and is described in detail by Hickey et al. [23]. **DNA topoisomerase II** activity was measured using an assay kit purchased from TopoGen, Inc.

Immunodetection of DNA Polymerases δ, ε, RP-A, RF-C, PCNA and DNA Primase. Denaturing polyacrylamide gel electrophoresis of the various protein fractions was performed as previously described [24]. The resolved polypeptides were transferred (15 volts, 16h, 4°C) to nitrocellulose membranes, and immunodetection of the respective DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham). A monoclonal antibody prepared against the C-terminal portion of DNA polymerase δ was used at a 1:100 dilution to probe membranes for the 125 kD polymerase δ polypeptide. The anti-polymerase ε antibody which recognizes the 140 and >200 kD forms of polymerase ε, was used at a 1:1000 dilution. Both the anti-RF-C monoclonal antibody (mAb-11), which recognizes the 140 kD subunit of the RF-C protein-complex, and the anti-RP-A antibody (p34-20), which recognizes the 34 kD subunit of RP-A, were generous gifts from Dr. Bruce Stillman. Both antibodies were used at a 1:500 dilution. The anti-DNA primase antibody, a gift from Dr. William Copeland, was used at a 1:500 dilution. The anti-PCNA antibody was used at a dilution of 1:1000. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots at a dilution of 1:5000.

Forward Mutagenesis Assay: Transfection and Plating. 12 ng of pBK-CMV plasmid DNA (Stratagene), encoding the *lac-Zα* gene, were incubated with 15-20 μg of protein fraction per *in vitro* DNA replication assay. The replicated pBK-CMV DNA was then Dpn I digested, precipitated as described [20] and used in the transfection of E. coli strain XL1-Blue MRF'. Bacterial stocks maintained in Luria broth, containing 10% glycerol, were mixed with 300 pg of the pBK-CMV DNA replicated *in vitro* and this mixture was incubated for 10 minutes on ice; subsequently, the DNA was electroporated into the cells

under the following conditions: 1.4 kV, 25 μF, 200 ohms. Immediately following electroporation, 960 μls of chilled, sterile SOC buffer (20 mM glucose in Luria broth media) were added to the reaction cuvette. The electroporated mixture was then incubated in a rotary shaker (250 rpm) at 35°C for 1h. An aliquot of the incubated culture, sufficient to yield 100-600 bacterial colonies per plate, was spread on top of 20 mls of solidified Luria broth agar containing 0.5 mg/ml kanamycin, 25 mg/ml IPTG and 25 mg/ml X-gal. (These plating conditions yield an intense blue colored bacterial colony when the bacteria express the unmutated plasmid and a light blue to white colored bacterial colony when the bacteria contain plasmids harboring mutations in the *lac-Zα* gene.)

Scoring of Mutant Phenotypes. Mutant phenotypes, resulting from the inactivation of the *lac-Zα* gene in the pBK-CMV plasmid, were scored after approximately 12-15 hours of incubation at 35°C. In order to reproducibly score the variable color intensities of the mutant phenotypes, a scale of blue color intensities has been established [25]. Unmutated pBK-CMV DNA generates a dark blue color which, on a scale of 0-4, is assigned a value of 4. The variable mutant phenotypes are distinguished as 0^+(white/colorless), 1^+(faint blue), 2^+(medium blue) and 3^+(almost wild type). In order to eliminate false positives resulting from plating artifacts, mutant colonies were picked from the plates, diluted in 50 mM sodium borate buffer (pH 9.0) and mixed with an equal dilution of bacteria containing unreplicated pBK-CMV plasmid. Plating of this mixture on the agar plates containing the color substrate X-gal (see above) enhances the contrast between the wild-type and mutant phenotypes as well as permits the scoring of subtle phenotypic differences arising from small variations in the position and number of point mutations within the *lac-Zα* gene.

RESULTS

Human Breast Cancer Cell DNA Replication Proteins Co-fractionate as a Readily Sedimentable Form.

In order to determine whether a sedimentable complex of DNA replication proteins could be isolated from human breast cancer cells, as previously demonstrated for HeLa [13,14] and FM3A cells [15], we subjected MDA MB-468 cells to the fractionation scheme outlined in Figure 1. The PEG NE/S-3, S-4 and P-4 fractions were collected and assayed for DNA polymerase α activity. The majority of the enzyme's activity partitioned with the sedimentable P-4 fraction following polyethylene glycol precipitation and discontinuous gradient centrifugation of the NE/S-3 fraction (Table 1). This result is consistent with our earlier work on the purification of the DNA synthesome from HeLa and FM3A cells [13-15], in which the DNA polymerase α activity contained in the DNA synthesome partitioned to the P-4 fraction at the sucrose interface.

In addition to determining DNA polymerase α activity, we assayed the PEG NE/S-3, S-4 and P-4 fractions for *in vitro* SV40 DNA replication activity (Materials and Methods). DE81 filter binding analysis was used to quantitate the level of [α-^{32}P]dCMP incorporation into SV40 DNA replication products. Following subfractionation of the PEG NE/S-3 fraction into the S-4 and P-4 fractions, the ability to support SV40 DNA replication *in vitro* partitioned exclusively with the sedimentable P-4 fraction (Table 1). This pattern of partitioning of DNA replication activity, is also consistent with our earlier work on the purification of the synthesome from HeLa and FM3A cells [13-15]. Only negligible amounts of radiolabel were incorporated into DNA replication products when reactions lacked SV40 large T-antigen. These data indicate that all of the activities required to execute large T-antigen dependent SV40 DNA replication reside in the human breast cancer cell sedimentable P-4 fraction.

Further Purification of the Human Breast Cancer Cell DNA Synthesome.

We further purified the breast cancer cell DNA synthesome from the sedimentable P-4 fraction by Q-Sepharose anion-exchange chromatography; a method successfully employed for the purification of the DNA synthesome from HeLa cells [13,14]. The P-4 fraction was applied to a 1 ml Q-Sepharose column and the DNA synthesome eluted by a linear gradient of KCl (50-500 mM). Figure 2 shows the profile of DNA polymerase α activity as it eluted from the Q-Sepharose column. The DNA polymerase α activity eluted from the column as an initial sharp peak at lower salt concentrations (fractions 6-10), with an additional small peak of activity at higher salt concentrations (fractions 21-23). Negligible amounts of enzyme activity were found in the column flow-through and wash fractions (data not shown).

