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In order to continue to determine the degree to which the accumulation of mutations in breast cancer cells is due to a change in the fidelity of the cellular DNA replication machinery. We have continued and expanded our experiments utilizing the multiprotein DNA synthesome isolated from malignant, non-malignant, and normal breast cells from both tissue culture and surgically resected human breast tissue. The DNA synthesome has been extensively demonstrated to carryout full length DNA replication *in vitro* and has been demonstrated by our lab to accurately depict the DNA replication process as it occurs in the intact cell. By utilizing a target gene in a forward mutagenesis assay, we have observed that the DNA synthesome from malignant breast cells replicates DNA with a significantly diminished fidelity as compared to the synthesome from non-malignant or normal breast cells. Furthermore, nucleotide sequencing has demonstrated that the types of replication errors created by the DNA synthesome derived from the malignant breast cells include nucleotide insertions, deletions, and mispairs. To begin to explore the mechanisms responsible for the diminished fidelity of DNA synthesome-mediated replication, we have initiated studies to map the possible interactions of the DNA synthesome with several DNA repair proteins. Initial data suggests that at least six key DNA repair proteins are tightly associated with the DNA synthesome.

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Ben Weeks Sekowski 8/31/97

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Introduction

To date it has been nearly impossible to distinguish whether the cellular transformation process gives rise to conditions that cause cancer cells to accumulate mutations (2) , or whether an increase in the rate of mutation within cells gives rise to the transformation process that culminates in the formation of cancer cells (3,4). Currently, a strong case can be made relating the number of unrepaired mutations in the cell with the development of a cancerous phenotype (5-7). The mutations noted to correlate with the expression of a cancerous phenotype could result from an increase in errors made during both DNA replication and DNA repair (8-17). In order to begin to assess the degree to which errors created during DNA replication contribute to the overall mutation frequency observed in cancer cells, I proposed to compare the fidelity of the DNA replication process in malignant breast cells and non-malignant breast cells. Several studies have reported that the activity of DNA β polymerase, an enzyme implicated in gap filling DNA synthesis during DNA repair (17,18) is decreased in cancer cells. These and other investigations also indicate that cancer cells generally have a higher error rate during the repair of gapped DNA and report that at least some of the common mutations include frame shifts and deletions of DNA sequence (17-22). However, most of these investigations were performed using either crude cell extracts or purified enzymes. *In vitro* assays using crude cell extracts contain nucleases and proteases, which may alter the integrity of the replication or repair enzymes in the extract or the DNA templates used in these assays. These factors may subsequently affect our interpretation of the data obtained using crude cell extracts. Those studies that use purified enzymes do not take into account that DNA repair in intact cells generally occurs in a highly controlled environment (13), with both the DNA and key enzymes organized into higher-order structures. These assays also do not adequately consider the potential contributions of accessory factors present in the intact cell that may enhance the fidelity of the DNA repair process. The observations reported by Kunkel's group (17,18) reinforce the idea that the maintenance of high fidelity DNA synthesis and repair requires at least some of the proteins used during DNA replication. Assays that ignore the possible involvement of the DNA replication proteins in the repair process are not capable of presenting an accurate picture of intact cell DNA repair, and also ignore the possible role played by the fidelity with which DNA replication is initially carried out and the overall contribution of the fidelity of DNA replication to the development of a "mutator" phenotype.

In order to better understand the extent to which the intact DNA replication machinery contributes to the overall mutation frequencies observed in malignant and non-malignant breast cells, I have designed experiments to examine the degree of fidelity exhibited during the DNA replication process in both normal and cancerous breast cells. To accomplish this goal I have isolated a multiprotein DNA replication complex (which we have designated the DNA synthesome) from both normal breast tissue cells and malignant breast cancer cells and have begun to determine the ability of the DNA synthesome from both cell types to faithfully copy a target gene used in our *in vitro* replication assay system. We have previously shown that the DNA synthesome isolated from mammalian cells is fully competent to carry out large T-antigen-dependent DNA synthesis *in vitro* (23-25). The DNA synthesome has been purified to about 30-40 polypeptides and is fully competent to replicate DNA bidirectionally from a defined origin of DNA replication, producing semi-conservatively replicated DNA. The rate of DNA replication and the products of the *in vitro* reaction suggest that the DNA synthesome faithfully mimics the replication process carried out in intact cells. Using this multiprotein DNA replication complex we have initiated experiments intended to determine whether cancer cells exhibit a higher mutation frequency due to a defect in the fidelity of the DNA synthetic process. Our data suggest that there is a higher frequency of mutation in our assay when the target DNA sequence is replicated by the DNA synthesome from the breast cancer cells than when it is replicated by the DNA synthesome from normal breast cells. Our most recent data suggest that the types of mutations created by malignant breast DNA synthesome-mediated replication include mismatches, and insertions and deletions of nucleotides.

Although we have observed decreased fidelity of the DNA replication as mediated by the DNA synthesome from malignant human breast cell lines and breast tissue, it is important to also consider the

potential contribution of the extensive repair activities present in the cell. In order to begin to explore the possible interaction of the DNA synthesome with the cellular DNA repair proteins, we have recently initiated a series of experiments designed identify the structural and functional interactions of several key DNA repair proteins with the breast cell DNA synthesome. We decided to look for the association of mismatch repair proteins hMSH2, hMLH1, hPMS2, and GTBP with the DNA synthesome due to their suggested interaction with the DNA replication process (40) and evidence which links them to the development of several types of cancer (41-48). We have also begun to examine the association between the DNA synthesome and MYH (homolog to the bacterial MutY protein), an adenine glycosylase which removes A/G and A/GO errors (49-51). Most recently, we have begun examining the association of the protein kinase Ku with the DNA synthesome, as it has also been suggested to participate in DNA replication and repair (52, 53).

To date, these experiments have yielded exciting data which suggest a direct interaction between the DNA synthesome and key DNA mismatch repair proteins. Experiments are ongoing to further map the relationship of these and other repair proteins associated with the DNA synthesome of the human breast cell.

Materials and Methods

Replication template (phagemid pBK-CMV) and bacteria culture.

a. The pBK-CMV vector (Stratagene) contains the full 298 bp SV40 origin of DNA replication, including both large T-antigen binding sites I and II. It also contains the eucaryotic promoter for the cytomegalovirus (CMV), the procaryotic RNA start sequence (at position 1221), the lacP gene (at position 1300-1220), which codes for the lacZ gene promoter, the α-lacZ gene (at position 1183-810), the start site for the β-gal gene (ATG) (at position 1183), and the stop site for the gene (TAA) (at position 799).

b. The XL1- Blue MRF' strain of E. coli This strain was purchased from Stratagene as the optimal strain of E. coli for the growth and expression of the pBK-CMV vector. It's genetic composition is: [(mcrA) 183, d(mcr(B-hsdSMR-mrr) 173, endA1, supE44, thi 1, recA1, gyrA96, relA1, lac[F'proAB, $lacI^{a}Z$ (m15, $Tn10(tet^{R})$)] Cells were transfected by electroporation (38,39).

c. Expression of the non-mutated β-galactosidase gene in the transformed E.coli growing in the presence of both the chromogenic substrate of the β-galactosidase gene product, 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal), and the inducer for the β-galactosidase gene, isopropylthio-β-D galactosidase (IPTG), will produce dark blue colonies. Errors in the sequence encoding the β-galactosidase gene result in white colonies. Intermediate phenotypes (light blue) may result from less severe mutations of the gene encoding β-galactosidase.

Cell culture.

The malignant breast cancer cell line, Hs578T (Homo sapiens No. 578, tumor cells), is an aneuploid, mammary myo-epithelial cell line derived from a mammary tumor that does not express the estrogen receptor protein (32). The Hs578T cells are grown in suspension in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 10 units /ml bovine insulin, and 10% fetal bovine serum (FBS). The non-malignant breast cell line Hs578Bst (Homo sapiens No. 578, breast cells) is diploid and is, most likely, of myoepithelial origin (32,41). It is derived from breast tissue found peripheral to the Hs578T tumor. The Hs578Bst cells are grown in monolayer culture with modified Dulbecco's medium, 30 ng/ml epidermal growth factor (EGF), and 10% FBS. The malignant MCF7 cell line is derived from a human breast adenocarcinoma. Cells from the plural effusion were used to establish the line. It has retained many characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes (64) It is maintained in Eagles' medium with non-essential amino acids, 1 nM sodium pyruvate, bovine insulin (10ug/ml), 90%, and fetal bovine serum, 10%.

The non-malignant MCF10A cell line is an immortalized cell line from human fibrocystic breast disease tissue of a 34 year-old Caucasian patient (65). It has a near normal karyotype, and by electronmicroscopy the cells display characteristics of luminal ductal cells but not myoepithelial cells. The cell line is maintained in a 1:1 mixture of Dulbecco modified Eagle's medium and Ham's F12 medium with 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone, 95%; and horse serum, 5%. All cell lines were purchased from the ATCC.

Fractionation scheme for the isolation of breast cell DNA synthesome.

Protein fractions from all breast cell types were prepared on ice or at 4°C following modified procedures of Malkas et al. (1990) and Coll et al (1996). The fractionation procedure for human breast tissue samples was modified to include freezing and pulverization in liquid nitrogen prior to Dounce homogenization. Cell homogenate fractions from all breast cell sources were centrifuged at 2,500 rpm (1,740 x g) for 10 minutes in order to separate the crude nuclear (P-1) and cytosolic fractions (S-1). Mitochondria (P-2) are pelleted from the S-1 fraction by centrifugation at 12,500 rpm (18,000 x g) for 15 minutes. The resultant supernatant (designated the S-2) fraction is then subjected to ultracentrifugation at 100,000 x g for 1 hour to remove microsomes (P-3), and the supernatant are designated the S-3 fraction. The crude nuclear pellet (P-1) are resuspended in buffer and gently rocked for 2 hours. After a 10-minute centrifugation at 15,000 x g the supernatant (designated NE), containing soluble protein extracted from the nuclei, is collected, combined with the S-3 fraction and made 2M in KCl and 5% in polyethylene glycol (PEG 6000). The mixture is stirred gently for one hour at 4°C and pelleted by centrifugation for 15 minutes at 16,000 rpm (30,900 x g). The resultant supernatant (PEG NE/S-3) is collected and layered onto a 2M sucrose cushion and subjected to centrifugation at 40,000 rpm (100,000 x g) for 16-18 hours at 4°C. The material above the sucrose cushion (top 70% of the tube) is collected and designated the S-4 fraction. The material collected at the sucrose interface (bottom 30%) is designated the P-4 fraction.

The P-4 fraction is then applied to a Q-Sepharose column (Pharmacia) (25 mg protein/1 ml of matrix) which is pre-equilibrated with loading buffer containing 50 mM Tris-HCl, pH 7.5/ 1 mM DTT/ 1 mM EDTA/ 10% glycerol/ 50 mM KCl. Unbound protein is washed from the matrix with 8 volumes of column-loading buffer. The matrix-bound protein is eluted by an increasing KCl gradient (50 mM - 1 M) in 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 10% glycerol. The column fractions will then be assayed for their ability to support *in vitro* SV40 DNA replication. The column fractions able to support *in vitro* SV40 DNA replication are pooled and layered onto an 11-ml 10-30% sucrose gradient containing 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 0.5 M KCl. The tubes containing the sucrose gradients are centrifuged at 100,000 x g for 16 hours, and the sucrose gradient fractions containing the replication-competent DNA synthesome are pooled, aliquoted, and stored at -80°C.

In Vitro DNA Replication Assay.

The DNA replication reactions (50ul) were carried out as described in Sekowski et al., 1997 (37).

DNA polymerase α and δ Assays.

DNA polymerase α activity was measured as described by and Lamothe et al., 1981 (55) and Vishwanatha et al., 1986 (56). One unit of polymerase α activity is equivalent to the incorporation of 1nmol of [^{3}H]-TMP into DNA per hour per mg protein at 37°C. The polymerase δ activity was measured according to procedures previously described by Lee et al, 1991 (57).

Precipitation of the Replicated DNA.

The DNA in the remaining 40 µl from each in vitro DNA replication reaction was precipitated as described in detail in Sekowski et al., 1997 (37). Briefly, after extraction and precipitation, the DNA was subjected to Dpn I digestion.

Forward Mutagenesis Assay: Transfection and Plating.
The Dpn I digested, *in vitro* replicated pBK-CMV DNA (fig. 3) was used to transfect the E.coli host (strain XL1- Blue MRF') as described in detail in Sekowski et al., 1997 (37). The transfection and plating conditions give intense blue color for the wild-type plasmid which facilitates the visualization of mutant phenotypes. The mutant colonies range from white to intermediate (relatively blue) phenotypes.

Scoring of Mutants.
The inactivation of the α-complementation gene (the product of which is the catalytic subunit of β-galactosidase) due to a mutation in the lac Zα gene in pBK-CMV will give a variety of mutant phenotypes, due to the lack of a fully functional β-galactosidase gene product. The mutant phenotypes were scored as described in Sekowski et al. (1997) (37). Calculations described in the figure legend of Table I have been made to convert the percentage mutant colonies to a number able to reflect the average number of nucleotide errors in the replicated plasmid.

Determination of the types of nucleotide errors in the DNA replicated in vitro.
Two flanking primers of 27 bp each (fig.8) were used to carry out nucleotide sequencing of the lacZα gene in the pBK-CMV plasmids extracted from clonal bacterial colonies expressing the mutant and wildtype β-galactosidase enzyme. Thus, the specific types of mutations that result from DNA replication mediated by the malignant and non-malignant human breast DNA synthesomes were obtained.

DNA template for intact cell DNA replication : pCEP4βgal.
The episome pCEP4 (Amersham Co.) (Fig. 4) was used as a vector for the insertion of the complete lacZα gene (encoding the β-galactosidase enzyme) into the multiple cloning site (MCS). This template will not only replicate inside a mammalian cell (utilizing the E-B virus origin), and confer selection in hygromycin, but will also contains the lac Z α gene for translation in a bacterial host.

Intact cell DNA replication fidelity: transfection of episome. Breast culture cells (MCF 7 and MCF10A) were grown as described above, harvested during logarhymic growth using 10mM EDTA/saline buffer and scraping, diluted in media, counted, and electroporated in the presence of the episome pCEP4βgal. Successful transfection of pCEP4βgal into a cell allows selection by Hygromycin (Sigma Co.) Thus, only those cells containing a functional episome survive in hygromycin containing media. Cells were allowed to grow for approximately two weeks, after which the episome was extracted from the cells using the procedure described by Hirt et al.,1967 (33). The DNA was purified, DpnI digested, and transfected into the E.coli host, XL1-Blue MRF'. The relative percentage of mutant colonies resulting from the transfection was measured and the mutation frequency resulting from intact cell replication calculated as mentioned above for the *in vitro* DNA replication fidelity assay.

Intact cell DNA replication fidelity: BrdU incorporation. To confirm that the episome underwent at least one round of semi-conservative DNA replication, transfected breast cells were grown in hygromycin-media containing the nucleotide analog Bromodeoxyuridine (BrdU). After the cells were allowed to grow for approximately two weeks, the episomal (incorporated with BrdU) was extracted from the cells. The extracted DNA was then subjected to cesium chloride density gradient analysis. Serial fractions of the DNA were collected from the CsCl gradient and the absorbance measured. The correct proportion of extracted DNA was comprised of heavy-light and heavy-heavy BrdU labeled DNA suggesting that at least one round of semi-conservative DNA replication of the episome had been completed in the breast

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting Analyses.
Thirty micrograms or 50 microliters (if protein concentration less than 0.5μg/μl) of sucrose gradient fractions were loaded per lane of the gels, and after resolution through an 8% SDS-PAGE the resolved

proteins were elctrophoretically transferred to nitrocellulose membranes. Immunodetection was carried out using a light-enhanced chemiluminescent (ECL) detection system according to manufacturers instructions (Amersham, Arlington Heights, IL) The antibodies directed against hMSH2, hPMS2, and hMLH1, hMSH6 (GTBP) were used at a dilution of 1ug/mL (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody directed against the bacterial protein MutY was a gift from Dr. A-L Lu and was used at a dilution of 1:200. The anti-Ku was used at a dilution of 1:1000 (Oncogene Science). The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots. Prestained SDS-PAGE molecular size markers were obtained from New England Biolabs (Boston, MA).

Gel Shifts Demonstrate Specific Binding to Mispaired and IDL containing DNA templates.

Oligonucleotides of 40 base pairs containing a single G/T mispair, or an single insertion-deletion loop of 2 or 4 nucleotides were constructed by the Biopolymer Facility (UMAB). The annealed oligonucleotides were 3'end labeled with Klenow fragment of DNA polymerase I for 30 min. at 25°C on the presence of [α-^{32}P]dCTP (50μCi at 3,000Ci/mmol), 20 μM dTTP, 20 μM dATP, and 20 μM dGTP. The resulting blunt-ended 40 bp duplex DNA mixture was passed through a 1mL P-60 column to remove unincorporated nucleotides. One microgram of Q-sepharose purified DNA synthesome was incubated with 1.9 fmol of the labeled template for 20 minutes at 37°C, after which 0.1% glutaraldehyde was added and the reaction incubated for an additional 10 minutes at 25°C. Sucrose was added to 14% in the reactions after which the reactions were resolved through a 5% non-denaturing polyacrylamide gel at 125V for 1 hr at 4°C. Loading dye was run in separate parallel lane in the gel. After drying the gels were exposed to Kodak XAR film (Kodak, Inc. Rochester, NY) at -80°C for 12-19 hr.

Co-localization of DNA synthesome-associated mismatch repair proteins with the mismatch-containing DNA template.

Large scale (5 fold greater) DNA synthesome-mismatch binding reactions were prepared as described for the gel-shift experiments above except that the target mismatch template was unlabeled and that no competitor DNA was added to the reactions. After the reactions were resolved on a 5% non-denaturing polyacrylamide as described above, the protein-DNA complexes were electrophoretically transferred to nitrocellulose using a semi-dry XL transfer apparatus (BioRad), 20 V for 20 min at 25°C. The nitrocellulose membranes were immunoblotted using the antibodies as described above for the SDS-PAGE immunoblotting.

Co-immunoprecipitation Analyses

One hundred micrograms of the Q-Sepharose peak fraction was subjected to co-immunoprecipitations according to a modified procedure of the protocol described in Coll et.al.1997 (to be published elsewhere). Briefly, the pre-cleared protein fractions were incubated overnight at 4°C with antibodies directed against hMSH2 (Q-20, Santa Cruz Biotechnology, 2.5 μg/reaction), PCNA (mAb-10, 2.5 μg/reaction), or polymerase α (SJK 132-20) 20μg/reaction. Thirty microliters of protein A or G agarose beads, pre-coated with BSA, were then added to the reactions containing the antigen-antibody complex, and the reactions were continued for one hour at 4°C. The antigen-antibody-bead complexes were pelleted by low-speed centrifugation at 3,500 rpm for 5 minutes at 4°C, and the supernatant and pellet fractions were resolved on a 8% SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with antibodies directed against PCNA, polymerase α, hMSH2, hMLH1, hPMS1, hPMS2, GTBP (hMSH6), MYH, and Ku as described above.