The peak of DNA polymerase α activity that eluted from the Q-Sepharose column (fractions 6-10), was designated the Q-Sepharose peak. Both the peak and the column flow-through fractions were assayed for *in vitro* SV40 DNA replication activity. The Q-Sepharose peak contained over 80% of the large T-antigen dependent *in vitro* DNA replication activity; while the column flow-through fraction supported significantly less DNA synthesis (Table 2).

Velocity Sedimentation Analysis of the Breast Cancer Cell DNA Synthesome.

We determined the sedimentation coefficient of the breast cancer cell DNA synthesome by subjecting the Q-Sepharose peak fraction to velocity sedimentation analysis in a 10-30% sucrose gradient containing 0.5M KCl [15]. The sucrose gradient fractions were collected and assayed for DNA polymerase α and *in vitro* SV40 DNA replication activities. Both activities co-sedimented in the sucrose gradient with a sedimentation coefficient of 18S (Figure 3; Table 2). This 18S sedimentation coefficient for the breast cell DNA synthesome corresponds to the S-value obtained for the HeLa cell DNA synthesome. Presumably, the 18S value of the human breast cancer cell DNA synthesome accounts for its ready sedimentation to the sucrose interphase following the centrifugation of the PEG NE/S-3 fraction (Figure 1).

The DNA Replication Proteins that Copurify with the Breast Cancer Cell DNA Synthesome.

We performed Western blot analyses and enzyme assays to identify the DNA replication proteins that copurify with the breast cancer cell DNA synthesome during its various stages of purification. As numerous studies have shown that DNA polymerase δ plays an integral role in the *in vitro* synthesis of SV40 origin containing DNA [8,26,27], we probed the PEG NE/S-3, P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions for the presence of the protein. Utilizing a monoclonal antibody prepared against the C-terminal peptide of DNA polymerase δ [28], we found that the protein exclusively co-purified with the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 4). The enzyme was not detectable in the replication-deficient S-4 and Q-Sepharose flow-through fractions.

In addition to DNA polymerase δ, we examined the human breast cancer cell fractions for the presence of RF-C [27,29] and DNA primase [27]. Immunoblot analyses, using antibodies that recognize either the 140 kDa subunit of the RF-C protein complex or the 58 kDa subunit of DNA primase, revealed that RF-C and DNA primase resided only in the replication-competent protein fractions (Figure 4).

Western blot analysis also shows that the DNA replication protein, PCNA, was present in the replication-competent breast cancer cell fractions, as well as the S-4 and Q-Sepharose flow-through fractions (Figure 4). This result suggests that PCNA may not be as tightly associated with the DNA synthesome as compared to DNA polymerases α, δ, RF-C and DNA primase. Furthermore, immunodetection of RP-A [30,31] with a monoclonal antibody to the 70 kDa subunit of the protein, reveals that the polypeptide fractionated with both the replication-competent and -deficient fractions (Figure 4). These results suggest that only a fraction of the cellular pools of PCNA and RP-A copurify with the breast cancer cell DNA synthesome.

Furthermore, we determined whether the breast cancer cell DNA synthesome possesses DNA topoisomerase I activity by assaying several breast cancer fractions for

their respective enzymatic activity (Materials and Methods). In Figure 5, lanes 1-3 show the conversion of supercoiled form I DNA to relaxed, open circular form II DNA by the topoisomerase I activity present in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Importantly, the relaxation of supercoiled plasmid DNA by the Q-Sepharose peak fraction was inhibited by 200 μM camptothecin (lane 4), a specific inhibitor of DNA topoisomerase I [32]. This indicates that the conversion of supercoiled plasmid DNA to form II DNA was mediated specifically by topoisomerase I.

As with PCNA, RP-A and DNA topoisomerase I, only a fraction of the total cellular pool of DNA topoisomerase II co-purifies with the breast cancer cell DNA synthesome. We assayed the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions for DNA topoisomerase II activity. The decatenation of interlocked aggregates of *Crithidia fasisculata* kinetoplast DNA to monomeric, open circular DNA by the topoisomerase II enzyme present in all three fractions is shown in Figure 6 (lanes 2-4). In addition, we determined that the Q-Sepharose peak, flow-through and sucrose gradient peak fractions were devoid of nuclease contamination because they did not support the relaxation of kinetoplast DNA to the linear DNA fragments (Figure 6). Moreover, as DNA topoisomerase II requires ATP for catalytic activity, incubation of the Q-Sepharose peak with a reaction buffer lacking ATP did not support the relaxation of kinetoplast DNA (Figure 6, lane 1).

Isolation of the DNA synthesome from Breast Tumor Tissue.

In order to verify that the DNA synthesome could be isolated from breast cancer tissue as well as breast cancer cells, we subjected biopsied human breast tumor tissue to a modified version of the purification scheme depicted in Figure 1 (Materials and Methods). The alterations to the purification protocol were made to facilitate the isolation of the DNA synthesome from small quantities of breast tumor tissue. We collected and assayed the NE/S-3, S-4 and P-4 fractions for DNA polymerase α and large T-antigen dependent SV40 DNA replication activities. Table 3 shows that the majority of both

activities partitioned exclusively with the sedimentable P-4 fraction after discontinuous gradient centrifugation of the NE/S-3 fraction.

We further purified the DNA synthesome that was isolated from the human breast tumor tissue, using ion exchange chromatography (Materials and Methods). We collected and assayed the column fractions for both DNA polymerase α and *in vitro* SV40 DNA replication activities. A peak of DNA polymerase α activity (fractions 2,3) was found to elute from the column in the presence of 1M KCl (Table 4). In contrast, only a minor amount of DNA polymerase α activity was found in the column flow-through fraction (Table 4). We also tested the fractions containing the peak polymerase α activity (fractions 2,3) as well as the column flow-through for *in vitro* SV40 DNA replication activity. Only fractions 2 and 3 supported SV40 DNA replication; the column flow-through did not contain DNA replication activity (data not shown).