Results

I. Results of Task I : Preparation of the replication template DNA, months 1-12.

Since submitting the original proposal, in which we describe using the M13mp2 bacteriophage as the DNA template for the replication assay, we have discovered that M13mp2 bacteriophage has an intrinsic mutation rate of approximately 2-5 x 10^{-4} mutant colonies. In order to minimize the background mutation frequency rate we will use the pBK-CMV plasmid (Stratagene), for which the detectable mutation rate has been observed to be less than 1 x 10^{10} colonies (Sekowski and Hickey, unpublished data). As shown in figure 3, the pBK-CMV contains the SV40 origin of replication, including large T-antigen binding sites I and II, the full LacZα gene, and the kanamycin resistance gene. We have successfully grown this plasmid in XL1-Blue MRF' E.coli, a strain selected for its ability to support optimal growth and expression of this plasmid (Stratagene), and isolated and purified the supercoiled form I plasmid DNA for use in the DNA replication assay.

II. Results of Task 2: Purification of the DNA synthesome, Months 1-24.

Our laboratory has isolated a multiprotein DNA replication complex (the DNA synthesome) from human cervical carcinoma cells (HeLa) (27,35), from mouse mammary cells (FM3A) (28) and most recently, from MDA MB 468 human breast cancer cells (36), from the genetically matched human breast cell lines, Hs578Bst (non-malignant) and Hs578T (cancerous), and from the human breast epithelial cell lines MCF7 (malignant) and MCF10A (non-malignant) (40). In the past year, we have also successfully isolated the DNA synthesome from genetically matched malignant and non-malignant human breast tissue, as well as from normal human breast reduction tissue (36,40).

The complex is isolated using a series of steps that includes ultracentrifugation, ion-exchange chromatography, and sucrose gradient centrifugation as shown in the schematic figure 1. The sedimentation coefficient of the multiprotein complex from the MDA MB 468 breast cancer cells is approximately 18S as measured by sucrose gradient density analysis (36). The sedimentation coefficients of DNA synthesome from Hs578Bst (non-malignant), Hs578T (malignant), MCF10A (non-malignant), and MCF7 (malignant), as well as from the malignant, non-malignant, and normal human breast tissue are currently under analysis in our laboratory. The integrity of the multiprotein complex is maintained after treatment with DNase, RNase, 2M KCl, NP40/butanol, and Triton X-100, and after chromatography on DE52-cellulose and Q-Sepharose, suggesting that the association of proteins with one another is independent of nonspecific interaction with other cellular macromolecular components (28).

Most importantly, we have demonstrated that the DNA synthesome from the MDA MB-468 (36), and from the Hs578BSt, Hs578T, MCF10A, and MCF7 cell lines, as well as from the malignant, non-malignant, and normal human breast tissue (40), are fully competent to replicate DNA *in vitro* in a variation of the assay described by Li and Kelly (1984) (30). The demonstrated replication ability of the isolated multiprotein form of DNA polymerase suggests that all of the cellular activities required for large T-antigen-dependent *in vitro* papovavirus (i.e., SV40 and polyoma virus) DNA synthesis are present within the isolated DNA replication apparatus. Our lab has previously found that the mammalian DNA synthesome includes DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), RP-A (a.k.a. RF-A, and HSSB), topoisomerases I and II, helicases I and IV, RF-C or Activator 1 (A-1), and poly(ADP)-ribose polymerase (PARP) (27,28,35,36,39). The presence of these enzymes in the DNA synthesome has been verified by both Western blotting and, when possible, enzyme activity assays (e.g., RP-A, RF-C, PCNA do not have intrinsic enzymatic activity). The most current model of the DNA synthesome is shown in figure 2 in the appendix.

III. Results of Task 3: Isolation and analysis of the DNA synthesome-mediated DNA replication products, months 1-48.

DNA synthesome from Hs578Bst, Hs578T, MCF10A, MCF7, and human breast tissue cells have been utilized to mediate *in vitro* DNA replication assays. The purified DNA product from these assays was been subjected to Dpn I digestion and separated on a 1% neutral agarose gel. The products (visualized by autoradiography) demonstrate that the replication reactions mediated by the DNA synthesome from all cell types are capable of producing a full length DNA replication product (Sekowski, unpublished data). The level of DNA replication in each reaction has also been examined by measuring the incorporation of [^{32}P]- dCTP into the newly replicated DNA collected on DE81 filters and counted by liquid scintillation counting.

Measurement of the DNA polymerase α and the *in vitro* DNA replication activities.

As shown in the table (Figure 5), the polymerase α activity (pmoles incorporated nucleotides/hr/mg protein) of the DNA synthesome from the malignant cultured breast cells sources is enhanced 7 fold (MCF7 over MCF10A) and 18 fold (Hs578T over Hs578Bst). The increase of the polymerase α activity of the DNA synthesome from the malignant breast tissue appears to be quite variable ranging form 1.3 (tumor A), 1.4 (tumor D), and 1.7 (tumor C) fold up to 3.5 (tumor B) and 6.5 (tumor E) fold.

Interestingly, the DNA replication activity (pmol/hr/μg) (Fig.5) of the DNA synthesome from malignant breast cell culture is after a 3 hour incubation period is approximately 3.6-5.6 times higher in the reactions mediated than in those mediated by the synthesome from the non-malignant breast culture cells. However, the level of DNA replication of the DNA synthesome from any of the malignant breast tumor tissue samples to date does not differ by more than 0.3 fold above the activity of the DNA synthesome from the genetically matched non-malignant tissue samples.

IV. Results of Task 4: Transfection and expression of the replicated DNA product in E. coli, months 12-48.

The XLl-Blue MRF' strain of E.coli was successfully transfected with each of the following : the wild type pBK-CMV, the fully replicated/Dpn I digested pBK-CMV, and an equimolar concentration of pUC19 DNA (negative control) as described in the methods. The entire electroporation mixture containing the transfected E.coli and SOC medium was plated (100 ul per plate) onto LB agar plates containing optimal concentrations of kanamycin, X-gal, and IPTG. The transfected E.coli containing the wild type pBK-CMV expressed a dark blue phenotype (100%). The E.coli transfected with the negative control DNA (pUC19) consistently created mutant (white) colonies (100%). As shown in the table in figure 7, E.coli transfected with pBK-CMV replicated by the DNA synthesome from the Hs578T (malignant) cell line demonstrated a frequency of 86.5 x 10^{-6} mutations per nucleotide of the replicated plasmid. Similarly, E.coli transfected with DNA produced in replication reactions mediated by the MCF7 DNA synthesome resulted in a frequency of 51.5 x 10^{-6} mutations created per nucleotide of the replicated plasmid. Plasmids replicated by the DNA synthesome of the non-malignant Hs578Bst and the MCF10A cells were only found to have mutation frequencies of 15 x 10^{-6} and 11.8 x 10^{-6}, respectively.

To date, three separate experiments have been completed, under our empirically determined optimal conditions, all of which suggest that the frequency of replication errors in the malignant Hs578T and the MCF7 DNA synthesome -mediated replication reaction appear to be approximately 5.7 times (Hs578T) and 4.4 times higher (MCF7) that of the plasmid replicated by the DNA synthesome from the non-malignant Hs578Bst and MCF10A cells (see figure 6 (table) in the appendix).

Within the past year, I have begun to examine the replication fidelity of the DNA synthesome extracted from malignant and non-malignant human breast tissue, as well as from normal breast reduction tissue. Thus far, two separate genetically matched sets of tissue have been processed and tested for replication fidelity. The DNA synthesomes from genetically matched breast tumors A and B, were found to replicate DNA with a mutation frequency of 25.2 x 10^{-6} and 11.8 x 10^{-6} mutant nucleotides per

replicated plasmid, respectively. This represents a 3.6 and 3.8 fold decrease in fidelity below that demonstrated by the genetically matched tissues A and B (A and B mutation frequencies of 6.97×10^{-6} and 9.62×10^{-6}, respectively.

Lastly, recent results from two experiments examining the DNA synthesome from surgically resected normal human breast reduction tissue, demonstrated the fidelity with which the replication is carried out is relatively high. The mutation frequency per nucleotide is 6.43×10^{-6} ; therefore it is not significant different than the fidelity of the non-malignant breast tissue from cancer patients.

Studies examining the DNA replication fidelity of the DNA synthesome from other genetically matched breast tumor and tissue samples, from other normal breast reduction tissue, and from several types of benign tumors are ongoing.

Mutant and wildtype colonies were collected and grown separately in LB broth containing tetracycline and kanamycin over night. The clonal populations of mutant and normal E.coli were then collected by centrifugation, resuspended in fresh LB broth, made 10% in glycerol, and frozen at -80^0C for later extraction and nucleotide sequencing of the lacZα gene in the plasmid.

V. Results of Task V: DNA sequencing and analysis of the mutant DNA replication products, Months 12-48.

I have built an extensive library of clonal mutants isolated from the blue/white mutant selection assay. In order to determine whether the mutations observed in the bacterial colonies isolated from the mutant selection assay occur randomly or are located to specific segments of the plasmid DNA template we have completed sequencing β-galactosidase gene (Lac Zα) extracted from clonal mutant (white) and normal (blue) colonies. To this date, we have been able to successfully sequence four mutant and four normal plasmid extracted from bacteria transfected with the pBK-CMV replicated by the breast cancer DNA synthesome (MCF7). We have utilized the automated sequencing (UMAB sequencing facility) to sequence the β-galactosidase gene (Lac Zα). Using flanking primers (27 bp each) shown in figure 89 of the appendix, we were able to identify the types of errors which occur as a result of the DNA synthesome-mediated DNA replication (figure 9). To date, three types of mutations have been observed: 1) insertions of a single nucleotide, 2) deletions of a single nucleotide, and 3) nucleotide misincorporations (i.e. G/T mispairs).

VI. Results of additional experiments: Months 36-48.

Intact cell DNA replication fidelity studies.

Studies are ongoing. We are unable to report data at this time.

Identification of DNA repair proteins that co-fractionate with the DNA synthesome. by SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting Analyses

As shown in figure 11 the DNA repair proteins hMSH2, hMLH1, hPMS2, GTBP (hMSH6), MYH, and Ku were detected exclusively in the sucrose gradient fractions numbers 4 through 7. More importantly, these are the same fractions in which nearly all of the DNA replication activity and activities from DNA polymerases α and δ have been detected (figures 10a-c). Thus, these data provide compelling evidence toward the hypothesis that the DNA synthesome is tightly associated with at least some of the proteins responsible for carrying out DNA mismatch repair. Furthermore, the direct association of the DNA repair proteins with the DNA synthesome may make a significant contribution toward the overall fidelity of the DNA replication machinery.

Specific Binding to Mispaired and IDL containing DNA templates

As shown in figures 12a-12d, reactions which allowed the human breast cell DNA synthesome to bind a radiolabelled DNA template containing either a G/T mispair, or an insertion-deletion loop of 2 or 4 nucleotides demonstrated that the human breast DNA synthesome has a high specific binding activity as they were not able to be competed away by high concentrations (up to 900 fold) of unlabeled homopolymer DNA (fig.12d). (containing no errors). Competition created in the same manner demonstrated that synthesome binding to the mismatched templates can be competed away almost completely by an unlabeled mismatch containing DNA template (figs. 12a-c). Studies comparing the relative binding affinities of the DNA synthesome for the G/T, IDL_2 and IDL_4 are ongoing.

Co-localization of DNA synthesome-associated mismatch repair proteins with the mismatch-containing DNA template.

Studies are ongoing. We are unable to report data at this time.

Co-immunoprecipitation Analyses

Studies are ongoing. We are unable to report data at this time.

Discussion

Our original proposal described experiments in which an M13 vector containing the SV40 viral origin of DNA replication and the β–galactosidase gene were covalently linked. This M13 vector was used in an *in vitro* DNA replication assay in which DNA synthesis was mediated by a multiprotein DNA replication complex (the DNA synthesome) isolated from both malignant and non-malignant human breast cells. Our goals were to determine whether the DNA synthetic machinery (the DNA synthesome) of breast cancer cells was more error-prone than the DNA synthetic machinery of normal breast cells, and whether our results supported the hypothesis that the higher incidence of mutations observed in breast cancer cells, was due to a reduction in the fidelity of the breast cancer cell DNA synthesome relative to the fidelity of the normal breast cell DNA synthesome .

In setting up the mutation selection assay, we discovered that M13 had an inherent mutation frequency of 2-5 x 10^{-4}. mutant colonies relative to total transfectants. We believed that this high spontaneous rate of mutation, when the M13 was simply transfected into bacterial cells, would potentially mask the true mutation rate arising from errors created by the breast cancer cell DNA synthesome. If this were correct it would make it impossible to accurately assess whether the breast cancer cell DNA synthesome was error-prone. To overcome this potential difficulty we developed a strategy to covalently link the SV40 viral origin of replication and the β-galactosidase gene into a regular plasmid. During our initial planning of the details to construct this plasmid vector, we discovered that Stratagene had already constructed such a vector (pBK-CMV), and that it could be purchased from the company. We rapidly discovered that the Stratagene plasmid could be replicated *in vitro* by the DNA synthesome, and that the level of DNA replication could be optimized to that observed in the *in vitro* DNA replication assay employing another routinely used DNA template, pSVO+. The spontaneous mutation frequency of the unreplicated plasmid transfected into the XL1-Blue MRF' strain of E.coli was found to be less than 1x 10^{-10}.

While this aspect of the project was being developed, our laboratory group isolated the multiprotein DNA replication complex (the DNA synthesome) from two genetically matched human breast cell lines,

Hs578BSt (non-malignant) and Hs578T (malignant), and two other human breast cell lines, the MCF 10A (non-malignant) and MCF7 (malignant), using the method previously described by our lab for other mammalian cell lines (27,28,35,36). The DNA synthesome from each of these human breast cell lines is fully competent to complete full length, semiconservative, large T-antigen dependent *in vitro* DNA replication. This fact, as well as results described previously by our lab (27,28,35,36) suggest that all of the cellular protein activities necessary for *in vitro* SV40 DNA synthesis are present within the DNA synthesome isolated from the Hs578Bst, Hs578T, MCF10A, and MCF7 human breast cell lines. Additionally, the requirements for SV40 DNA replication *in vitro* by the isolated human breast cell DNA synthesome are comparable to the requirements that have been observed with crude cell extracts from permissive cells (30); namely, the initiation of SV40 DNA synthesis is dependent on the presence of both large T-antigen and a functional SV40 replication origin sequence.

An initial assessment of the level and fidelity of DNA replication carried out by the DNA synthesome of each of the human breast cell culture lines suggests a relationship between the relative rate with which the DNA template is replicated by the DNA synthesome and the relative frequency of mutational sequence errors that are created in the replicated DNA. Thus far, we have found that the level of DNA replication is between 3.6 -5.6 fold higher (fig.5) in the replication reactions mediated by the DNA synthesome from the cultured breast cancer cells than those mediated by the DNA synthesome from cultured non-malignant breast cells. The apparent increased rate of replication carried out by the breast cancer cell DNA synthesome correlates with an 4.4-5.7 fold increase (fig. 7)in the frequency of mutations in the plasmid DNA replicated by the DNA synthesome from the malignant cultured breast cells than when it is replicated by the synthesome from the non-malignant cultured breast cells.

I have also begun to examine the fidelity of the DNA synthesome extracted from genetically matched malignant and non-malignant human breast tissue, as well as from normal human breast reduction tissue. Excitingly, the initial DNA replication fidelity data we have collected form these samples supports the fidelity data gathered from the DNA synthesome from malignant and non-malignant cell lines.
However, the direct correlation between the specific replication activity and mutation frequency that was observed for the DNA synthesome from the breast cell lines has not been observed for the DNA synthesome from the breast tissue samples. Although the mutation rate of the DNA synthesome from the malignant breast tumors was observed to be significantly elevated (3.5-3.6 fold above the non-malignant mutation level)(fig. 7), the level of specific replication activity (pmol/hr/μg) (fig.5) was not significantly higher than that observed for the synthesome from non-malignant breast tissue. Thus, the level with which the DNA synthesome replicates DNA does not necessarily dictate the level of fidelity with which it will carry out this task. Studies are ongoing on other tumor/tissue samples in order to confirm these initial conclusions.

After recently identifying that the primary type of replication errors made by the malignant DNA synthesome were nucleotide insertions, deletions, and mismatches I have designed and initiated a series of experiments to explore the possible involvement of several of the DNA repair proteins in the DNA synthesome-mediated replication process. Since the human homologues to the bacterial MutHLS mismatch repair system are known to repair these types of replication errors, it seems evolutionarily advantageous that they would be closely linked to the DNA replication machinery, in order to quickly and efficiently repair these types of errors. In fact, compelling evidence exists to suggest that such an interaction exists between the DNA repair and the DNA replication systems (40). Additionally, there has been a significant amount of recent literature reporting that alterations in proteins hMSH2, hMLH1, hPMS2, and GTBP may play a role in the initiation and /or progression of several types of cancer. For these reasons, we have chosen to look for an association of these repair proteins with the human breast cell DNA synthesome. We have also begun to explore the interaction of the DNA synthesome with MYH (homolog to the bacterial MutY protein), an adenine glycosylase which removes A/G and A/GO errors (49-51). Most recently, we have also begun examining the association of the protein kinase Ku with the DNA synthesome, as it has also been suggested to participate in DNA replication and repair (52, 53).

To begin to define the involvement of these proteins with the human breast cell DNA synthesome, I performed Western immunoblot analyses on SDS-PAGE resolved DNA synthesome proteins from the MCF7 cell line. The DNA synthesome fractions used had been purified through the sucrose gradient step (the most enriched for DNA synthesome proteins of our fractionation) (see fig. 1). Excitingly, all of the major mismatch and two other repair proteins (46,47) for which we have probed were detected exclusively in the fractions which support peak DNA polymerase α, polymerase δ, and most importantly, complete *in vitro* DNA replication (Fig 10) (27, 28, 35). Thus, we our initial structural data suggests that these DNA repair proteins may be tightly associated with the human breast cell DNA synthesome.

Gel shift studies have also been initiated in order to define the ability of the intact breast cell DNA synthesome to bind specifically to common types of DNA errors such as insertion-deletion loops of 2 to 4 nucleotides and mispairs (i.e. a G/T mispair). Our data suggests that the DNA synthesome binds with relatively high affinity to all of these types of mismatches, as competition with at least 900 fold homopolymer DNA (matched non-mismatch containing DNA) does not significantly disrupt the binding of the synthesome to any of the mismatch containing DNA templates. Competition studies are ongoing to determine to relative binding affinity of the human breast DNA synthesome to these and other types of common DNA errors.

During this upcoming funding year we plan to continue to expand our DNA replication fidelity studies to examine DNA synthesome from many different types of breast cancer and breast disease, as well as examine changes in the DNA synthesome fidelity in relation to stage of breast cancer.

Although, to date, we cannot yet describe the actual differences in the breast cancer cell DNA synthesome and the normal breast cell DNA synthesome, responsible for the altered replication rate and replication fidelity of the breast cancer cell DNA synthesome, it is likely that alteration in specific components of the DNA synthesome are responsible for these differences. We are planning a detailed investigation into specific molecular alterations of the DNA synthesome from malignant breast cells, including the exploration of the structural and functional relationship of the DNA repair components of the DNA synthesome from malignant and non-malignant human breast cells.

To date, our results support the hypothesis that the cellular transformation process is, at least partially, a consequence of an increased rate in the accumulation of certain types of mutations and that these mutations arise, in part, due to a decrease in the fidelity of the DNA replication machinery (i.e. DNA synthesome) of the cancer cell.

References

1.Kunkel, T.A. Mutational specificity of depurination. Proceedings of the National Academy of Science, USA 81:1494-1498, 1984.

2.Speyer, J.F. Mutagenic DNA polymerase. Biochemical and Biophysical Research Communications 21:6, 1965.

3.Farber, E. and Rubin, H. Cellular adaptation in the origin and development of cancer. Cancer Research 51:2751-2761, 1991.

4.Prehn, R.T. Cancers beget mutations versus mutations beget cancers. Cancer Research 54:52976-5300, 1994.

5.Klibanski, A. Editorial: further evidence for a somatic theory in the pathogenesis of human pituitary tumors. Journal of Endocrinology and Metabolism 71:1415A-1415C, 1990.

6.Morris, D.W. and Cardiff, R.D. Multistep model of mouse mammary tumor development. Avd. (sic) Viral Oncology 7:123-140, 1987.

7.Liu, E.T. Oncogenes, breast cancer, and chemoprevention. Journal of Cellular Biochemistry 17G:161-166, 1993.

8.Ponten, J. Natural history of breast cancer. Acta Oncologia 29:325-329, 1990.

9.Skolnick, M.H. and Cannon-Albright, L.A. Genetic predisposition to breast cancer. Cancer Supplement 70:1747-1754, 1992.

10.Smith, H.S., Lu, Y., Deng, G., et al. Molecular aspects of breast cancer. Journal of Cellular Biochemistry 17G:144-152, 1993.

11.Shiu, R.P.C., Watson, P.H. and Dubil, D. C-myc oncogene expression in estrogen-dependent and -independent breast cancer. Clinical Chemistry 39:353-355, 1993.

12.Mazars, R., Spinardi, L., BenCheikh, M., Simony-Lafontaine, J., Jeanteur, P. and Theillet, C. p53 mutations occur in aggressive breast cancer. Cancer Research 52:3918-3923, 1992.

13.Dawkins, H.J.S., Robbins, P.D., Smith, K.L., et al. What's new in breast cancer?: Molecular perspectives of cancer development and the role of the oncogene c-erb-2 in prognosis and disease. Path. Research Pract. 189:1233-1252, 1993.

14.Sutherland, R.L., Watts, C.K.W. and Musgrove, E.A. Cyclin gene expression and growth control in normal and neoplastic human breast epithelium. Journal of Steroid Biochemistry and Molecular Biology 47:99-106, 1993.

15.Goodman, M.F., Creighton, S., Bloom, L.B. and Petruska, J. Biochemical basis of DNA replication fidelity. Critical Reviews in Biochem.

16.Boosalis, M.S., Petruska, J. and Goodman, M.F. DNA polymerase insertion fidelity-gel assay for site-specific kinetics. Journal of Biological Chemistry 262:14689-14696, 1987.

17.Kunkel, T.A. Frameshift mutations by eucaryotic DNA polymerase in vitro. Journal of Biological Chemistry 29:13581-13587, 1986.

18.Kunkel, T.A. and Soni, A. Exonucleolytic proofreading enhances the fidelity of DNA synthesis by chick embryo DNA polymerase-gamma. Journal of Biological Chemistry 263:4450-4459, 1988.

19.Copeland, W.C., Lam, N.K. and Wang, T.S.-F. Fidelity studies of the human DNA polymerase α. Journal of Biological Chemistry 268:11041-11049, 1993.

20.Copeland, W.C. and Wang, T.S.-F. Mutational analysis of the human DNS polymerase α. Journal of Biological Chemistry 268:11028-11040, 1993.

21.Thomas, D.C., Roberts, J.D., Sabatino, R.D., et al. Fidelity of mammalian DNA replication and replicative DNA polymerases. Biochemistry 30:11751-11759, 1991.

22.Kunkel, T.A. The mutational specificity of DNA polymerase-β during *in vitro* DNA synthesis. Journal of Biological Chemistry 260:5787-5796, 1985.

23.Roberts, J.D. and Kunkel, T.A. Fidelity of a human cell DNA replication complex. Proceedings of the National Academy of Science USA 85:7064-7068, 1988.

24.Roberts, J.D., Nguyen, D. and Kunkel, T.A. Frameshift fidelity during replication of double-stranded DNA in HeLa cell extracts. Biochemistry 32:4083-4089, 1993.

25.Roberts, J.D., Thomas, D.C. and Kunkel, T.A. Exonucleolytic proofreading of leading and lagging strand DNA replication errors. Proceedings of the National Academy of Science USA 88:3465-3469, 1991.

26.Hackett, A.., Smith, H.S., Springer, E.L., et al. Two syngenic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs 578T) and the diploid myoepithelial (Hs578Bst) cell lines. Journal of the National Cancer Institute 58:1795-1806, 1977.

27.Malkas, L.H., Hickey, R.J., Li, C., Pederson, N. and Baril, E.F. A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*. Biochemistry 29:6362-6374, 1990.

28.Wu, Y., Hickey, R.J., Lawlor, K., et al. A 17S multiprotein form of murine cell polymerase mediates polyomavirus DNA replication *in vitro*. Journal of Cellular Biochemistry 54:32-46, 1994.

29.Sanger, F., Nicklen, S. and Coulson, A.R. DNA sequencing with chain terminating inhibitors. Proceedings of the National Academy of Science USA 74:5463-5467, 1977.

30.Li, J.J. and Kelly, T.J. Simian virus 40 DNA replication *in vitro*. Proceedings of the National Academy of Science USA 18:6973-6977, 1984.

31.Hanahan, D. Studies on transformation of E. coli with plasmids. Journal of Molecular Biology 166:557-565, 1983.

32.Kingston, R.E. Introduction of DNA into mammalian cells. In: Current Protocols in Molecular Biology, edited by Anon.. Cold Spring Harbor, NY:Cold Spring Harbor Press, 1993, pp. 9.0.1-9.5.6.

33.Hirt, B. Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology 26:365-369, 1967.

34.A.T.C.C. Hs578Bst normal: a non-estrogen dependent breast cell line. Journal of the National Cancer Institute 58:1795-1806, 1977.

35. Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Lee, M., Malkas,L.H. Further characterization of the human cell multiprotein form of DNA polymerase. Journal of Cell Biochemistry 59(1), pp. 91-97, 1995.

36. Coll, J.M., Sekowski, J.W., Hickey, R.J., Schnaper, L., Yue, W., Brodie, A.M.H., Uitto, L., Syvaoja, J.E., and Malkas, L.H. The Human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncology Research, Vol. 8, Nos.10/11, pp.435-447, 1996.

37. Sekowski, J.W., Malkas, L.H., Wei, Y., Hickey, R.H. Mercuric ion inhibits the activity and fidelity of the human cell DNA synthesome. Toxicology and Applied Pharmacology 145, 268-276, 1997.

39. Applegren, N., Sekowski, J.W., Hickey, R.H., Tuteja, N., Wilson, S., Uitto, L., Syvaoja, J., and Malkas, L.H. (1997). The human cell DNA synthesome: Evaluation of its DNA replication mechanism and the identification of additional components. J. Cell Biochem., submitted, 1997.

40. Sekowski, J.W., Malkas, L.H., Schnaper, L., and R.J. Hickey. Human breast cancer cells contain an error-prone DNA replication apparatus. Nature Medicine, in preparation, 1997.

41. Umar, A. and Kunkel, T.A. DNA replication fidelity, mismatch repair, and genome stability in cancer cells. Eur. J. Biochem. 238, 297-307, 1996.

42. Leach, F.S., N.C. Nicolaides, N. Papadopoulos, B. Liu, J. Jen, R. Parsons, P. Peltomaki, P. Sistonen, L. A. Aaltonen, M. Nystrom-Lahti, X.-Y. Guan, J. Zhang, P.S. Meltzer, J.-W. Yu, F.-T. Kao, D.J. Chen, K.M. Cerosaletti, R.E. Keith Fournier, S. Todd, T. Lewis, R.J.Leach, S.J.Naylor, J. Weissenbach, J.-P. Mecklin, H. Jarvinen, G.M. Petersen, S.R. Hamilton, J. Green, J. Jass, P. Watson, H.T. Lynch, J.M. Trent, A. de la Chapelle, K. Kinzler, and B. Vogelstein. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 75: 1215-1225, 1993.

43. Fishel, R.A., M.K.. Lescoe, M.R.S. Rao, N.G.Copeland, N.A. Jenkins, J. Garber, M. Kane, and R. Kolodner. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 75: 1027-1038, 1993.

44. Nicolaides, N., N. Papadopoulos, B. Liu, Y-F Wei, K.C. Carter, S. M. Ruben, C.A.Rosen, W. A. Haseltine, R.D. Fleishmann, C. M. Fraser, M.D. Adams, J. C. Venter, M.G. Dunlop, S.R. Hamilton, G.M. Peterson, A. de la Chapelle, K.W. Kinzler, and B. Vogelstein. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature 371: 75-80, 1994.

45. Papadopoulos,N., N. Nicolaides, Y-F Wei, S. M. Ruben, K.C. Carter, C.A. Rosen, W. A. Haseltine, R.D. Fleishmann, C. M. Fraser, M.D. Adams, J. C. Venter, S.R. Hamilton, G.M. Peterson, P. Watson, H. T. Lynch, P. Peltomaki, J.-P. Mecklin, A. de la Chapelle, K. Kinzler, and B. Vogelstein. Mutation of *mut*L homolog in hereditary colon cancer. Science 263: 1625-1629, 1994.

46. Palombo, F., P. Gallinari, I. Iaccarino, T. Lettieri, M. Hughes, A. D'Arrigo, O. Truong, J.J. Hsuan, and J. Jiricny. GTBP, a 160-Kilodalton protein essential for mismatch-binding activity in human cells. Science 268: 1912-1914, 1995.

47. Papadopoulos,N., N. Nicolaides, B. Liu, R. Parsons, C. Lengauer, F. Palombo, A. D' Arrigo, S. Markowitz, J.K.V. Willson, K. W. Kinzler, J. Jiricny, and B. Vogelstein. Mutations of GTBP in genetically unstable cells. Science 268: 1915-1917, 1995.

48. Alani, E., T. Sokolsky, B. Studamire, J.J. Miret, and R.S. Lahue. Genetic and biochemical analysis of Msh2p-Msh6p: role of ATP hydrolysis and Msh2p-Msh6p subunit interactions in mismatch base pair recognition. Molec. and Cell. Biol. 17(5): 2436-2447, 1997.

49. Meyers, M., M. Theodosiu, S. Acharya, E. Odegaard, T. Wilson, J.E. Lewis, Davis, C. Wilson-Van Patten, R. Fishel, and D.A. Boothman. Cell cycle regulation of the human DNA mismatch repair genes hMSH2, hMLH1, and hPMS2. Cancer Research 57: 206-208 1997.

50. Lu, A.-L. and D-Y Chang. A novel nucleotide excision repair for the conversion of A/G mismatch to C/G base pair in E.coli. Cell 54: 805-812, 1988.

51. Lu , A.-L., J.J. Tsai-Wu, and J. Cillo. DNA determinants and substrate specificities of *Escherichia coli* MutY. J. Biol. Chem. 270 (40): 23582-23588, 1995

52. McGoldrick, J.P., Y.-C. Yeh, M. Solomon, J. M. Essigmann, and A.-L. Lu.. Characterization of a mammalian homolog of the *Escheriachia coli* MutY mismatch repair protein. Mol. and Cell. Biol. 15(2): 989-996, 1995.

53. Stuvier, M.H, Coenharts, F.E.J., van der Vliet, P.C. The autoantigen Ku is indistinguishable from NFIV, a protein forming multimeric protein-DNA complexes. J. Exp. Med. 172: 1049-1054, 1990.

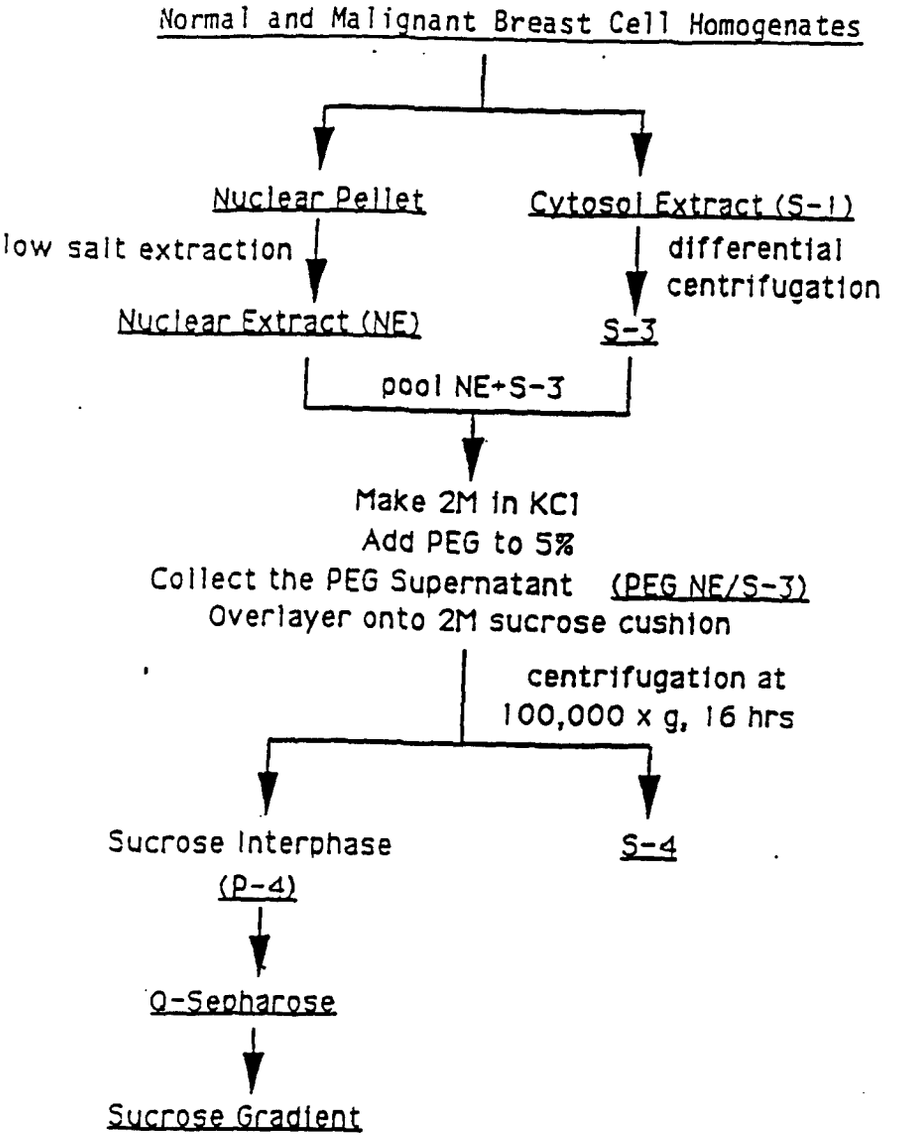
54. Anderson, C.W. DNA damage and the DNA-activated protein kinase. Trends in Biochem. Sci. 18: 433-437, 1993.

55. LaMothe, P., Baril, B., Chi, A., Lee, L., and Baril, B. Accessory protein for DNA polymerase α activity with single stranded DNA template. Proc. Natl. Acad. Sci USA78: 4723-4727, 1981.

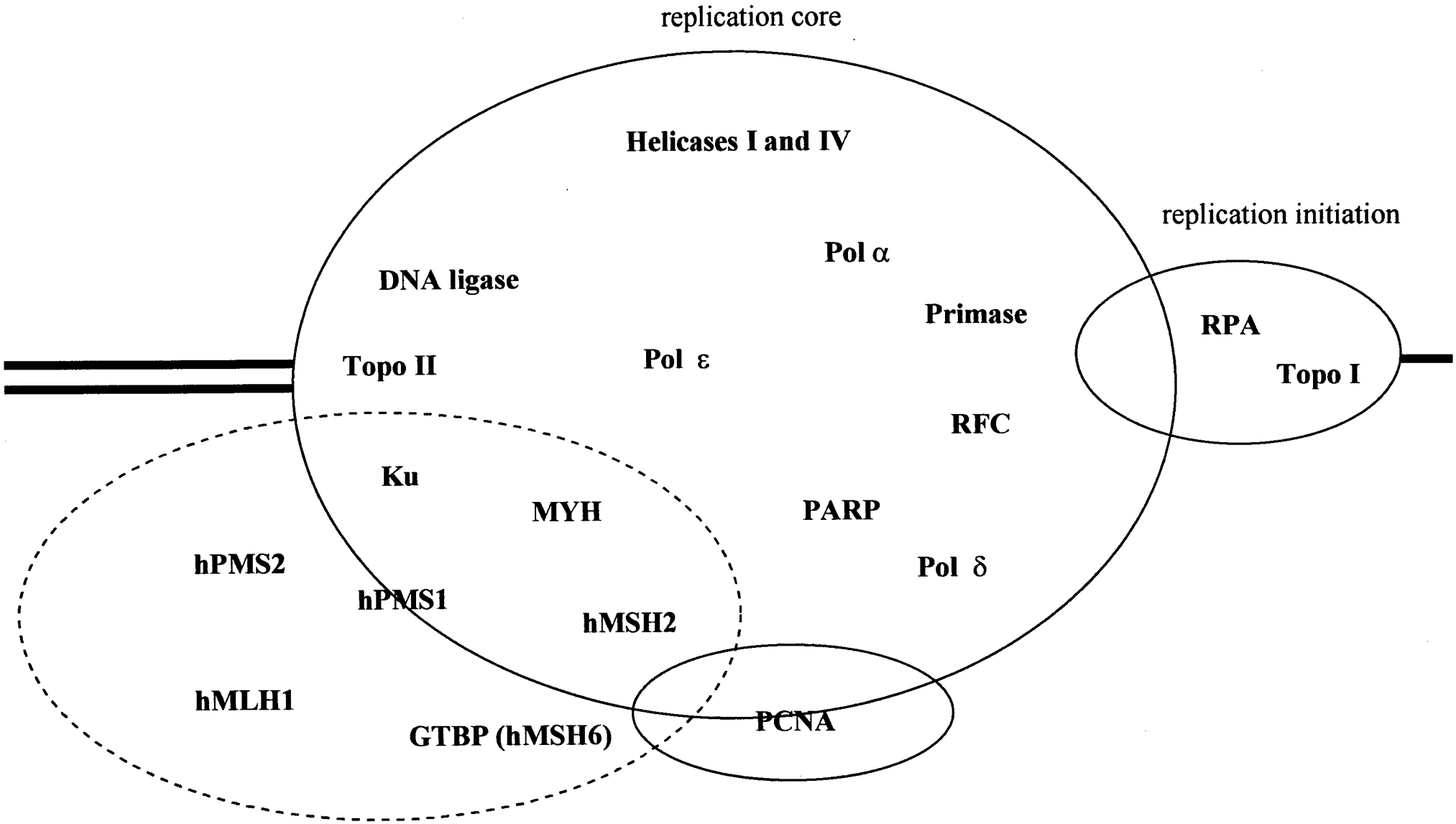
56. Vishwatha, J.K, Yamaguchi, M., DePamphilis, M.L., Baril, E.F. Selection of the template initiation sites and the lengths of RNA primers synthesized are strongly affected by its organization in a multiprotein polymerase α complex. Nucl. Acids. Res. 14: 7305-7323, 1986.