We also fractionated breast cancer tissue-derived from a xenograft nude mouse model [17]. Homogenous breast tumors were surgically excised from nude mice subcutaneously injected with MCF-7 breast cancer cells. Using the modified purification protocol, it was found that most of the DNA polymerase α and DNA replication activities resided with the sedimentable P-4 fraction following discontinuous gradient centrifugation of the NE/S-3 fraction (Table 5). These results suggest that the DNA synthesome exists as a functional complex within human breast cancer cells *in vivo*.

DNA Polymerase ε Copurifies with the Breast Cancer Cell DNA Synthesome.

Several lines of evidence support a role for DNA polymerase ε in cellular DNA replication. First, DNA polymerase ε is more abundant in proliferating tissues than in non-proliferating tissues [33]. Second, when quiescent human fibroblast cells are stimulated to proliferate, the mRNA levels of DNA polymerase ε, like those of polymerase α, dramatically increase just prior to S-phase [33]. Third, when the gene encoding the yeast homologue of DNA polymerase ε is mutated, the yeast cells fail to proliferate; suggesting a critical role for this polymerase in cell proliferation [34]. In order to determine whether DNA polymerase ε copurifies with the breast cancer cell DNA

synthesome, we probed the MDA MB-468 derived protein fractions with an antibody that recognizes the >200 kDa polypeptide. Immunoblot analysis reveals that DNA polymerase ε was present in the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 7). Only a minor amount of DNA polymerase ε was present in the replication-deficient S-4 fraction (Figure 7), while none was detected in the Q-Sepharose flow-through (data not shown).

DNA Replication Fidelity of the Breast Cancer Cell DNA Synthesome.

The fidelity of DNA synthesis is mediated in part by the proof-reading capacity of the intrinsic 3'-5' exonuclease activity of DNA polymerase δ [25]. We employed a forward mutagenesis assay to measure the fidelity of the *in vitro* DNA synthesis process carried out by the breast cancer cell DNA synthesome (Materials and Methods) [35]. In this assay we utilized the DNA synthesome isolated from MDA MB-468 breast cancer cells and human breast tumor tissue to replicate plasmid DNA containing the SV40 origin of replication and the *lac-Zα* gene. The results of the fidelity assay were quantitated using the blue/white selection protocol described in the Materials and Methods [25]. These results were compared to the replication fidelity of the DNA synthesome isolated from non-malignant Hs587Bst breast cells. We determined that the DNA synthesome from MDA MB-468 cells possessed a replication fidelity approximately 6 fold lower than that of the synthesome from Hs587Bst cells (Table 6). Similarly, the DNA synthesome purified from human breast tumor tissue possessed an approximately 5-fold lower DNA replication fidelity than the Hs587Bst synthesome (Table 6). These differences in replication fidelity between the malignant and normal breast cell DNA synthesome suggest that transformation to the malignant phenotype alters the process by which the synthesome from normal cells replicates DNA.

Discussion

In this report, we have described for the first time the purification of a multiprotein DNA replication complex isolated from human breast cancer cells and breast tumor tissues. The integrity of the breast cancer cell DNA synthesome was maintained after treatment with high salt, polyethylene glycol precipitation, anion-exchange chromatography and sucrose gradient sedimentation. These results suggest that the co-purification of the synthesome's proteins with one another is independent of non-specific interactions with other cellular macromolecules. In addition, upon velocity sedimentation analysis of the breast cancer cell DNA synthesome, both the DNA polymerase α and DNA replication activities co-migrated in the sucrose gradient with a coefficient of 18S. This 18S sedimentation coefficient is comparable to that obtained for the HeLa cell DNA synthesome [13,14].

Our data show that the DNA polymerase α and DNA replication activities of the synthesome isolated from breast cancer cells and breast tumor tissues, was enriched by the successive steps of the purification process. Furthermore, the P-4 as well as the Q-Sepharose peak fractions from the breast cancer cells and tissues possessed comparable levels of *in vitro* SV40 DNA replication activity. Overall, the isolation of the DNA synthesome as a fully functional complex from human and nude mouse xenograft breast tumor tissues, strongly suggests that the synthesome mediates DNA replication *in vivo*.

We have identified several of the key DNA replication proteins comprising the breast cancer cell DNA synthesome utilizing immunoblot analyses and enzymatic assays; these proteins include: DNA polymerase δ, PCNA, DNA polymerase α, DNA primase, RF-C, RP-A, DNA topoisomerases I, II, and DNA polymerase ε. All of these polypeptides, excluding DNA polymerase ε, have been shown to be required for the faithful replication of SV40 DNA *in vitro* [8,9,10]. Moreover, the functions that each of these proteins performs during DNA replication have been determined by utilizing the SV40 system. Recent studies demonstrate that DNA polymerase α-primase synthesizes RNA-DNA primers required for the initiation of leading strand and Okazaki fragment

synthesis [36,37]. On the other hand, DNA polymerase δ conducts the replication of the leading strand during DNA chain elongation [36,38]. According to a current model for eukaryotic DNA replication, the activities of both DNA polymerases α and δ are coordinated in part by RF-C, which serves as a connector or hinge between the proteins [27]. Additionally, PCNA--an accessory factor for polymerase δ--may participate in the coordination of leading and lagging strand synthesis by functioning as part of a molecular switch from the initiation to the elongation phase of DNA replication [38,39]. The co-purification of DNA polymerases α, δ, DNA primase, PCNA and RF-C with the breast cancer cell DNA synthesome indicates that the synthesome may act as a coordinated dipolymerase replication complex.

RP-A functions during SV40 DNA synthesis to stabilize newly formed single-stranded regions created in replicating DNA by the helicase activity of the large T-antigen [40]. Topoisomerase I, also a component of the breast cancer cell DNA synthesome, relaxes positive DNA supercoils as they accumulate ahead of the replication fork [41]. Such an action is necessary for translocation of the replication machinery along template DNA during DNA synthesis. In addition to topoisomerase I, topoisomerase II can carry out the unwinding activity required for the progression of the replication fork during SV40 DNA synthesis [41]. Furthermore, studies in which intact cells were incubated with topoisomerase II inhibitors demonstrate that the topoisomerase II is necessary for the decatenation of newly replicated daughter DNA molecules [42] following DNA synthesis. Presumably the enzyme functions in these roles as a component of the DNA synthesome. We are presently characterizing the breast tumor tissue-derived synthesome with respect to its protein components. Although not yet identified as components of the breast cancer cell DNA synthesome, DNA helicase and DNA ligase I were found to copurify with the synthesome isolated from HeLa and FM3A cells [14,15]. Both of these enzymes have been shown to be required for eukaryotic DNA replication *in vivo* [43,44]. Presumably, all of the proteins comprising the breast cancer cell DNA synthesome copurify with the tumor tissue-derived synthesome, as it is fully capable of supporting SV40 DNA replication *in vitro*.

In order to preserve the integrity of the information contained in DNA, normal mammalian cells must replicate their DNA with an error frequency as low as 10^{-10} [33]. Such a high fidelity for DNA replication must be maintained by the DNA synthesis and DNA repair systems functioning within the cell. We utilized a forward mutagenesis assay [25] to examine the fidelity with which the breast cancer cell and human breast tumor tissue DNA synthesome replicates plasmid DNA containing the *lac-Zα* gene. This assay detects point mutations occurring within the *lac-Zα* gene as well as frame-shift mutations occurring in other positions on the plasmid. We found a 5-6 fold decrease in the replication fidelities of the DNA synthesome isolated from malignant breast cells and tissue compared to that of the normal breast cell DNA synthesome (Table 6). Our results are consistent with the observation that mammary cancer cells accumulate extensive genetic damage [45,46]. The significant difference in the replication fidelities between the malignant and normal breast cell DNA synthesome suggests that transformation alters the process by which the latter replicates and/or participates in the repair of DNA. Indeed, it has been demonstrated that specific DNA replication proteins are targets for molecular modification during cellular transformation [4]. For example, DNA polymerases α and ε purified from Novikoff hepatoma cells have altered physicochemical and catalytic properties compared to the respective polymerases isolated from normal liver cells [4]. During DNA synthesis, these altered molecular and catalytic properties may contribute to a decreased specificity for nucleotide selection by the polymerases, which in turn leads to an increased mutation rate. Importantly, we have determined by two-dimensional polyacrylamide gel electrophoresis that significant physical differences exist between the protein components of the DNA synthesome purified from malignant and normal breast cells.[8] We are currently conducting experiments to determine the precise molecular changes that occur to the components of the breast cell DNA synthesome during transformation. We fully expect these studies to advance our understanding of how DNA replication fidelity is reduced in breast cancer cells.

We previously described a model for the organization of the proteins comprising the DNA synthesome isolated from mouse mammary carcinoma cells and HeLa cells

[14,15]. We can now extend this model to include the breast cancer cell DNA synthesome, based on the fractionation and column chromatographic profiles of its protein components (Figure 8). As DNA polymerases α, δ, ε, DNA primase and RF-C were observed to copurify primarily with the replication-competent DNA synthesome, we propose that these proteins form the core of the DNA synthesome. The "tight" association of DNA polymerase ε with the DNA synthesome suggests that the protein may play a role in mammalian cell DNA replication *in vivo*. It has been postulated that DNA polymerase ε links the replication machinery with the S-phase checkpoint by acting as a sensor that coordinates transcriptional responses to DNA damage in yeast [47]. Such a role for the protein may exist in mammalian cells as well. In addition, we have included DNA ligase I as a member of the tightly associated components of the complex as it was observed to exclusively copurify with the DNA synthesome from FM3A and HeLa cells [14,15].

Unlike the other components, PCNA, RP-A and topoisomerases I and II were observed to co-fractionate and co-elute, following column chromatography, with those fractions containing the breast cancer cell DNA synthesome as well as with fractions lacking DNA replication activity. These results suggest that only a fraction of the cellular pools of PCNA, RP-A, and topoisomerases I, II, copurify with the DNA synthesome. This is consistent with the recognition that these proteins have additional roles in mediating cellular functions such as transcription, recombination and repair. During the initial stages of SV40 DNA replication, both topoisomerase I and RP-A facilitate the melting of SV40 DNA [48]. Therefore, we propose that both of these proteins constitute the "initiation" components of the breast cancer cell DNA synthesome. We are currently performing co-immunoprecipitation studies to determine the exact physical interactions of the synthesome's proteins with each other. Their physical association depicted in Figure 8 is consistent with data from our laboratory as well as with several reports on SV40 and eukaryotic DNA replication [7,8,27,38].

. In this report, we have isolated and described a multiprotein complex for DNA replication from breast cancer cells and breast tumor tissues. The isolation of a fully

functional DNA synthesome from tumor tissues strongly suggests that the synthesome mediates breast cancer cell DNA replication *in vivo*. Furthermore, we have established that the human breast cancer cell and tumor tissue-derived DNA synthesome possess a lower fidelity for DNA replication than the synthesome purified from non-malignant breast cells. Breast cancer cells possess high rates of DNA synthesis and an accumulation of genetic damage [2, 45,46]. Understanding the process of DNA replication, as it occurs in breast cancer cells *in vivo,* will greatly facilitate the development of improved anti-breast cancer agents. We fully expect that the complete characterization of the breast cancer cell DNA synthesome will further our understanding of aberrant breast cell DNA replication as well as contribute to the development of these improved therapies.

Figure Legends

Figure 1. Flow diagram of the isolation scheme used to purify the DNA synthesome from MDA MB-468 human breast cancer cells. A detailed description of the isolation scheme is presented in the text and Materials and Methods.

Figure 2. Q-Sepharose chromatographic profile of DNA polymerase α activity in the MDA MB-468 derived P-4 fraction. A description of the column preparation and elution conditions are provided in the text and Materials and Methods.

Figure 3. Velocity sedimentation analysis of the DNA synthesome present in the MDA MB-468 Q-Sepharose peak fraction. 0.8 ml of the Q-Sepharose peak fraction were layered onto a 9 ml 10-30% sucrose gradient containing 0.5 M KCl. Centrifugation was performed as described in the Materials and Methods. The assay for DNA polymerase α activity was performed according to published procedures [21,22]; one unit of activity denotes 1 nmol of TMP incorporated into DNA per hour at 35°C.