57. Lee, M.Y.W.T., Jiang, Y., Zhang, S.J., and Toomey, N.L. Characterization of human polymerase δ and its immunochemical relationships with polymerases α and ε . J. Biol. Chem.266(4): 2433-2429.

Fig. 1


Normal and Malignant Breast Cell Homogenates
Nuclear Pellet
Cytosol Extract (S-1)
low salt extraction
differential centrifugation
Nuclear Extract (NE)
S-3
pool NE+S-3
Make 2M in KCl
Add PEG to 5%
Collect the PEG Supernatant (PEG NE/S-3)
Overlayer onto 2M sucrose cushion
centrifugation at 100,000 x g, 16 hrs
Sucrose Interphase (P-4)
S-4
Q-Sepharose
Sucrose Gradient

Current Model of the BREAST CELL DNA SYNTHESOME



potential DNA repair components of the synthesome

Fig. 2

Fig. 3

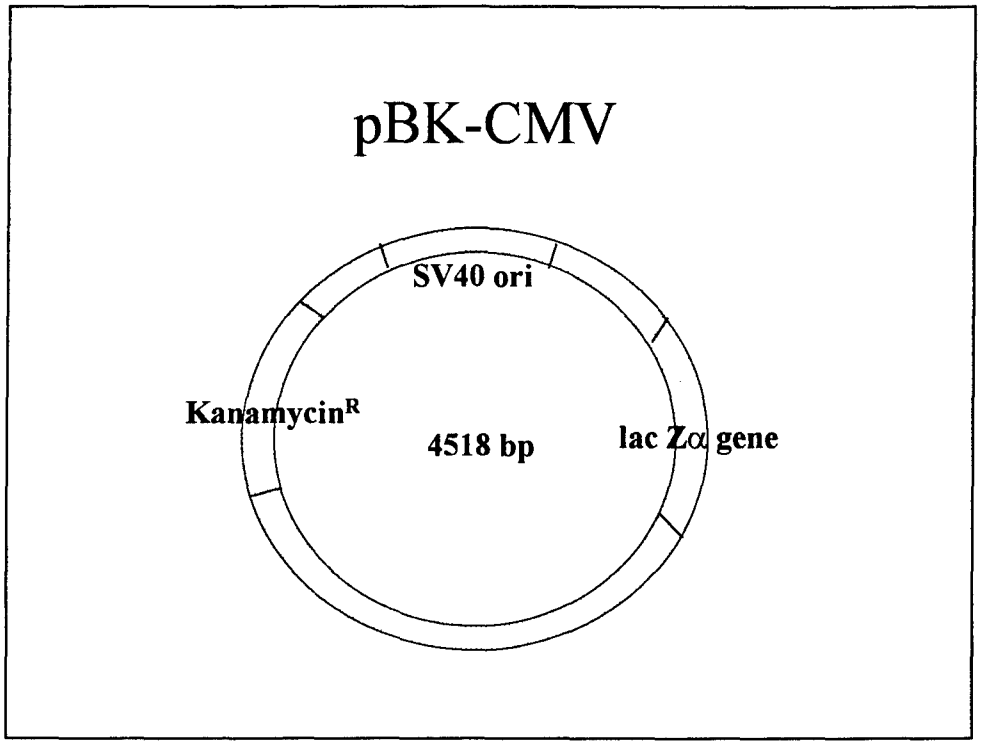

pBK-CMV
SV40 ori
Kanamycin^R
4518 bp
lac Zα gene

Fig. 4

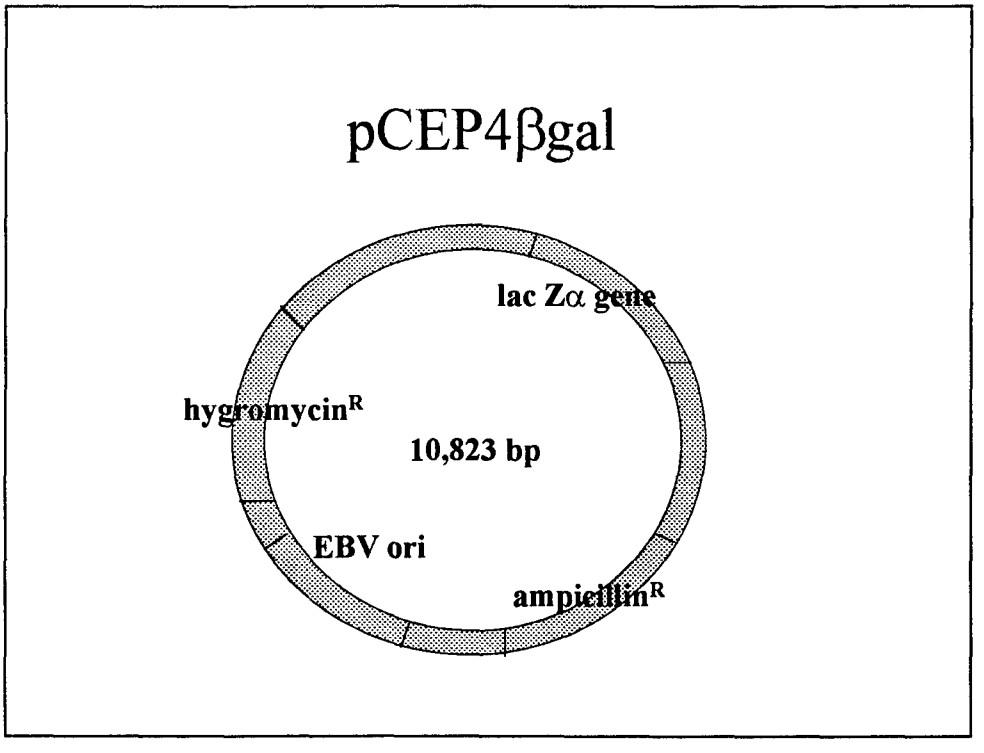

pCEP4βgal
lac Zα gene
hygromycin^R
10,823 bp
EBV ori
ampicillin^R

Figure 5

DNA synthesis and replication activities of the malignant and non-malignant human breast DNA synthesome.

Source of DNA synthesome	DNA polymerase α activity [c]	DNA replication (+TAg) [d]	DNA replication (-TAg) [e]	Fold TAg dependent replication activity [f]
MALIGNANT human breast cells				
MCF 7	5394 (7 fold)	9,110	1,730	5.6
Hs578T	7528 (18 fold)	5,724	1,257	3.6
MDA-MB468	N/A/	N/A	N/A	-
[a] tumor A	912 (1.3 fold)	1,480	333	1.0
[a] tumor B	352 (3.5 fold)	1,100	0	1.3
[a] tumor C	700 (1.7 fold)	2,500	165	2.0
[a] tumor D	417 (1.4 fold)	1,250	0	1.0
[a] tumor E	650 (6.5 fold)	N/A	N/A	N/A
[a] tumor F	N/A	N/A	N/A	N/A
[a] tumor G	N/A	N/A	N/A	N/A
NON-MALIGNANT human breast cells				
MCF10A	745	1,378	57	-
Hs578Bst	400	1,272	42	-
[a] tissue A	700	1,500	0	-
[a] tissue B	99	870	0	-
[a] tissue C	420	1145	0	-
[a] tissue D	300	1150	0	-
[a] tissue E	100	N/A	N/A	-
[a] tissue F	N/A	N/A	N/A	-
[a] tissue G	N/A	N/A	N/A	-
NON-MALIGNANT human breast tissue				
benign phylodes tumor	27	N/A	N/A	-
benign ductal hyperplasia	270	N/A	N/A	-
NORMAL breast cells				-
[b] normal A	43	1210	0	-

[a] Surgically resected female human breast tissue. Genetically matched samples are denoted by corresponding alphanumeric designations (tumor A , tissue A, etc.) Factors such as stage of malignancy, genetics, race, age, were double blind under after data collection.

[b] Surgically resected breast reduction tissue from healthy females.

[c] DNA polymerase activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to 1 picomoles [^{3}H]TMP incorporated in DNA per hour per microgram protein at 35°C. The values represent the average of two independent experiments.

[d] In vitro DNA replication activity assays were perfomed as described previously. Units represent amount of nascent DNA produced expressed as picomoles nascent DNA per hour per microgram synthesome protein. The values represent the average of two independent experiments. TAg= SV40 large T-antigen.

[e] Values represent T-antigen independent DNA synthesis (pmol DNA per hour per microgram)
[f] fold TAg dependent specific replication activity (+TAg values minus -TAg values) of DNA synthesome from malignant breast cells divided by those values from obtained from DNA synthesome from non-malignant breast cells.

FORWARD MUTAGENESIS ASSAY: PART I

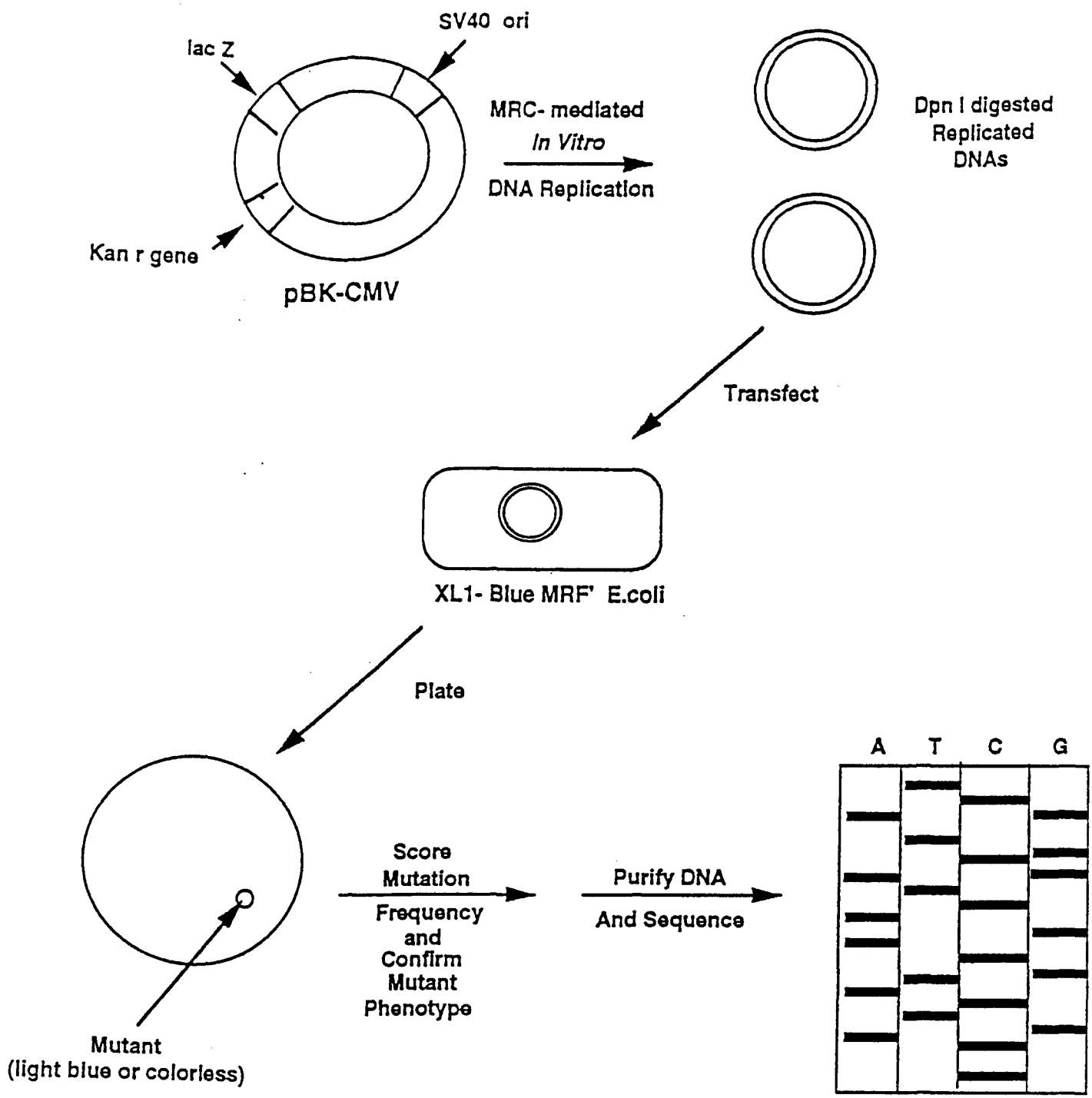


Figure 7

DNA replication fidelity estimates of the malignant and non-malignant human breast DNA synthesome

Source of DNA synthesome	total number of colonies scored	number of mutant colonies	mutant frequency ($\times 10^{-6}$) [c]	fold mutation rate [d]
MALIGNANT human breast cells				
MCF 7	6.0×10^4	576	51.5	4.4
Hs578T	6.0×10^4	960	86.5	5.7
MDA-MB468	6.0×10^4	762	68.1	NG
[a] tumor A	3.0×10^4	141	25.2	3.6
[a] tumor B	3.0×10^4	209	37.2	3.8
[a] tumor C	N/A	N/A	N/A	N/A
[a] tumor D	N/A	N/A	N/A	N/A
NON-MALIGNANT human breast cells				
MCF10A	4.0×10^4	66	11.8	-
Hs578Bst	4.0×10^4	113	15.0	-
[a] tissue A	1.0×10^4	13	6.97	-
[a] tissue B	1.0×10^4	18	9.62	-
[a] tissue C	N/A	N/A	N/A	-
[a] tissue D	N/A	N/A	N/A	-
NON-MALIGNANT human breast tissue				
benign fibroadenoma	N/A	N/A	N/A	-
benign phylodes tumor	N/A	N/A	N/A	-
benign ductal hyperplasia	N/A	N/A	N/A	-
NORMAL [b] breast cells				-
normal A	1.0×10^4	12	6.43	-

[a] Surgically resected female human breast tissue. Genetically matched samples are denoted by corresponding alphanumeric designations (tumor A , tissue A, etc.) Factors such as stage of malignancy, genetics, race, age, were double blind under after data collection.

[b] Surgically resected breast reduction tissue from healthy females.

[c] Values represent the relative number of errors created per nucleotide of the replicated plasmid. This derivation was based on the following formula described by Roberts and Kunkel (1988): The number of mutant colonies/total number of transformed colonies minus the background mutation rate (none detected in 5×10^{-8} colonies) divided by the chance of a nucleotide defect within in the lacZ α gene if the colony expresses a white phenotype (0.5) divided by the number of sites in the target gene (373 bp). The lacZ α gene comprises 8.25% of the total pBK-CMV plasmid (4518bp). Each value represents the average of 2-3 individual experiments.

[d] Values represent the fold increase in mutation frequency of the malignant synthesome as compared to its genetically matched non-malignant counterpart. NG = no genetically matched counter part available. N/A= data not available at this time.

Fig. 8

Sequencing primers (27 bp each) for the lacZα gene

Primer 1

5' CACTTAAAATTGGATCTCCATTCGCCC(TOP STRAND) ----------->
3' GTGAATTTT AAC CTAGAGG TAAGCGG TAAGTCCGACGCGTTGACAA

TOP STRAND----> ---------->
3'GGGAAGGGCGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGG

---------->
3'GGATGTGCTGCAAGGCGATTAAGTTGG GTAACGCCAG GGTTTTCCCA

---------->
3'GTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTAT

---------->
3'AGGGCGAATTGGGTACACTTACCTGGTACCCCACCCGGGTGGAAAATCGA

---------->
3'TGGGCCCGCGGCCGCTCTAGAAGTACTCTCGAGAAGCTTTTTGAATTCTT

---------->
3'TGGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCCAGCTTTTGTTCC

5'--- *GAAATCACTCCCAATTAAAGCT C GAA C CGCA TT AG TTCCA* ***3'***
3' CTTTAGTGAGG <---------- **GGCGTAATCAAGGT**

5' ATA G ATAC TGG
TAT CGATGACC 5' (BOTTOM STRAND)

Primer 2

Mutations in the lac Z α gene of pBK-CMV Replicated by the MCF7 DNA Synthesome

5' CACTTAAAATTGGATCTCCATTCGCC (TOP STRAND) ---------->
3' GTGAATTTTAACCTAGAGGTAAGCGGTAAGTCCGACGCGTTGACAA

TOP STRAND----> -------- ----------C---------- T---------->
3'GGGAAGGGCGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGG

insertion of additional C
---------- ----------ACCCCATT ---------->
GGGATGTGCTGCAAGGCGATTAAGTTGGGTAA CGCCAG GGTTTTCCCA

G/Tmispair
---------- G ---------->
3'GTCACGACGTTGTAATAACGACGGCCAGTGAATTGTAATACGACTCACTAT

insertion of additional T
G/T mispair
----------G ---------->
3'AGGGCGAATTGGGTACACTTACCTGGTACCCCACCCGGGTGGAAAATCGA

insertion of C
----------AAACAA ---------->
3'TGGGCCCGCGGCCGCTCTAGAAGTACTCTCGAGAAGCTTTTTGAATTCTT

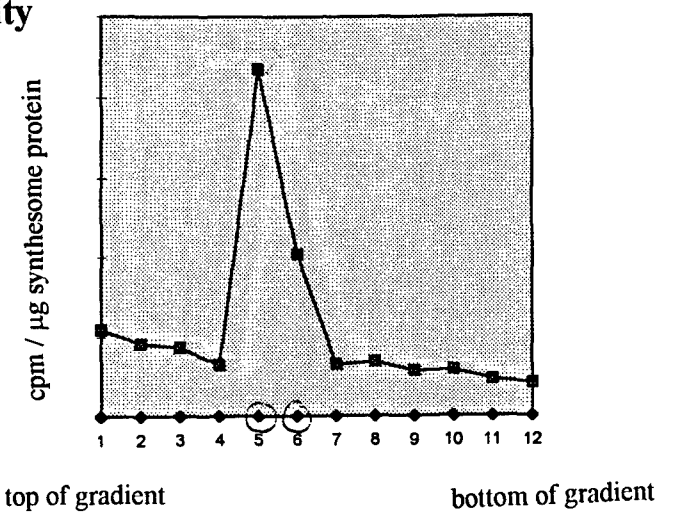
deletion of TA
------ " TA"---------->
3'TGGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCCAGCTTTTGTTCC

5'--- GAAATCACTCCCAATTAAA GCTC GAACC GCAT TAGTT CC A
3' CTTTAGTGAGG < ---------- **CGAGCTTGGCGTAATCAAGGT**

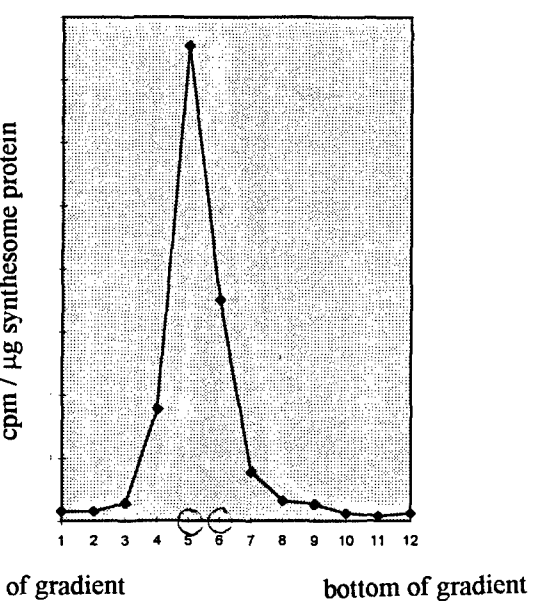
5' ATA GATA CT GG
3' **TAT CGATGACC** **5' BOTTOM STRAND**

Fig. 10- DNA polymerase α and δ and DNA replication activities are found between sucrose gradient fractions 4 and 7.