Figure 4. Western blot analysis of the MDA MB-468 breast cancer cell derived fractions. Thirty micrograms of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak (QS), Q-Sepharose flow-through (FT) and sucrose gradient peak (SG)) were resolved on 8% polyacrylamide gels, then transferred to nitrocellulose membrane filters. The membranes were incubated with primary antibodies against (a) DNA polymerase δ, (b) RP-A, (c) RF-C, (d) PCNA and (e) DNA primase. Following incubation with the appropriate species-specific secondary antibody conjugated to horseradish peroxidase, the immobilized proteins were detected using a light-enhanced chemiluminescence system (Amersham). The relative positions of the protein molecular weight markers are shown in the lane designated (M).

Figure 5. DNA topoisomerase I activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Reaction assays containing 8 μg of the Q-Sepharose peak (QS), 8 μg of the Q-Sepharose flow-through (FT) or 20 μg of the sucrose gradient peak (SG) were incubated for 30 minutes at 37°C with 0.3 μg of $pSVO^+$ plasmid DNA. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (0.5 μg/ml) staining of gels, topoisomers were visualized with an ultraviolet light source. Lanes 1-3 show the conversion of supercoiled, form I DNA to relaxed, open circle form II DNA by the topoisomerase I activity present in the SG, FT and QS fractions, respectively. Lane 4 shows the inhibition of QS topoisomerase I activity by 200 μM of camptothecin [32]. Lane 5 shows the position of supercoiled plasmid $pSVO^+$.

Figure 6. DNA topoisomerase II activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Decatenation reactions were performed in topoisomerase II buffer (TopoGen) with 0.15 μg of KDNA and 10μg of the respective protein fraction. Lane 1 shows the position of KDNA networks after incubation with Q-Sepharose peak (QS) in a buffer lacking ATP. Lanes 2-4 show the relaxation of KDNA to nicked, open circular DNA by the topoisomerase II activity present in the QS, flow-through (FT) and sucrose gradient peak (SG) fractions. Lane 5 shows the positions of the decatenated KDNA markers: nicked, open circular (top), linear (bottom). All reactions were stopped by the addition of a stop buffer containing 1% SDS. Reactions were loaded directly onto a 1% agarose gel containing 0.5 μg/ml ethidium bromide. After electrophoresis, DNA products were visualized with an ultraviolet light source.

Figure 7. Immunoblot analysis for the presence of DNA polymerase ε in the MDA MB-468 breast cancer cell derived fractions. 50 μg of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak (QS) and sucrose gradient peak (SG)) were resolved on a 8% polyacrylamide gel then transferred to a nitrocellulose membrane. The

membranes were incubated with a primary antibody against human DNA polymerase ε. Following incubation with an anti-mouse secondary antibody conjugated to horseradish peroxidase, the immobilized protein was detected using a light-enhanced chemiluminescent system (Amersham).

Figure 8. Model for the human breast cell DNA synthesome. A full description of the model is presented in the text.

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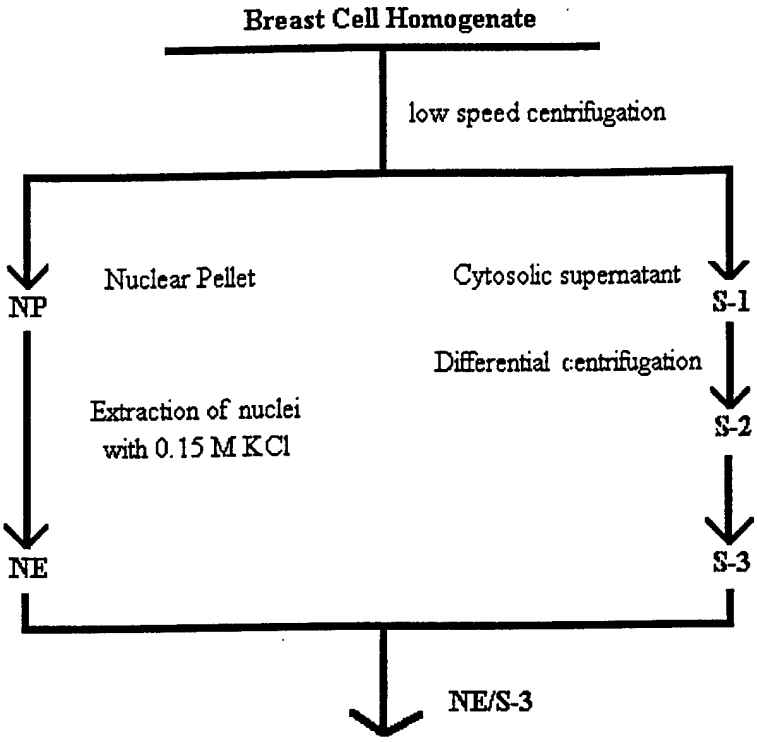
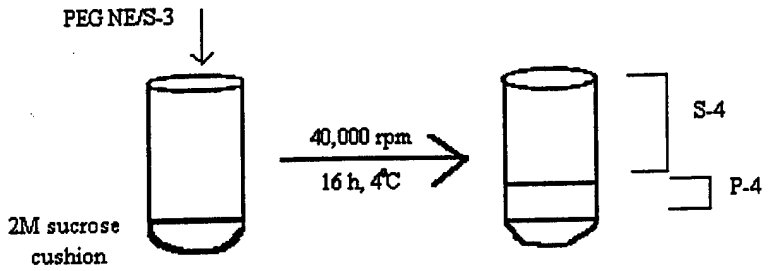


Adjust to 2M KCl and add PEG to 5%
Collect PEG supernatant = PEG NE/S-3
Layer PEG NE/S-3 onto 1 ml 2M sucrose cushion