10A. In vitro DNA replication activity



10B. DNA polymerase α



10C. DNA polymerase δ

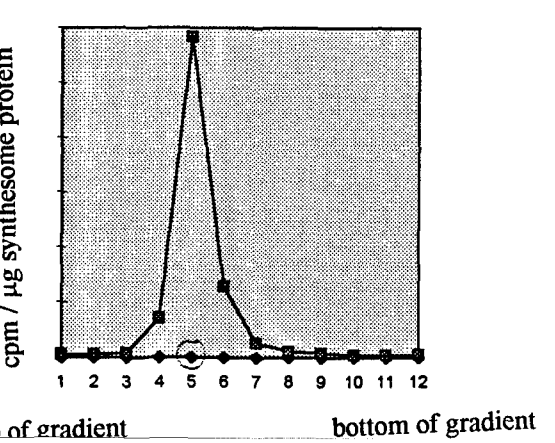


Figure 11. DNA repair proteins are found exclusively in those sucrose SG fractions which contain peak replication activity

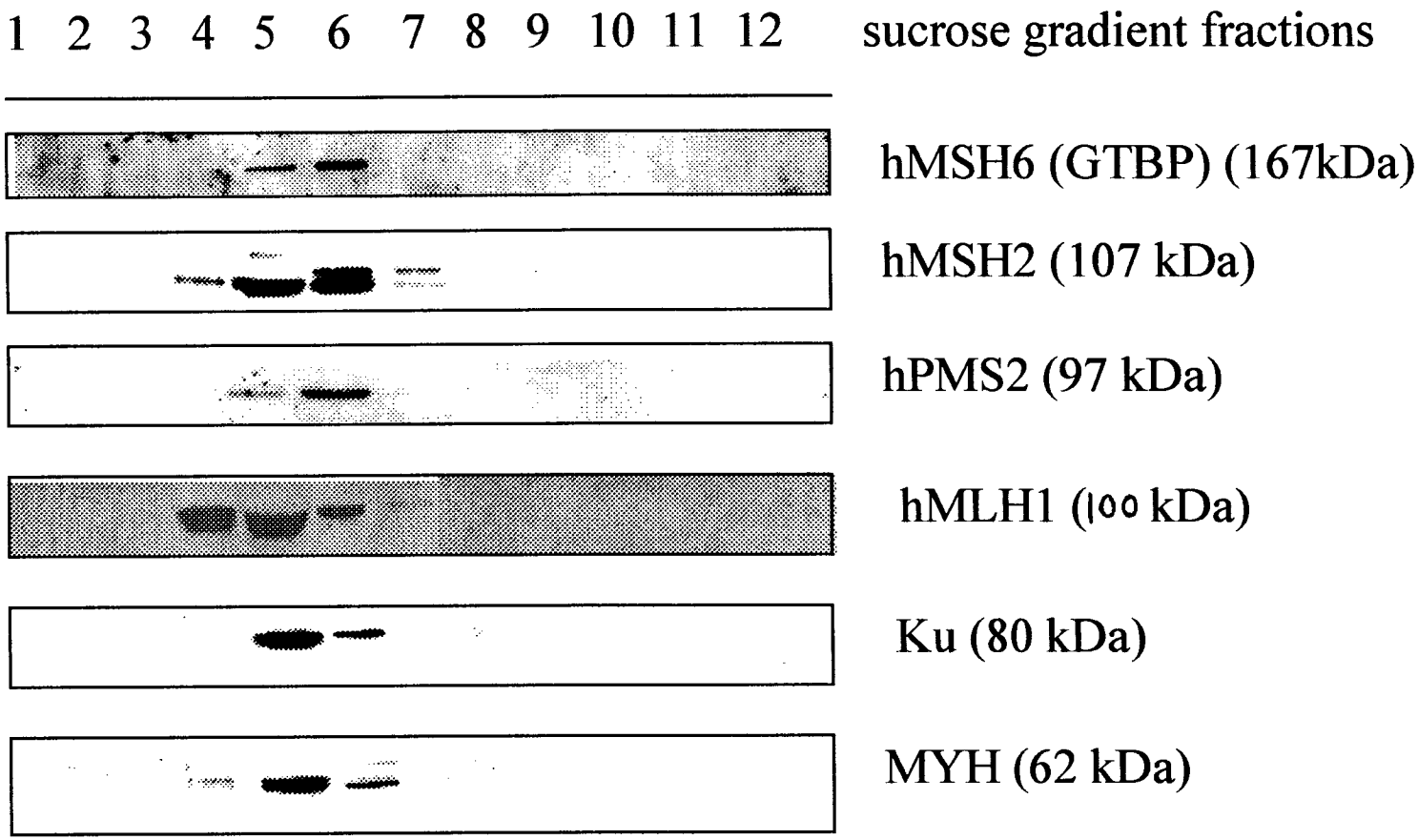


Fig. 11

Figure 12 a. Binding of the DNA synthesome to DNA containing a G/T mismatch

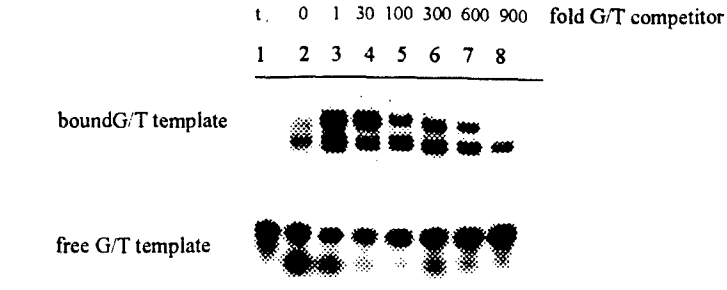


Figure 12 b. Binding of the DNA synthesome to DNA containing an IDL2

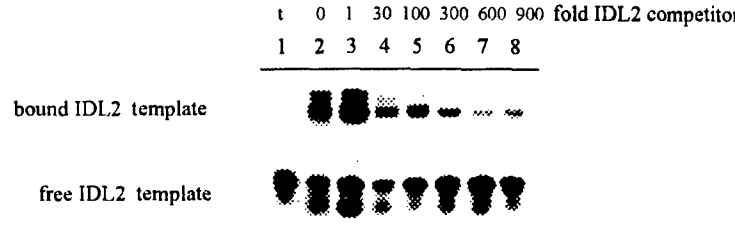


Figure 12 c. Binding of the DNA synthesome to DNA containing an IDL4

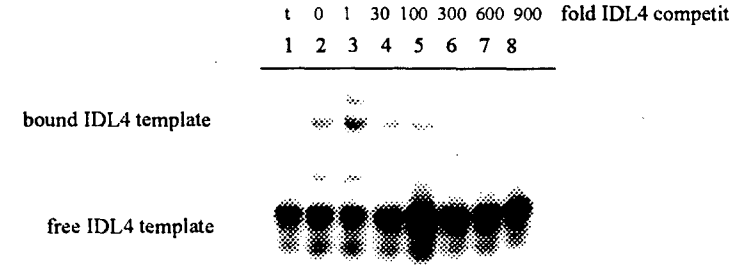
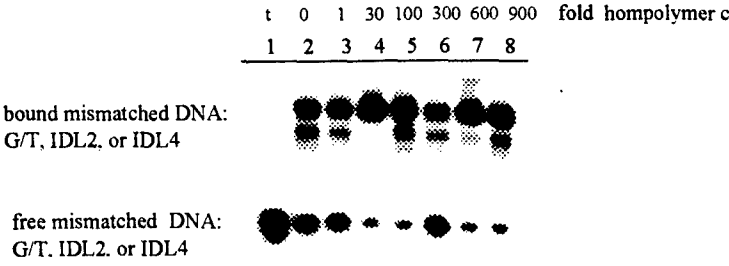


Figure 12d. Homopolymer DNA (without a mismatch or IDL) is not able to compete for binding when the DNA synthesome is bound to DNA containing a G/T mismatch, an IDL2, or an IDL4.



Addendum 1

Principal Investigator: JENNIFER WEEKS SEKOWSKI Grant # DAMD17-94-J-4151

PUBLICATIONS
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(ENCLOSED WITH REPORT)

Sekowski, J.W., Malkas, L.H., Wei, Y., Hickey, R.H. (1997) Mercuric ion inhibits the activity and fidelity of the human cell DNA synthesome. Toxicology and Applied Pharmacology 145, 268-276.
(ENCLOSED WITH REPORT)

Applegren, N., **Sekowski, J.W.**, Hickey, R.H., Tuteja, N., Wilson, S., Uitto, L., Syvaoja, J., and Malkas, L.H. (1997). The human cell DNA synthesome: Evaluation of its DNA replication mechanism and the identification of additional components. J. Cell Biochem., submitted.
(ENCLOSED WITH REPORT)

Sekowski, J.W., L.H. Malkas, and R. J. Hickey. (1997) The malignant human breast cell DNA synthesome contains an error-prone DNA replication apparatus. Nature Medicine, in preparation.

Sekowski, J.W., D.J. Hoelz, L.H. Malkas, A.-L. Lu, S. Han, and R. J. Hickey. (1997) Human Mismatch DNA Repair Proteins are Coupled to the Cellular DNA Replication Apparatus. Cell, in preparation.

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12/12-12/14/96.

J.W. Sekowski, L.H. Malkas, Y. Wei, R.J. Hickey (1996) Fidelity of DNA replication in malignant and non-malignant human breast cells. McGill DNA Replication Meeting, St.Sauveur, Quebec, Canada - 10/17-10/21/96.

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

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Abstract. In this report, we describe for the first time the isolation and purification of a multiprotein complex for DNA replication from MDA MB-468 human breast cancer cells. This complex, which we designate 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, replication protein A, replication factor 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 from 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 novel forward mutagenesis assay suggest that the DNA synthesome isolated from a nonmalignant 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*. *Copyright © 1996 Elsevier Science Inc.*

Key words: breast cancer; DNA replication; multiprotein complex; fidelity; DNA polymerase.

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 with nonmalignant 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[j] based cell-free DNA synthesis include DNA polymerase α, DNA primase, DNA polymerase δ, PCNA, RP-A, 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

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[j]*Abbreviations used:* SV40, simian virus 40; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; PMSF, phenylmethyl sulfonyl fluoride; AAN, aminoacetonitrile hemisulfate; TDEG, 50 mM Tris HCl, pH 7.5, 1 mM DTT, 1 mM Na_3EDTA, 10% glycerol; NE, nuclear extract; IPTG, isopropyl β-D-thiogalactopyranoside.

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 DNA products synthesized by the human cell multiprotein complex consist of monomeric circular form I and II DNAs as well as topological and replicative intermediates [13]. Furthermore, the majority (80–90%) of these products are resistant to *DpnI* digestion, which is consistent with the criteria for semiconservative replication of full-length DNA [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 nonspecific 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 novel forward mutagenesis assay, which establish that the DNA synthesome isolated from breast cancer cells and breast tumor 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, OH). 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.

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 newborn calf serum and FBS. Exponentially growing cells (5×10^5 cells/ml medium) were harvested and washed three times with 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 (1000 rpm, 5 min, 4°C), and the cell pellets stored at –80°C until fractionation. Hs587Bst cells were cultured in monolayer in DMEM supplemented with 30 ng/ml epidermal growth factor and 10% FBS. Subconfluent cells were harvested and washed three times with PBS. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 min, 4°C) and the pellets stored at –80°C until fractionation. MCF-7 cells were cultured in Eagle's minimum essential medium containing 5% FBS and 600 μg/ml neomycin sulfate, as described in Yue *et al.* [17]. Subconfluent MCF-7 cells were scraped into Hanks' solution and centrifuged at 1000 rpm for 2 min 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. 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 postmicrosomal supernatant (S-3). The nuclear extract and the postmicrosomal supernatant were combined and adjusted to 2 M 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 min (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 13,000 rpm for 15 min, 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. Five milliliters of the dialyzed MDA MB-468 P-4 fraction was loaded onto a 1-ml Q-Sepharose (Pharmacia, Piscataway, NJ) column (1 cm^3 bed volume/25 mg protein) preequilibrated 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 0.4 ml were collected and assayed for protein and enzymatic activity.

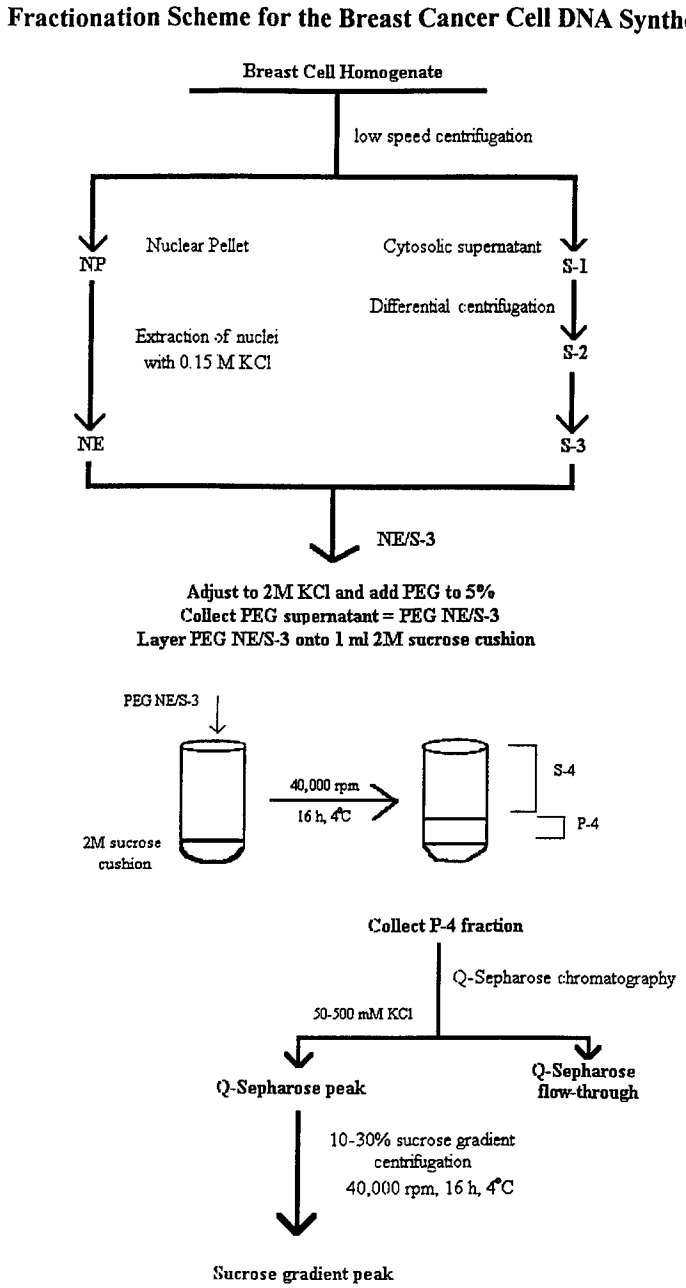

Figure 1. Flow diagram of the isolation scheme used to purify the DNA synthesome from MDA MB-468 human breast cancer cells. A detailed description of the isolation scheme is presented in the Materials and Methods.

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. Six hundred microliters (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 tumor tissue according to a modified version of the isolation scheme depicted in Figure 1 and as described in a previous section of these Materials and 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 min (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 min (4°C) using a TLA 100.3 rotor and the supernatant (NE) retained. 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 min and 59,700 rpm, 22.2 min, respectively. The final postmicrosomal supernatant (S-3) was collected. The NE was combined with the S-3 fraction; 4.5 ml of the NE/S-3 fraction was then layered over a 0.5-ml 2 M sucrose cushion. After centrifugation at 40,000 rpm for 16 h (4°C) using a Beckman (Columbia, MD) 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. Seven hundred microliters of the dialyzed human breast tumor P-4 fraction (3.3 mg protein) was loaded onto a 0.15-ml DEAE cellulose column, preequilibrated 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 preequilibration buffer. Matrix-bound protein was then eluted with 8 volumes of TDEG buffer containing 1 M 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 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 [α-^{32}P]dCTP; 40 mM creatine phosphate; 1 μg creatine kinase. Each reaction was incubated for 2 h at 35°C. The replication assay reaction products were processed using DE81 (Whatman, Maidstone, UK) 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 dNMP into SV40 origin-containing plasmid DNA per 2 h 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 1 nmol 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 (20 volts, 16 h, 4°C) to nitrocellulose membranes, and immunodetection of the respective DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham, Arlington Heights, IL). 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 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Both antibodies were used at a 1:500 dilution. The anti-DNA primase antibody, a gift from Dr. William Copeland (National Institute of Environmental Health Sciences, Research Triangle Park, NC), 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 immunoassays at a dilution of 1:5000.

Forward Mutagenesis Assay

Transfection and plating. Two hundred nanograms of pBK-CMV plasmid DNA (Stratagene, La Jolla, CA), encoding the *lac-Zα* gene, was incubated with 35–45 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 *Escherichia coli* strain XL1-Blue MRF′ (Stratagene): [*(mcrA) 183, δ(mcr(B-hsdSMR-mrr) 173, endA1, supE44, thi_1, recA1, gyrA96, relA1, lac[F′ proAB, lacI^{q}Z(m15, Tn10(tet^R)*]. Forty microliters of 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 min 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 μl chilled, sterile SOC buffer (20 mM glucose in Luria broth medium) was added to the reaction cuvette. The electroporated mixture was then incubated in a rotary shaker (250 rpm) at 35°C for 1 h. An aliquot

of the incubated culture, sufficient to yield 100–600 bacterial colonies per plate, was spread on top of 20 ml 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.) As a negative control for the forward mutagenesis assay, 2×10^9 bacterial cells were transfected with 200 ng unreplicated, *Dpn*I digested pBK-CMV DNA; virtually no antibiotic resistant wild-type colonies were produced under these conditions (data not shown). Also, the background mutation frequency for this assay was determined by transfecting 10^9 bacterial cells with 200 ng unreplicated pBK-CMV DNA. Only one mutant colony per 10^6 wild-type colonies was produced under these conditions (data not shown).

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 h of incubation at 35°C by a modification of a procedure described by the laboratory of Kunkel [25]. 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). 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 cofractionate as a readily sedimentable form. 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

Table 1. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from MDA MB 468 human breast cancer cells.

Fraction	PEG NE/S-3	S-4	P-4	QS Peak	FT	SG
DNA polymerase α[a]	132.5	0.3	188.3	[c]	1.01	[c]
DNA replication (+T)[b]	103.8	8.8	110.6	136.6	3.4	141.2
DNA replication (–T)[b]	3.1	0.0	0.2	8.5	1.8	10.2

[a]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 1 nmol [^{3}H]TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.
[b]*In vitro* SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol [^{32}P]dCMP into SV40 origin-containing DNA. These values represent the average of two independent experiments.
[c] Not determined.

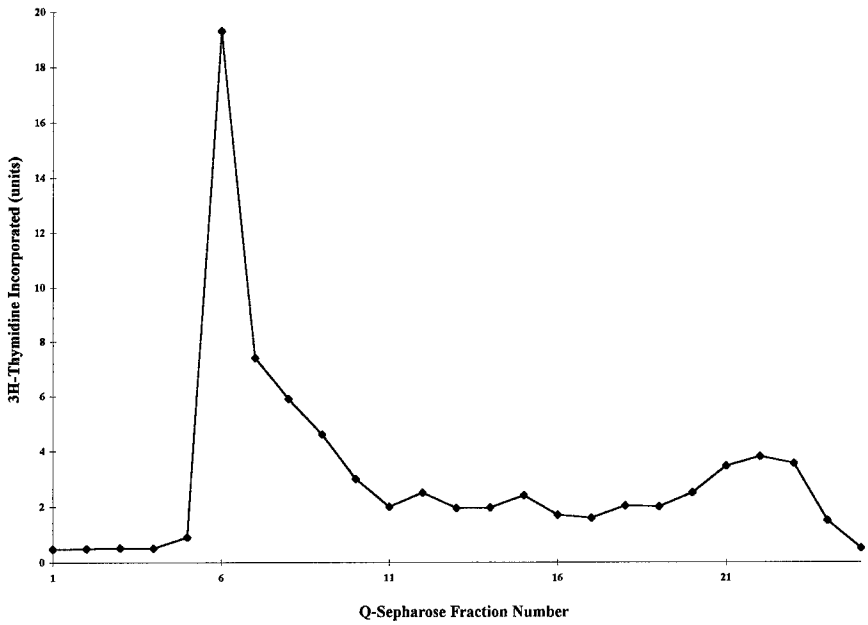

Figure 2. Q-Sepharose chromatographic profile of DNA polymerase α activity in the MDA MB-468 derived P-4 fraction. A description of the column preparation and elution conditions are provided in the Materials and Methods.

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; the column flow-through fraction supported significantly less DNA synthesis (Table 1).

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 0.5 M KCl [15]. The sucrose gradient fractions were collected and assayed for DNA polymerase α and *in vitro* SV40 DNA replication activities. Both activities cosedimented in the sucrose gradient with a sedimentation coefficient of 18S (Figure 3; Table 1). 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 interface 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 copurified 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 with 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.

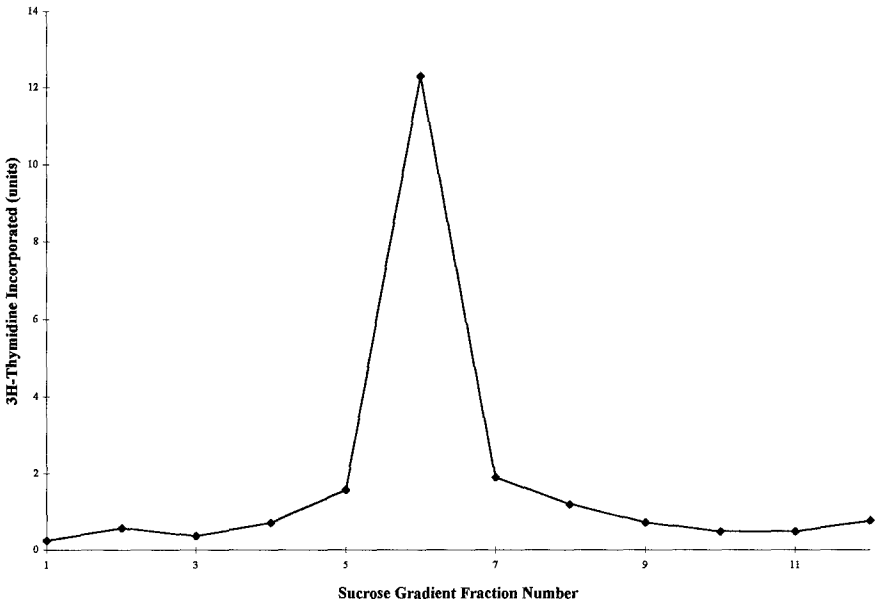

Figure 3. Velocity sedimentation analysis of the DNA synthesome present in the MDA MB-468 Q-Sepharose peak fraction. Eight hundred microliters of a milliliter of the Q-Sepharose peak fraction was layered onto a 9-ml 10–30% sucrose gradient containing 0.5 M KCl. Centrifugation was performed as described 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 TMP incorporated into DNA per hour at 35°C.

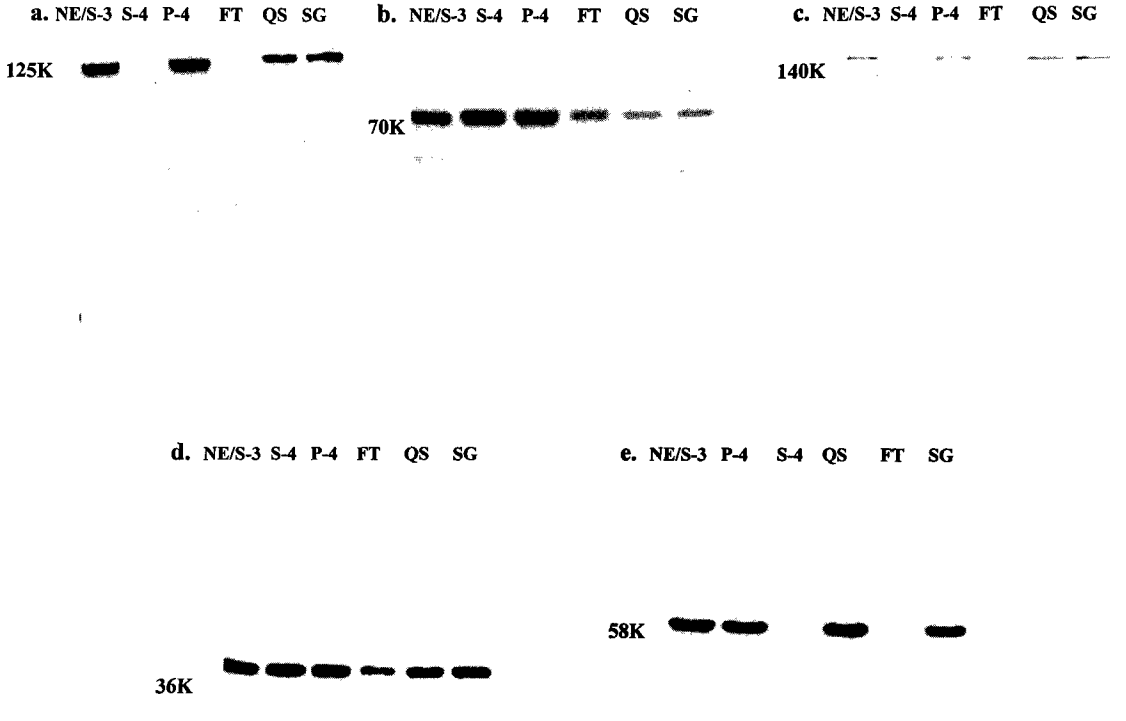

Figure 4. Western blot analysis of the MDA MB-468 breast cancer cell derived fractions. Thirty micrograms of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak [QS], Q-Sepharose flow-through [FT] and sucrose gradient peak [SG]) were resolved on 8% polyacrylamide gels, then transferred to nitrocellulose 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).

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 copurifies 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 fasciculata* 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).

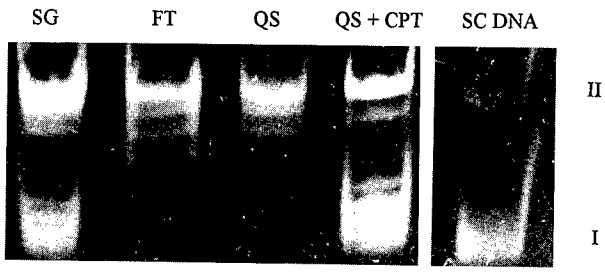

Figure 5. DNA topoisomerase I activity in the Q-Sepharose peak, Q-Sepharose flow-through, and sucrose gradient peak fractions. Reaction assays containing 8 μg of the Q-Sepharose peak (QS), 8 μg of the Q-Sepharose flow-through (FT), or 20 μg of the sucrose gradient peak (SG) were incubated for 30 min at 37°C with 0.3 μg 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 camptothecin [32]. Lane 5 shows the position of supercoiled plasmid pSVO$^+$.

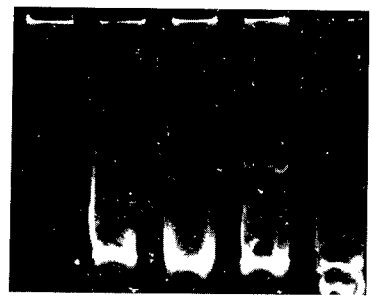

Figure 6. DNA topoisomerase II activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Decatenation reactions were performed in topoisomerase II buffer (TopoGen) with 0.15 μg kinetoplast DNA (KDNA) and 10 μg of the respective protein fraction. Lane 1 shows the 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.

Isolation of the DNA synthesome from breast tumor tissue. To verify that the DNA synthesome could be isolated from breast cancer tissue as well as from 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 2 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 anion 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 1 M KCl (Table 2). In contrast, only a minor amount of DNA polymerase α activity was found in the column flow-through fraction (Table 2). 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).

Table 2. 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	Column Peak	Flow-through
DNA polymerase α;[a]	27.8	1.7	37.5	77.3	1.3
DNA replication (+T)[b]	29.5	1.8	122.9	N.D.[c]	N.D.[c]
DNA replication (–T)[b]	5.74	0.5	12.1	N.D.[c]	N.D.[c]

[a]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 10^{-10} mol [^{3}H]TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.
[b]*In vitro* SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol [^{32}P]dCMP into SV40 origin-containing DNA. These values represent the average of two independent experiments.
[c]Not determined.

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. By 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 3). 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 nonproliferating 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]. 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 proofreading 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 nonmalignant Hs587Bst breast cells. We determined that the DNA synthesome purified from MDA MB-468 cells possessed a replication fidelity approxi-

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

Fraction	NE/S-3	S-4	P-4
DNA polymerase α[a]	40.6	2.0	123.2
DNA Replication +T[b]	57.2	11.2	158.7
DNA Replication –T[b]	5.5	4.1	9.6

[a]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 1×10^{-10} mol of ^{3}[H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.
[b]*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.

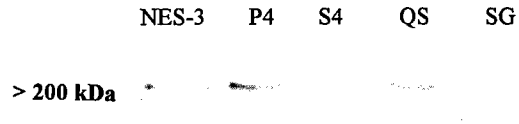

Figure 7. Immunoblot analysis for the presence of DNA polymerase ε in the MDA MB-468 breast cancer cell derived fractions. Fifty micrograms of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak [QS] and sucrose gradient peak [SG]) was resolved on a 8% polyacrylamide gel then transferred to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

mately 6-fold lower than that of the synthesome from Hs587Bst cells (Table 4). Similarly, the DNA synthesome purified from human breast tumor tissue possessed an approximately 5-fold lower DNA replication fidelity than the Hs587Bst synthesome (Table 4). These differences in replication fidelity between the malignant and nonmalignant 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 its treatment with high salt, polyethylene glycol precipitation, anion-exchange chromatography, and sucrose gradient sedimentation. These results suggest that the copurification of the synthesome's proteins with one another is independent of nonspecific 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 comigrated 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 were enriched by the successive steps of the purification process. Furthermore, the P-4 as well as the column 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*.

Table 4. DNA replication fidelity of the breast cell DNA synthesome.

Origin of DNA 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 [25] was performed to measure the fidelity with which the DNA synthesome from MDA MB 468 breast cancer cells, human breast tumor tissue and Hs578Bst cells replicates plasmid DNA. The replicated plasmid, containing the bacterial lac-Z gene, was *DpnI* digested and electroporated into E. coli. The bacteria 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 within the plasmid at location other than the lac-Z gene are not detected by this assay. Consequently, the reported percentages of white colonies provide a minimum estimate of the actual number of mutations arising during DNA synthesome mediated DNA replication. The percentage of mutant colonies expressed for the DNA synthesome is the average number taken from 3 separate assays of 10^4 transformed colonies each. The background mutation frequency for the forward mutagenesis assay was determined to be .0003% (Materials and Methods).

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* [8–10]. 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 and completes synthesis of the lagging 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 copurification 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 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. 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 [43, 44]. 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} [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 derived DNA synthesome replicates plasmid DNA containing the *lac-Zα* gene. This assay detects point mutations occurring within the *lac-Zα* gene as well as frameshift 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 with that of the DNA synthesome isolated from a nonmalignant breast cell line (Table 4). 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 DNA synthesome from malignant and nonmaligant breast cells 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 nonmalignant breast cells (Bechtel *et al.*, 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 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. 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 copurify exclusively with the DNA synthesome from FM3A and HeLa cells [14, 15].

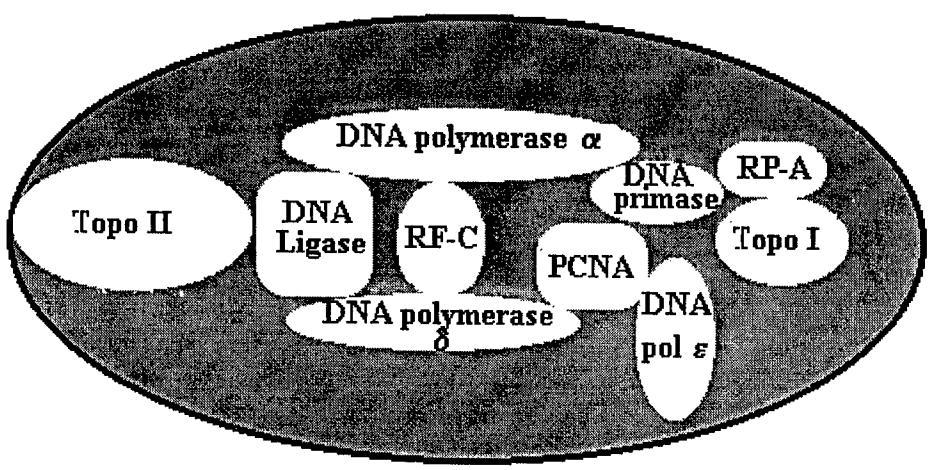

Figure 8. Model for the human breast cell DNA synthesome. A full description of the model is presented in the text.

Unlike the other components, PCNA, RP-A and topoisomerases I and II were observed to cofractionate 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 and 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 coimmunoprecipitation 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 study, 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 nonmalignant breast cells. Breast cancer cells often possess high rates of DNA synthesis and an extensive level of genetic damage [2, 45, 46]. Understanding the process of DNA replication as it occurs in breast cancer cells, will greatly facilitate the development of improved anti-breast cancer therapies. 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.