Collect P-4 fraction

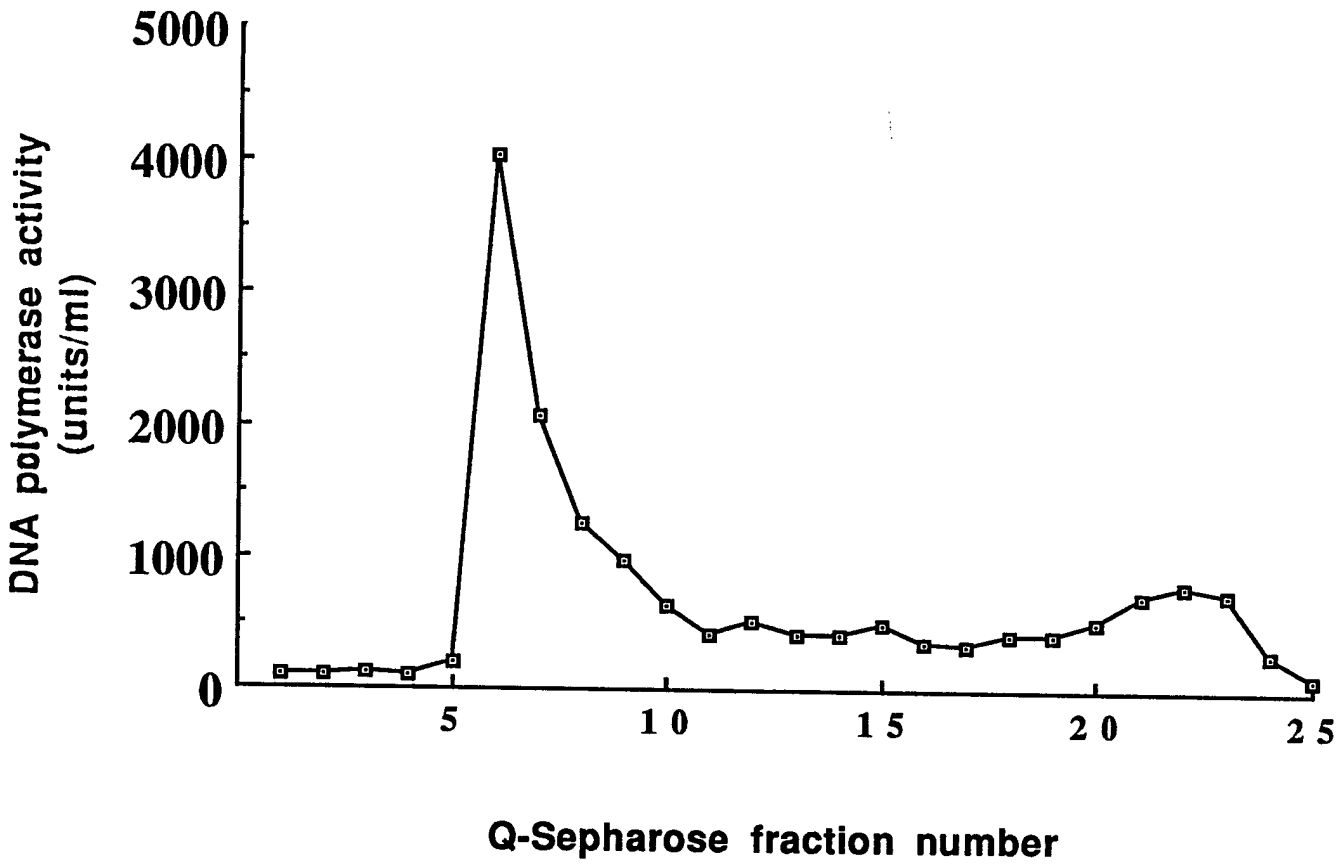
Q-Sepharose chromatography

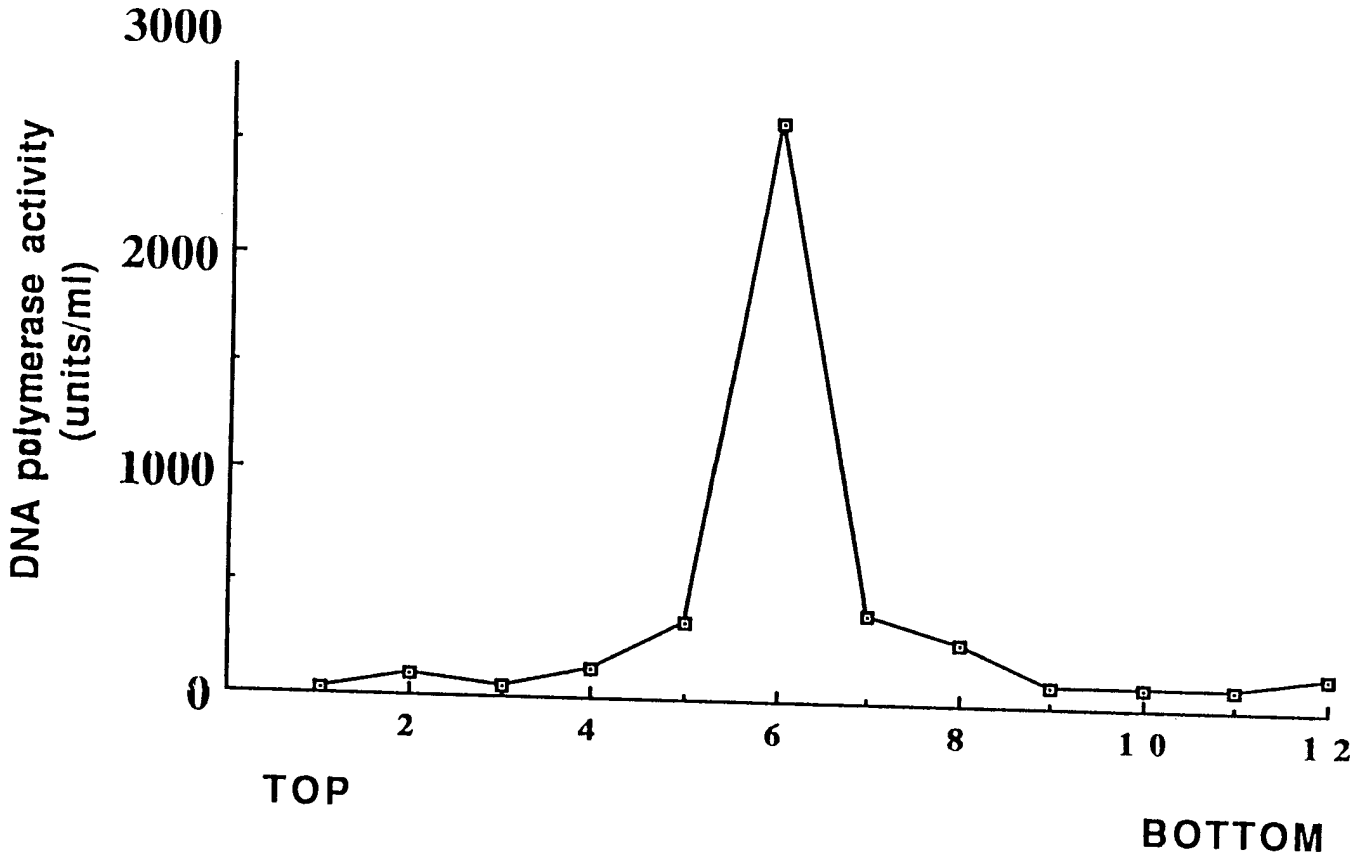
50-500 mM KCl

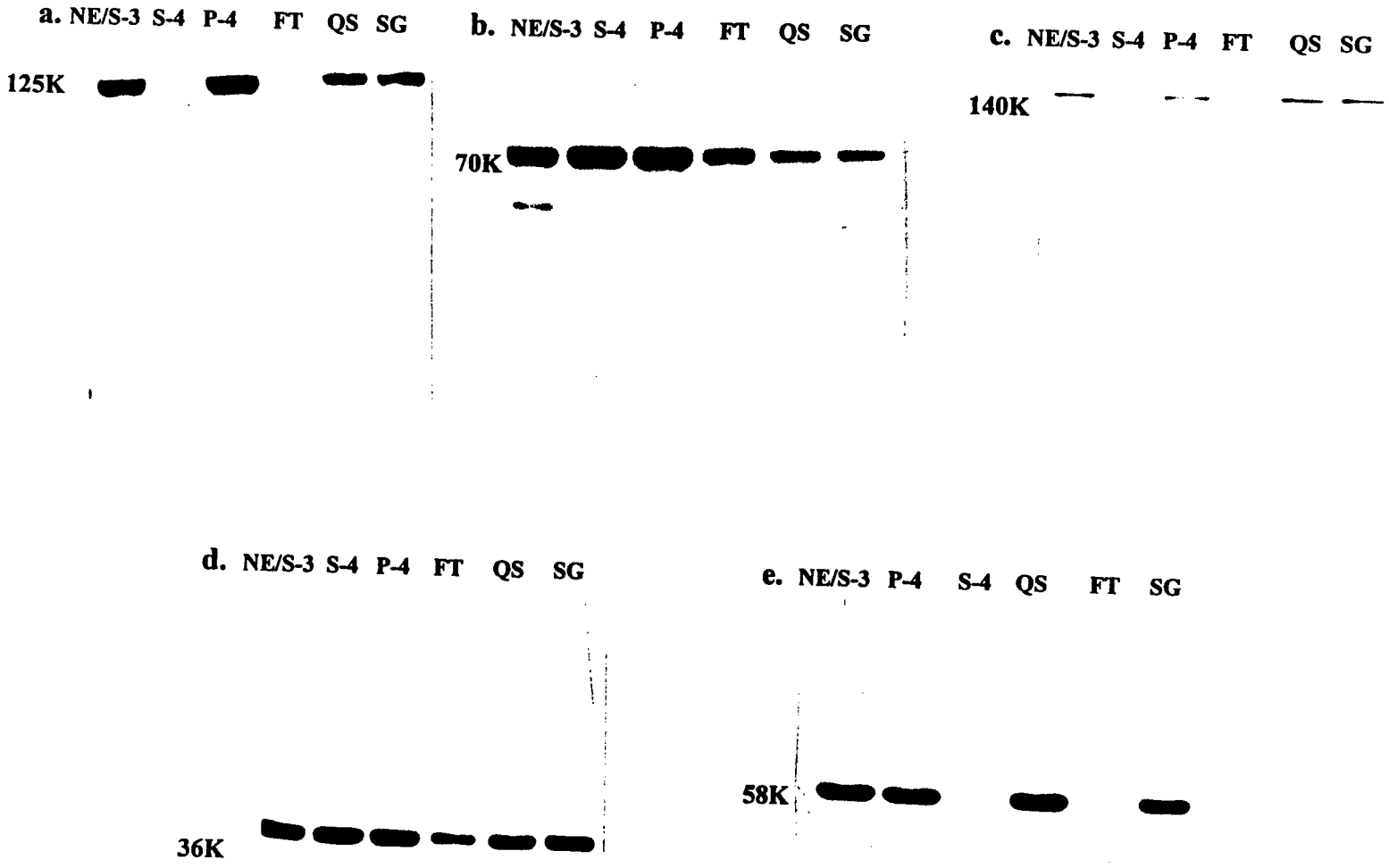
Q-Sepharose peak

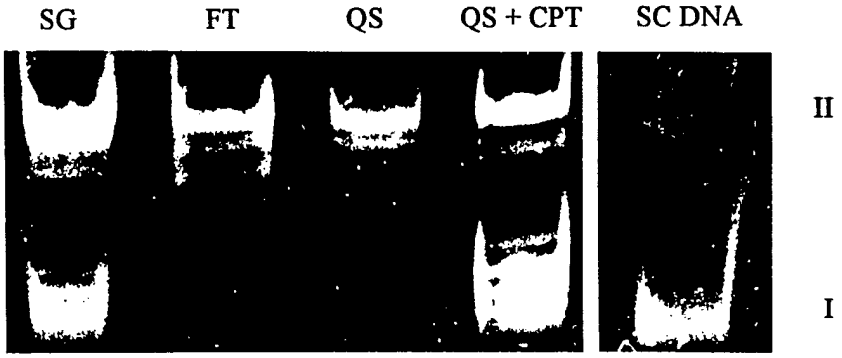
Q-Sepharose flow-through

10-30% sucrose gradient centrifugation
40,000 rpm, 16 h, 4°C


DNA polymerase activity
(units/ml)
5000
4000
3000
2000
1000
0
5
10
15
20
25
Q-Sepharose fraction number


3000
2000
1000
0
DNA polymerase activity
(units/ml)
2
4
6
8
10
12
TOP
BOTTOM

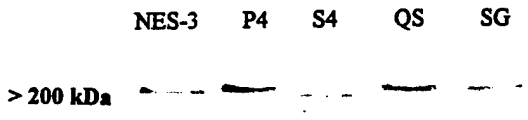

a. NE/S-3 S-4 P-4 FT QS SG
125K
b. NE/S-3 S-4 P-4 FT QS SG
70K
c. NE/S-3 S-4 P-4 FT QS SG
140K
d. NE/S-3 S-4 P-4 FT QS SG
36K
e. NE/S-3 P-4 S-4 QS FT SG
58K


SG
FT
QS
QS + CPT
SC DNA
II
I

KDNA networks

Decatenated DNA
Linear DNA

1 2 3 4 5


NES-3
P4
S4
QS
SG
> 200 kDa

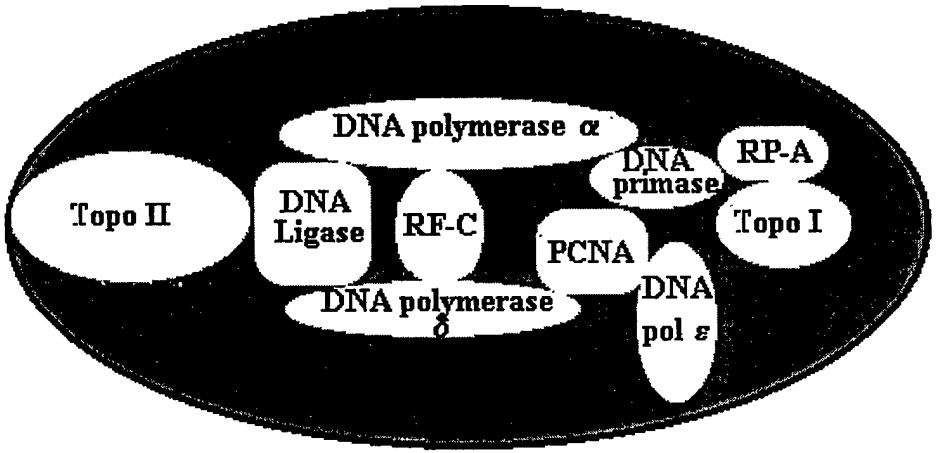

DNA polymerase α
DNA primase
RP-A
Topo II
DNA Ligase
RF-C
PCNA
Topo I
DNA polymerase δ
DNA pol ε

Table 1. DNA polymerase α and in vitro DNA replication activities fractionate with the P-4 fraction.

FRACTION	PEG NE/S-3	S-4	P-4
DNA Polymerase α*	132.5	0.3	188.3
DNA Replication(+T)#	103.8	8.8	110.6
DNA Replication (-T)#	3.1	0.0	0.2

*DNA polymerase activity with activated calf thymus DNA templates was assayed according to published procedures (21,22). One unit of DNA polymerase activity equals 1nmole of [^{3}H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of three experiments.

#In vitro SV40 DNA replication assays were performed as described in the Materials and Methods. One unit of SV40 replication activity is equal to the incorporation of 1pmole of [^{32}P]-dCMP into SV40 origin containing DNA *per* 2 hours at 35°C. These values represent the average of three experiments.

Table 2. In vitro DNA replication activities fractionate with the P-4 fraction.

FRACTION	Q-Sepharose Peak	Flow Through (FT)	Sucrose Gradient
DNA Replication (+T)#	136.6	3.4	141.2
DNA Replication (-T)#	8.5	1.8	10.2

#In vitro SV40 DNA replication assays were performed as described in the Materials and Methods. One unit of SV40 replication activity is equal to the incorporation of 1pmole of [^{32}P]dCMP into SV40 origin containing DNA per 2 hours at 35°C. These values represent the average of three experiments.

Table 3. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from human breast tumor tissue.

Fraction	NE/S-3	S-4	P-4
DNA polymerase α*	27.8	1.7	37.5
DNA Replication +T#	29.5	1.8	122.9
DNA Replication -T#	5.74	0.5	12.1

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [21,22] and as described in the Materials and Methods. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of [^{3}H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

In vitro SV40 DNA replication assays were performed as described in the Materials and Methods. One unit of replication activity equals the incorporation of 1 pmol of [^{32}P]-dCMP into SV40 origin containing DNA. These values represent the average of two independent experiments.

Table 4. DNA polymerase α activity of the column purified DNA synthesome from human breast cancer tissue.

Fraction	Peak	Flow-through
DNA polymerase a*	77.3	1.3

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [21,22] and as described in the Materials and Methods. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}H-TMP incorporated into DNA per hour at 35°C. Fractions 2 and 3 constitute the peak of DNA polymerase α activity. These values represent the average of two independent experiments.

Table 5. DNA polymerase α and *in vitro* SV40 DNA replication activities of the DNA synthesome from nude mouse tumor tissue.

Fraction	NE/S-3	S-4	P-4
DNA polymerase α*	40.6	2.0	123.2
DNA Replication +T#	57.2	11.2	158.7
DNA Replication -T#	5.5	4.1	9.6

* DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [21,22] and as described in the Materials and Methods. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of [^{3}H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

In vitro SV40 DNA replication assays were performed as described in the Materials and Methods. One unit of replication activity equals the incorporation of 1 pmol of [^{32}P]-dCMP into SV40 origin containing DNA. These values represent the average of two independent experiments.

Table 6. DNA replication fidelity of the breast cell DNA synthesome: Blue/White selection assay.

Origin of Synthesome	% mutants (ave. per 10^4 colonies)
MDA-MB 468 cell line	1.20% ± 0.2%
Human breast cancer tissue	0.93% ± 0.3%
Hs578 Bst (non-malignant)	0.19% ± 0.08%

An *in vitro* DNA replication fidelity assay [25] was used to measure the fidelity with which the synthesome from MDA MB 468 cells, human breast tumor tissue and Hs587Bst cells replicates plasmid DNA. The replicated plasmid, containing the bacterial lac-Z gene, was Dpn I digested and electroporated into E. coli, which were then plated onto LB agar containing the chromogenic substrates, X-gal and IPTG. Transformed bacteria expressing a non-mutated lac-Z gene (encodes the B-galactosidase enzyme) formed blue colonies on the plate, while bacteria containing DNA with mutations in the lac-Z gene formed white colonies. Mutations occurring in locations other than the lac-Z gene on the pBK-CMV plasmid are not detected by this forward mutagenesis assay. Consequently, the reported percentages of white colonies provide a minimum estimate of the actual number of mutations occurring in DNA synthesome mediated DNA replication. The percentage of mutant colonies expressed for each breast cell DNA synthesome is the average number taken from 3 separate assays of 10^4 transformed colonies each.