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REFERENCES

1. American Cancer Society, Basic Data, 1996.
2. Keshgegian, A.; Cnaan, A. Proliferation markers in breast carcinoma: mitotic figure count, S-phase fraction, proliferating cell nuclear antigen, Ki-67 and MIB-1 Anat. Pathol. 104:42–49; 1995.
3. Loeb, L. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res. 51:3075–3079; 1991.
4. Popanda, O.; Fox, G.; Thielmann, H. W. DNA polymerases α, δ and ε of Novikoff hepatoma cells differ from those of normal rat liver in physicochemical and catalytic properties. J. Mol. Med. 73:259–268; 1995.
5. Kelly, T. J. SV40 DNA replication. J. Biol. Chem. 263:17889–17892; 1988.
6. Challberg, M.; Kelly, T. Animal virus DNA replication. Annu. Rev. Biochem. 58:671–717; 1989.
7. Stillman, B. Initiation of eukaryotic DNA replication *in vitro*. Annu. Rev. Cell Biol. 5:107–245; 1989.
8. Hurwitz, J.; Dean, F. B.; Kwong, A. D.; Lee, S. H. The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. 265:18043–18046; 1990.
9. Malkas, L. H.; Hickey, R. J.; Baril, E. F. Multienzyme complexes for DNA synthesis in eukaryotes: P-4 revisited. In: Strauss, P. R.; Wilson, S. H., eds. The eukaryotic nucleus: molecular biochemistry and macromolecular assemblies. Caldwell: The Telford Press; 1990:pp. 45–68.
10. Stillman, B.; Bell, S. P.; Dutta, A.; Marahrens, Y. In: Marsh, J., ed. Regulation of the eukaryotic cell cycle. Ciba Foundation Symposium 170. Chichester: John Wiley and Sons; 1992:pp. 147–156.
11. Matthews, C. K.; Slabaugh, M. B. Eukaryotic DNA metabolism: are deoxynucleotides channeled to replication sites? Exp. Cell. Res. 162:285–295; 1986.
12. Reddy, G.; Fager, R. Replitase: a complex integrating dNTP synthesis and DNA replication. Crit. Rev. Eukaryot. Gene Exp. 3:255–277; 1993.
13. Malkas, L. H.; Hickey, R. J.; Li, C. J.; Pedersen, N.; Baril, E. F. A 21S enzyme complex from HeLa cells that functions in simian virus 40 (SV40) DNA replication *in vitro*. Biochemistry 29:6362–6374; 1990.
14. Applegren, N.; Hickey, R. J.; Kleinschmidt, A. M.; Zhou, Q.; Coll, J.; Wills, P.; Swaby, R.; Wei, Y.; Quan, J. Y.; Lee, M.; Malkas, L. H. Further characterization of the human cell multiprotein DNA replication complex (MRC). J. Cell. Biochem. 59:91-107; 1995.
15. Wu, Y.; Hickey, R. J.; Lawlor, K.; Wills, P.; Yu, F.; Ozer, H.; Starr, R.; Quan, J. Y.; Lee, M.; Malkas, L. H. A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication *in vitro*. J. Cell. Biochem. 54:32–46; 1994.
16. Tom, T.; Hickey, R. J.; Malkas, L. H. Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen dependent SV40 DNA replication activity. J. Cell. Biochem., in press.
17. Yue, W.; Zhou, D.; Chen, S.; Brodie, A. A nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene. Cancer Res. 54:5092–5095; 1994.
18. Simanis, V.; Lane, D. P. An immunoaffinity purification procedure for SV40 large T-antigen. Virology 144: 88–100; 1985.
19. Stillman, B.; Gerard, R. D.; Guggenheimer, R. A.; Gluzman, Y. T-antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. 4:2933–2939; 1985.
20. Sambrook, J.; Fritsch, E.; Maniatis, T. Commonly used techniques in molecular cloning. In: Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989:pp.E.1-E.39.
21. LaMothe, P.; Baril, B.; Chi, A.; Lee, L.; Baril B. Accessory proteins for DNA polymerase α activity with single-strand DNA templates. Proc. Natl. Acad. Sci. USA 78:4723–4727; 1981.
22. Vishwanatha, J. K.; Yamaguchi, M.; DePamphilis, M. L.; Baril, E. F. Selection of template initiation sites and the

lengths of RNA primers synthesized by DNA primase are strongly affected by its organization in a multiprotein DNA polymerase α complex. Nucl. Acids Res. 14:7305–7323; 1986.
23. Hickey, R. J.; Malkas, L. H.; Pedersen, N.; Li, C.; Baril, E. F. Multienzyme complex for DNA replication in HeLa cells. In: Moses, R. E.; Summers, W. C., eds. DNA replication and mutagenesis. Washington, D.C.: American Society for Microbiology; 1988:pp. 41–54.
24. Laemmli, UK Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685; 1970.
25. Roberts, J. D.; Kunkel, T. A. The fidelity of a human cell DNA replication complex. Proc. Natl. Acad. Sci. USA 85:7064–7068; 1988.
26. Prelich, G.; Tan, C. K.; Mikostura, M.; Matthews, B.; So, A. G.; Downey, K. M.; Stillman, B. Functional identity of proliferating cell nuclear antigen and a DNA polymerase δ auxiliary protein. Nature 326:517–520; 1987.
27. Tsurimoto, T.; Stillman, B. Replication factors required for SV40 DNA replication *in vitro*. J. Biol. Chem. 266:1961–1968; 1991.
28. Lee, M. Y. W. T.; Tan C. K.; Downey, K. M.; So, A. G. Further studies on calf thymus DNA polymerase δ purified to homogeneity by a new procedure. Biochemistry 23:1906–1913; 1984.
29. Lee, S. H.; Kwong, A. D.; Pan, Z. Q.; Hurwitz, J. Studies on the activator-1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase delta. J. Biol. Chem. 266:594–602; 1991.
30. Henricksen, L. A., Umbricht, C. B., Wold, M. S. Recombinant Replication Protein A: Expression, complex formation, and functional characterization. J. Biol. Chem. 269:11121–11132; 1994.
31. Fairman, M. P.; Stillman, B. Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J. 7:1211–1218; 1988.
32. Hsiang, Y. H.; Liu, L. F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. 48: 1722–1726; 1988.
33. Tuusa, J.; Uitto, L.; Syvaoja, J. Human DNA polymerase ε is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases. Nucl. Acids Res. 23:2178–2183; 1995.
34. Morrison, A.; Araki, H.; Clark, A. B.; Hamatake, R. B.; Sugino, A. A third essential DNA polymerase in *S. cerevisiae*. Cell 62:1143–1151; 1990.
35. Roberts, J. D.; Thomas, D. C.; Kunkel, T. A. Exonucleolytic proofreading of leading and lagging strand DNA replication errors. Proc. Natl. Acad. Sci. USA 88:3465–3469; 1991.
36. Stillman, B. Smart machines at the DNA replication fork. Cell 78:725–728; 1994.
37. Denis, D.; Bullock, P. Primer-DNA formation during simian virus 40 DNA replication *in vitro*. Mol. Cell. Biol. 13:2882–2890; 1993.
38. Waga, S.; Stillman, B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature 369:207–212; 1994.
39. Prelich, G.; Stillman, B. Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell 53:117–126; 1988.
40. Wobbe, C. R.; Dean, F.; Weissbach, L.; Hurwitz, J. *in vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA 82:5710–5714; 1985.
41. Yang, L.; Wold, M. S.; Li, J. J.; Kelly, T. J.; Liu, L. F. Roles of DNA topoisomerases in simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA 84:940–954; 1987.
42. Snapka, R. M. Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Biol. 6:4221–4227; 1986.
43. Budd, M. E.; Choe, W. C.; Campbell, A. L. *DNA2* encodes a DNA helicase essential for replication of eukaryotic chromosomes. J. Biol. Chem. 270: 26766–26769; 1995.
44. Petrini, J. H.; Xiao, Y.; Weaver, D. T. DNA ligase I mediates essential functions in mammalian cells. Mol. Cell. Bio. 15:4303–4308; 1995.
45. Thorgeirsson, S. S. Endogenous DNA damage and breast cancer. Cancer 71:2897–2899; 1993.
46. Nutter, L. M.; Wu, Y. Y.; Ngo, E. O.; Sierra, E. E.; Gutierrez, P. L.; Abul-Hajj, Y. J. An *o*-quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA damage. Chem. Res. Toxicol. 7:23–28; 1994.
47. Navas, T. A.; Zhou, Z.; Elledge, S. J. DNA polymerase ε links the DNA replication machinery to the S phase checkpoint. Cell 80:29–39; 1995.
48. Boroweic, J. A.; Dean, F. B.; Bullock, P. A.; Hurwitz, J. Binding and unwinding: how T-antigen engages the SV40 origin of DNA replication. Cell 60:181–184; 1990.

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Mercuric Ion Inhibits the Activity and Fidelity of the Human Cell DNA Synthesome

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Mercuric ion is cytotoxic and mutagenic to cells; however, the mechanisms of mercuric ion-induced cytotoxicity are not well understood. Numerous studies have suggested that these effects may be due in part to the alteration and inhibition of a variety of cellular processes including DNA replication, DNA repair, RNA transcription, and protein synthesis. Studies utilizing whole cells to examine these activities are not able to specifically identify the precise mechanism or site of the effect. Other studies carried out using whole cell extracts and variously purified DNA polymerases are not able to adequately represent the highly ordered environment in which DNA replication occurs in the intact cell. We report here, for the first time, the use of an intact human cell multiprotein complex (which we have termed the DNA synthesome) to carry out full-length DNA replication and DNA synthesis in the presence of Hg^{2+} ion *in vitro*. In this study we report that DNA replication and DNA polymerase activity, as well as DNA replication fidelity of the human cell DNA synthesome, are specifically inhibited by physiologically attainable concentrations of mercuric ion. © 1997 Academic Press

Mercuric ion is extremely cytotoxic to both procaryotic and eucaryotic cells (Umeda *et al.*, 1969; Umeda and Nishimura, 1979; Cantoni *et al.*, 1982, 1984; Goldberg *et al.*, 1983). In humans, mercuric ion is known to accumulate in the thyroid gland and to potentially lead to the formation of premalignant and malignant thyroid nodules (Zalchick *et al.*, 1985). Patients with chronic exposure to mercuric ion have also been found to have greatly elevated levels of mercuric ion in their kidney, as well as renal tumors containing elevated levels of mercuric ion (Boffetta *et al.*, 1993). The precise mechanism(s) mediating the carcinogenic effects of mercuric ion is not well defined, but appears to be related to the ingestion or inhalation of the metal, conversion of the metal to mercuric ion, and the translocation of the ion to the thyroid (Zalchick *et al.*, 1985). Part of the cytotoxic and carcinogenic effects of mercuric ion may be due to the ability of the metal ion to alter the DNA synthetic machinery of the cell (Goldberg *et al.*, 1983; Robinson *et al.*, 1984; Williams *et al.*, 1986). Alterations of the activity of the DNA replication machinery are suggested to have a role in mediating the mutagenic effects of mercuric ion (Ariza and Williams, 1996).

Mercuric ion has been reported to alter both the extent of DNA synthesis and the type of DNA replication products formed in experiments using intact mammalian cells, (Ariza and Williams, 1996; Robinson *et al.*, 1984; Oberly *et al.*, 1982; Christie *et al.*, 1984), crude mammalian cell extracts (Oberly *et al.*, 1982; Robinson *et al.*, 1984), and purified enzymes (Caldentey *et al.*, 1992; Williams *et al.*, 1986; Oberly *et al.*, 1982; Niyogi *et al.*, 1981; Hsie *et al.*, 1979). The inhibition of DNA polymerase activity by mercuric ion is postulated to be at least partially responsible for the observed inhibition of intact cell DNA synthesis. Furthermore, mercuric ion not only inhibits the activity of the DNA polymerase, but alters the fidelity with which DNA synthesis is carried out by this polymerase. *In vitro* mutagenesis assays utilizing purified *Escherichia coli* DNA polymerase have demonstrated that several divalent metals, which are known to be carcinogenic (e.g., lead, cadmium, and nickel) produce significant increases in the number of nucleotide misincorporations during the DNA synthesis process (Sirover and Loeb, 1976; Miyaki *et al.*, 1977; Sirover *et al.*, 1979; Tkeshelashvili *et al.*, 1979, 1980). Because mercuric ion is a suspected carcinogen and a member of the group BII elements (which also contains cadmium ion, a known carcinogen) we decided to examine whether the mercuric ion could potentially act as a carcinogen by altering the activity of the DNA synthetic machinery of the cell.

Mercuric ion has been postulated to use one or more of the following mechanisms to alter the fidelity of the DNA synthesis process. First, mercuric ion can alter substrate conformation (i.e., through metal-nucleotide interaction); second, it can alter the conformation of proteins essential for replication and for repair (Williams and Crothers, 1975) (i.e., through metal–protein interactions); third, mercuric ion can alter template-base specificity (Zakour *et al.*, 1981). Mercu-

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ric ion also exhibits two properties which dramatically contribute to the development of alterations contained in cells exposed to mercuric ion. First, mercuric ion has been shown to have potent DNA strand scission activity (Cantoni *et al.*, 1984; Robinson *et al.*, 1984; and Williams *et al.*, 1986). This property allows the ion to induce changes in the DNA template which can alter the ability of the DNA replication machinery to bind to the template properly. This disruption in template binding is also postulated to distort at least some of the components of the replication machinery, and subsequently alter the binding of deoxynucleotides by DNA polymerase and cause misincorporation of nucleotides into the growing DNA strand. Second, mercury has a strong affinity for thiol bonds, which are present in virtually all of the replication proteins, and many other cellular enzymes (Hayes, 1983). The binding of mercuric ion to these thiol groups can severely distort the structural integrity and activity of these proteins. To examine whether mercuric ion can alter the activity and fidelity of the DNA synthetic apparatus of human cells we isolated the cellular DNA synthesizing machinery from human cervical cancer cells (HeLa) and tested the effects of a range of mercuric ion concentrations on *in vitro* DNA replication activity, DNA polymerase activity, and fidelity with which this complex carries out DNA synthesis.

We chose to use the isolated DNA synthetic machinery from HeLa cells for these studies because our characterization of this highly organized complex of proteins (which we have termed the DNA synthesome) (Lin *et al.*, 1996) is fully competent to support all phases of simian virus 40 (SV40) origin-specific DNA replication *in vitro* (Malkas *et al.*, 1990). The biochemical characterization of this isolated multiprotein form of DNA polymerase has resulted in the identification of several protein components of the synthesome. These proteins include DNA polymerases α, δ, and ϵ, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), DNA helicase, DNA methyltransferase, poly(ADP)ribose polymerase, and DNA ligase I (Malkas *et al.*, 1990; Applegren *et al.*, 1995; Coll *et al.*, 1996). In addition, the DNA replication process mediated by the human cell synthesome *in vitro* has been shown to exhibit all of the features of the replication process as it is carried out by the intact cell (Malkas *et al.*, 1990; Applegren *et al.*, 1995). The DNA synthesome has been isolated and characterized from human and murine cells (Malkas *et al.*, 1990; Applegren *et al.*, 1995; Wu *et al.*, 1994), human breast tissue cells (Sekowski, unpublished data), primary human breast tumors (Coll *et al.*, 1996), human leukemia cells (Lin *et al.*, 1996), and human pancreatic cells (Hickey, unpublished data). Our model describing the DNA synthesome is based on the sedimentation and chromatographic profiles of the individual proteins found to copurify with one another as a fully functional DNA replication complex (Wu *et al.*, 1994; Applegren *et al.*, 1995; Coll *et al.*, 1996).

The results presented here strongly suggest that the human DNA synthesome can serve as a useful and highly novel *in vitro* model system for testing whether heavy metal ions can directly induce changes in the activity and fidelity of the cellular DNA synthetic apparatus.

METHODS AND MATERIALS

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 newborn bovine serum. Exponentially growing cells (5×10^5 cells/ml of medium) were harvested and washed three times with phosphate-buffered saline (PBS): 8.4 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (200*g*, 5 min, 4°C) and the cell pellets were stored at −80°C prior to initiating the isolation of the DNA synthesome.

Monolayer cultures of HeLa cells were grown in DME containing 5% each of irradiated calf and newborn bovine serum. The cultures were maintained at 37°C.

Preparation of the DNA synthesome. The HeLa cell DNA synthesome was isolated as described by Malkas *et al.*, (1990). The protein fraction designated Q-Sepharose (Malkas *et al.*, 1990), which contains the DNA replication-competent synthesome, was used in the experiments described in this report.

Preparation of the SV40 large T-antigen. The SV40 large T-antigen protein was prepared as described previously by Simanis and Lane (1985).

Measurement of intact cell DNA synthesis. Monolayer cultures of 0.5×10^5 HeLa cells were seeded in 60-mm cell culture dishes and allowed to grow overnight. The stock concentrations of mercuric chloride to be tested were dissolved in 18.3 Megaohm MilliQ H_2O and then added to the cell cultures to yield final concentrations of 1–100 μM. Following 2, 4, or 8 hr of exposure to the mercuric chloride, [^{3}H]thymidine was added to individual cell cultures, and the amount of [^{3}H]thymidine incorporated into macromolecular material in 1 hr was determined as described by Horwitz *et al.* (1971). The number of cells in plates containing $ZnCl_2$ and NaCl increased by approximately 25% over the 8 hr period in which the assay was performed. Plates containing $HgCl_2$ were treated identically to the control plates, and no cells were lost during the assay.

In vitro DNA replication assay. The reaction mixtures (50 μl) contain 30 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 0.5 mM DTT, 50 μM dATP, 100 μM each dGTP, dCTP, and dTTP, either 5 μCi [α-^{32}P]dCTP (4000 cpm/pmol) or 100 μM dCTP, 200 mM each CTP, UTP, and GTP, 4 mM ATP, 40 mM phosphocreatinine, 100 μg of creatinine phosphokinase, 15 mM sodium phosphate (pH 7.5), approximately 1.0 μg of large T-antigen, and 200 ng of the plasmid pBK-CMV (available from Stratagene, La Jolla, CA). The reaction mixture was incubated at 37°C for 3 hr. After 3 hr, 10 μl of the reaction mixture was pipetted onto Whatman DE81 filters and allowed to air-dry. The filters were then washed 1× (5 min) with 0.1 M NaPPi buffer (pH 7.0), and 3× (5 min) with 0.3 M ammonium formate (pH 7.4), air-dried, and counted in a liquid scintillation counter (30).

DNA polymerase assay. DNA polymerase activity was measured as described by Malkas *et al.*, (1990).

DNA strand scission assay. Two hundred nanograms of supercoiled pSVO+ plasmid DNA (Malkas *et al.*, 1990) was incubated with various concentrations of $HgCl_2$ in the *in vitro* DNA replication assay buffer for 2.5 hr at 37°C. The treated DNA was extracted and precipitated as described below, then visualized by nondenaturing agarose gel electrophoresis and ethidium bromide staining.

Precipitation of the newly replicated DNA. Following the spotting of 10 μl of the reaction mixture onto DE81 filters, the DNA in the remaining 40 μl of assay mixture was treated with *DpnI* (0.02 U, 30 min) to remove any unreplicated plasmid template DNA present in the reaction mixture, and after the digestion of nonreplicated template DNA, the replicated plasmid surviving digestion was extracted using the phenol/chloroform extraction procedure described by Sambrook *et al.* (1989). The extracted DNA was precipitated by the addition of ammonium acetate to a final concentration of 0.2 M along with 2 vol of 100% isopropanol. After centrifugation at 12,000 rpm (14,956*g*), the resulting DNA pellets were washed 3× with 70% ethanol, air-dried or dried in a speed-vacuum (Savant Instruments), and then resuspended in 20 μl TE buffer (10 mM Tris-HCl, pH 8.0/1 mM Na_2 EDTA).

Forward mutagenesis assay: Transformation and plating. The forward mutagenesis assay employed by our laboratory uses the DNA template pBK-CMV (available from Stratagene). This 4518-bp plasmid contains the SV40 origin of DNA replication, a neomycin- and kanamycin-resistance gene, the *lacZ* promoter (IPTG inducible), and the *lac Za* gene (encoding the complementation protein for β-galactosidase). We have determined that transformation of the unreplicated, unmodified pBK-CMV into the *E. coli* host results in the formation of less than one mutant colony per 5×10^5 transformants. Thus, the pBK-CMV template has a spontaneous mutation frequency that is approximately 10^4-fold lower than that of the M13mp2 phage DNA template, which is used in other mutagenesis assays.

The *in vitro*-replicated pBK-CMV (following *DpnI* digestion) was used to transform electrocompetent *E. coli* host cells (XL-1 Blue MRF'). The transformation was carried out by the addition of 30 μl of electrocompetent bacteria (approximately 9.0×10^8 cells) to approximately 100–200 ng of *DpnI*-treated pBK-CMV (10 μl). The mixture was incubated for 10 min on ice before electroporating the cells (at a setting of 1.4 kV, 25 μF, and 200 ohms) in a Bio-Rad Gene Pulser apparatus. Immediately following the electroporation, 1 ml of chilled sterile SOC buffer (20 mM glucose in LB media) was added to the electroporation cuvette. The electroporated mixture was then incubated on ice for 10 min, and then in a rotary shaker (250 rpm) at 37°C for 1 hr. An amount of the culture sufficient to yield 500–1000 bacterial colonies per plate was placed onto 20 ml of LB agar containing 1.25 mg kanamycin, 4 μl IPTG (200 mg/ml), and 40 μl X-gal (20 mg/ml). These plating conditions give intense blue color for the wild-type plasmid which facilitates the visualization of mutant phenotypes. The mutant colonies range from pure white to intermediate (light blue) phenotypes (Roberts *et al.*, 1988).

Scoring of mutants. The inactivation of the α-complementation gene (the product of which is the catalytic subunit of β-galactosidase), due to a mutation in the *lac Za* gene in pBK-CMV, gives a variety of mutant phenotypes, resulting from the lack of a fully functional β-galactosidase gene product. These mutant phenotypes were scored after incubating the plates at 37°C for approximately 12–15 hr. Using the plating conditions described above, expression of the wild-type pBK-CMV DNA in the *E. coli* host generates a dark blue bacterial colony. Bacterial colonies expressing the mutated pBK-CMV plasmid express an altered β-galactosidase gene, which can be distinguished from those colonies expressing the wild-type plasmid by their light blue to yellowish white color phenotypes. In order to reproducibly and precisely score the variable mutant phenotypes, only the pure white colonies (i.e., colonies lacking any trace of blue or green color) were scored as mutant. The light blue colonies compose 25% (±2%) of the total mutant population regardless of mercuric chloride treatment.

Reagents. Creatinine phosphokinase, phosphocreatinine, and all of the inorganic metal salts were purchased from the Sigma Chemical Co. The ribonucleotides and deoxyribonucleotides used in the assays were purchased from Pharmacia Biotechnology Inc., and the radiolabeled nucleotides used in our assays ([^{3}H]thymidine, 20 Ci/mmol; [^{3}H]dTTP, 20 Ci/mmol; and [^{32}P]dCTP, 3000 Ci/mmol) were purchased from DuPont-New England Nuclear.

RESULTS

Mercuric ion inhibits in vitro DNA replication (Fig. 1A, Table 1). To determine whether mercuric ion can directly affect the ability of the isolated human cell DNA synthesome to support *in vitro* DNA synthesis, we performed DNA synthesome-mediated *in vitro* DNA replication assays in the absence and presence of a variety of mercuric ion concentrations (Materials and Methods), and then calculated the specific activity of the DNA synthesome in units of picomoles of nascent DNA per hour per milligram of DNA synthesome (pmol/hr/mg). The results of these experiments (Fig. 1A) demonstrate that even low concentrations of mercuric ion significantly inhibit the specific DNA replication activity of the DNA synthesome. For example, reactions performed in the presence of 2 μM mercuric ion inhibited DNA replication by more than 50% (60 pmol/hr/mg) of the level of replication reactions performed in the absence of mercuric ion or in the presence of equimolar sodium chloride (control). When higher concentrations of mercuric ion (4, 6, and 10 μM) were added to the *in vitro* DNA replication reaction, the level of DNA replication decreased by more than 80% (17, 16, and 19 pmol/hr/mg, respectively). In fact, the level of DNA replication activity carried out with T-antigen at these concentrations of mercuric ion is similar to the level of background, or T-antigen-independent, DNA synthesis (13,13, and 11 pmol/hr/mg). This similarity suggests that mercuric ion (at 4, 6, and 10 μM) may have a direct effect on the activity of the T-antigen protein, or a DNA synthesome component, decreasing the T-antigen-inclusive DNA replication activity of the DNA synthesome to a level near that of a replication reaction carried out in the absence of T-antigen. At the higher concentrations of mercuric ion (20, 60, and 100 μM), a slight increase in T-antigen-dependent replication activity (from 20 to 25 pmol/hour/mg) was observed. This may reflect a slight precipitation of mercuric ion from the reaction mixture or the binding of mercuric ion to the DNA synthesome, to protein components of the replication buffer, or to the template DNA. Thus, the amount of mercuric ion available to inhibit the DNA synthesome may become decreased. To ensure that the observed increase in incorporation is not due to a gap-filling or DNA repair-type activity of the synthesome, we tested the ability of mercuric chloride to create single or double-strand breaks in a DNA template. In Fig. 1C, we show that incubation of a double-stranded DNA replication template, pSVO+ (see Materials and Methods), with various concentrations of mercuric chloride under DNA replication assay conditions does not cause an increase in single-strand nicks in the DNA (Form II), nor does it stimulate the formation of double-strand cuts, or small fragments of DNA.

To verify that the inhibition of synthesome-driven DNA replication was due solely to the interaction of the mercuric ion with the synthesome and not to the chloride component

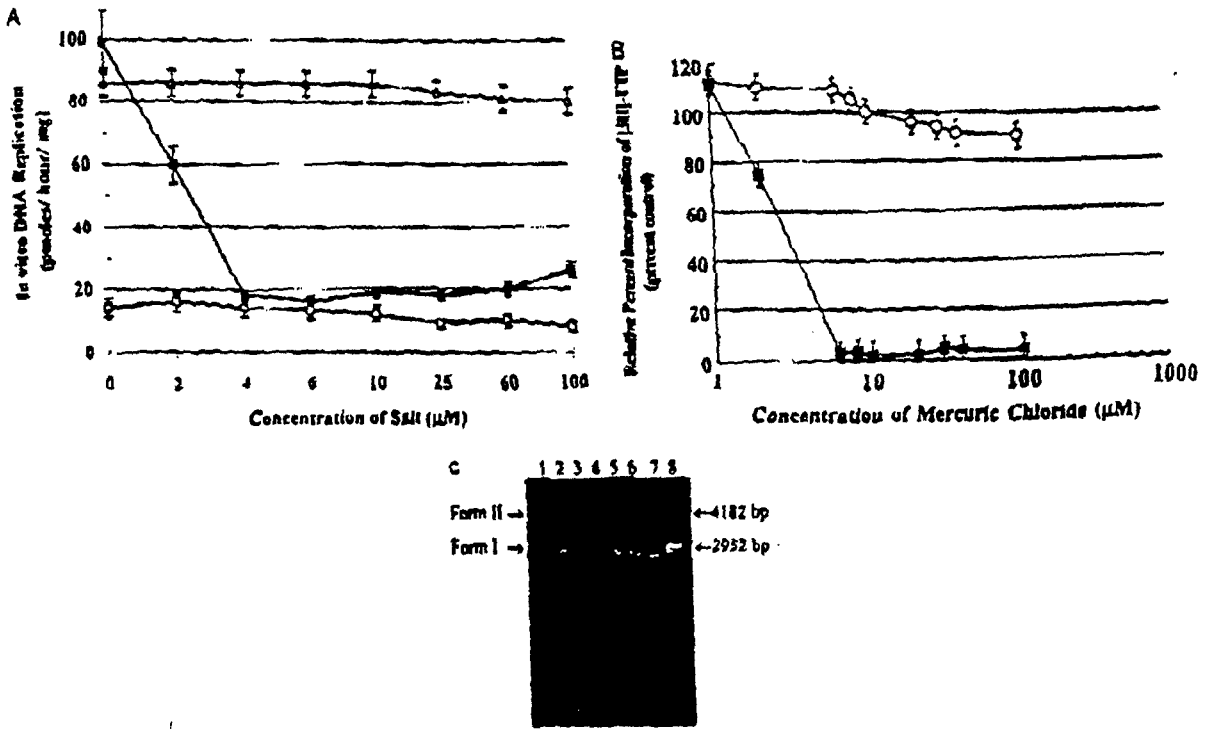

FIG. 1. (A) The effect of a range of mercuric chloride concentrations on DNA synthesome-mediated *in vitro* DNA replication. *In vitro* DNA replication reactions were performed as described under Materials and Methods, in the absence and presence of a range of concentrations of mercuric chloride (■, plus T-antigen DNA synthesis; □, minus T-antigen DNA synthesis) or sodium chloride (▲Δ, T-antigen-dependent DNA synthesis). The values reported are the average of three independent assays. The average of two control (sodium chloride) reactions carried out in the presence of various concentrations of sodium was used to calculate the specific activity of the control treated DNA synthesome. The sodium chloride values fell between 95 and 100% of the specific activity observed in DNA replication reactions carried out in the absence of metal salts. Reactions conducted in the presence of sodium chloride served as a positive control for comparing the effect of chloride ion on *in vitro* DNA synthesis. The mean specific activity of two control reactions deviated less than 3% from each other. The units of sodium chloride (control) specific activity represent T-antigen-dependent activity (units of DNA replication activity in the presence of T-antigen minus the units of DNA replication activity in the absence of T-antigen). (B) The effect of a range of mercuric chloride concentrations on DNA synthesome-associated DNA polymerase activity. *In vitro* DNA synthesome-associated DNA polymerase assays were performed as described under Materials and Methods, in the absence and presence of a range of concentrations of mercuric chloride (■) or sodium chloride (○). The values reported are the average of three independent assays. The average of two control reactions carried out in the absence of metal salts was used to calculate the activity of the nontreated DNA synthesome. (C) The effect of mercuric chloride on DNA template integrity. The interaction of mercuric ion and template DNA was examined using the strand scission assay as described under Materials and Methods. Mercuric chloride concentrations are indicated for each lane. Lane 1, 0 μM; lane 2, 2 μM; lane 3, 10 μM; lane 4, 25 μM; lane 5, 50 μM; lane 6, 75 μM; lane 7, 100 μM; lane 8, 123-bp marker (Sigma Co.).

of the salt or the divalent character of mercuric ion, we performed *in vitro* DNA replication reactions in the presence of various concentrations of sodium, zinc, or nitrate ions (Table 1). Our results indicate that sodium chloride did not significantly inhibit T-antigen-dependent *in vitro* DNA replication (i.e., less than 5% inhibition) at the concentrations tested. It was also observed that sodium nitrate had little effect on synthesome-driven DNA replication over the range of concentrations examined. To determine whether the effect of mercuric ion on *in vitro* DNA replication could be attributable to the divalent nature of the mercuric ion, we added zinc chloride to the replication reaction. We observed that zinc chloride slightly inhibited T-antigen-dependent *in vitro* DNA replication at the highest concentration tested (100 μM). Inhibition of the DNA synthesome-mediated *in vitro* DNA replication by 100 μM zinc chloride was only about 5% greater than that observed in reactions run in the presence of 100 μM sodium chloride or sodium nitrate, and though statistically significant, the decrease in synthetic activity ran well within the expected experimental error for the assay. Taken together, these results suggest that the effects of mercuric ion on synthesome-mediated DNA replication are due specifically to the mercuric ion itself, and not the counter ion or the divalent character of the metal ion.

Mercuric ion inhibits synthesome-associated DNA polymerase activity in a manner that mirrors the inhibition of synthesome-mediated in vitro DNA replication (Fig. 1B). A recent report has suggested that mercuric ion inhibits DNA

TABLE 1

Effect of Sodium Chloride, Sodium Nitrate, and Zinc Chloride on DNA Synthesome-Mediated *in Vitro* DNA Replication

Concentration (μM)	Percent control incorporation of [^{32}P]dCTP[a,b]		
	NaCl	$NaNO_3$	$ZnCl_2$
0	100	100	100
10	97	98	93
100	96	96	92

[a] DNA replication assays were conducted as described under Materials and Methods in the absence or presence of the metal salts indicated.

[b] Percent inhibition was calculated relative to reactions performed in the absence of salts.

[c] The values reported represent the average of three independent assays conducted at each of the indicated salt concentrations. These values deviated from the average by no more than 3%.

polymerase activity (Caldentey *et al.* 1992). We initiated studies to investigate whether mercuric ion would inhibit the activity of synthesome-associated DNA polymerase. DNA synthesome-associated polymerase activity of the DNA synthesome was measured in the absence or presence of various concentrations of mercuric chloride (1–100 μM) (Fig. 1B). Inhibition of DNA polymerase activity below that of the no metal control began at 2 μM mercuric ion. Over 95% of the DNA polymerase activity inhibition was inhibited at concentrations of mercuric ion in excess of 2 μM. Very low concentrations of mercuric ion (1 μM) and sodium ion (2–6 μM) appear to slightly stimulate DNA synthesome-associated polymerase activity.

We also observed that the DNA synthesome-associated DNA polymerase activity was only slightly affected by sodium chloride in excess of 40 μM, resulting in an inhibition in the activity of the DNA polymerase of less than 10% as compared to the no metal control. The inhibitory effects of mercuric ion on the activity of the DNA synthesome-associated DNA polymerase are therefore specifically attributable to the heavy metal ion, and closely parallel the mercuric ion-induced inhibitory profile observed for synthesome-mediated *in vitro* DNA replication.

Correlation of the effect of mercuric ion on synthesome-driven in vitro DNA replication with that of intact cell DNA synthesis (Fig. 2, Table 2). To correlate the effect of mercuric ion on *in vitro* DNA replication with that of exponentially growing cell DNA synthesis, we examined the ability of intact HeLa cells to incorporate [^{3}H]thymidine into DNA following their exposure to various concentrations of mercuric ion. Exponentially growing HeLa cells were incubated with increasing concentrations of mercuric ion for 2, 4, 6, or 8 hr. At each of these times the level of cell DNA synthesis was determined as described under Materials and Methods. Relative to the no metal controls, the results depicted in Fig. 2 indicate that intact cell DNA synthesis was inhibited by greater than 80% at the higher concentrations of mercuric ion (10–100 μM) during the first 2 hr of the experiment, while lower concentrations of mercuric ion (1–10 μM) reduced intact cell DNA synthesis by 25–80%. The effect of mercuric ion on intact cell DNA synthesis occurs rapidly. Within 2 hr of exposure to 1 μM mercuric ion cellular DNA synthesis is reduced to 64% of that exhibited by control cells not exposed to the metal. In addition, exposure to 1 μM mercuric ion for an additional 2 hr decreased intact cell DNA synthesis to only 20% of the unexposed control cells (Table 2). Higher concentrations of mercuric ion (10 and 100 μM) resulted in over an 85% inhibition in intact cells after only a 2-hr exposure to mercuric ion (Fig. 2), and the level of inhibition closely parallels that observed after prolonged exposure to 1 μM mercuric ion, and exposure to mercuric ion for up to 4 hr at 10 and 100 μM concentrations of the metal (Table 2). Exposure of cells to 100 μM mercuric ion for 8 hr completely abolished intact cell DNA synthesis. High

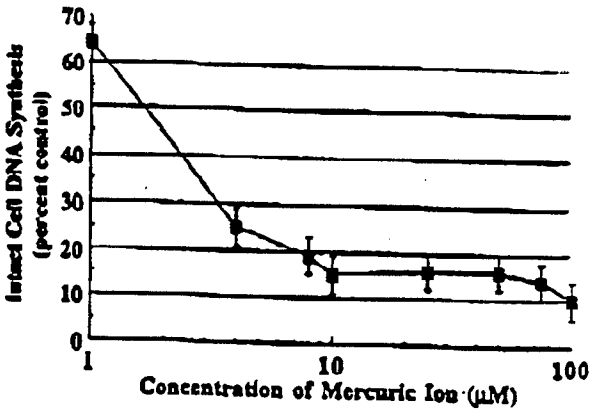

FIG. 2. The effect of a range of concentrations of mercuric chloride and sodium chloride on intact cell DNA synthesis. Intact cell DNA synthesis assays were determined as described under Materials and Methods, in the absence and presence of a range of concentrations of mercuric chloride (■). The values reported are the average of three independent assays. Control reactions were carried out in the absence of metal salts. The values for the incorporation of [^{3}H]thymidine into acid-insoluble material from the individual cultures of cells containing 1 × 10^5 cells/plate deviated from one another by less than 3% and were used to calculate the percent inhibition of [^{3}H]thymidine incorporated into cells incubated with each of the indicated concentrations of mercuric chloride. HeLa cells were seeded at a density of 5 × 10^5 per 60-mm cell culture plate. Twenty-four hours later the cultures were incubated with several concentrations of mercuric chloride (1, 4, 8, 10, 25, 50, 75, and 100 μM) for periods of up to 8 hr. At the time intervals indicated the cultures were labeled for 1 hr with [^{3}H]thymidine (1 μCi/ml) in fresh media, after which the cells were washed three times with PBS and lysed, and the level of nucleotide incorporated into DNA was measured by liquid scintillation counting as described by Horwitz *et al.* (1971). The level of intact cell DNA synthesis is expressed as a percentage of the level of DNA synthesis observed in cells incubated with 1 mM sodium chloride. The results shown in this figure are the average of three separate experiments; error bars denote the standard error of the mean.

TABLE 2
Effect of Sodium Chloride, Zinc Chloride, and Mercuric Chloride on Intact Cell DNA Synthesis

Salt Concentration (μM)	Percent control cell DNA synthesis[a,c,d]								
	2 hr			4 hr			8 hr		
	HgCl	NaCl	$ZnCl_2$	HgCl	NaCl	$ZnCl_2$	HgCl	NaCl	ZnCl
1	64	100	100	20	100	100	20	100	100
10	15	99	98	19	98	99	8	92	90
100	10	97	96	15	96	101	2	91	89

[a] Using the procedure described under Materials and Methods intact cell DNA synthetic activity was determined by measuring the incorporation o [^{3}H]thymidine into acid-precipitated material.

[c] The values reported represent the average of three independent experiments each of which was conducted using three tissue culture plates per sal condition indicated. These values deviated from the average by no more than 5%.

[d] Percent inhibition of the DNA synthetic activity was calculated relative to the control plates containing no metal salts.

[e] Cell cultures were exposed to the range of salt concentrations for periods of 2–8 hr prior to the addition of [^{3}H]thymidine. At the times indicatec on the table the media containing metal ion were replaced with fresh media lacking metal ion. 5 μl [^{3}H]thymidine was added to the cultures, and the cultures were incubated for 1 hr at 37°C prior to acid precipitation of the cellular DNA.

concentrations of mercuric ion affect intact cell DNA synthesis in a manner which correlates with the effect of mercuric ion on synthesome-mediated *in vitro* DNA replication and DNA synthesome-associated DNA polymerase activity (Figs. 1A and 1B).

In contrast to the inhibition of intact cell DNA synthesis by mercuric ion, the ability of intact cells to carry out DNA synthesis was maintained in the presence of sodium chloride or zinc chloride, and did not decrease over the course of the entire experiment (Table 2). This observation strongly suggests that the inhibition of intact cell DNA synthesis, like synthesome-driven DNA replication, was due solely to the mercuric ion, and not the divalent nature of the ion or the presence of the counterion.

Physiologically attainable concentrations of mercuric ion decrease in vitro DNA replication fidelity (Figs. 3, 4). In order to determine whether physiologically attainable concentrations of mercuric ion could alter the DNA synthetic product formed following exposure of the cellular DNA synthetic apparatus to very low concentrations of mercuric ion (i.e., between 1 and 10 μM) (Iyengar *et al.*, 1978; Nakada and Imura, 1980; Svensson *et al.*, 1995; Zalchick *et al.*, 1995), we incubated the purified HeLa cell DNA synthesome with increasing concentrations of mercuric ion in our forward mutagenesis assay (see Materials and Methods). Figure 3 shows the results of plating bacteria transformed with the *Dpn*I-resistant products of the DNA synthesome-mediated *in vitro* DNA replication reaction. We have optimized the *Dpn*I digestion of the replication products in order to eliminate colonies produced by the nonreplicated DNA template. As shown in Fig. 4 concentrations of mercuric ion between 6 and 8 μM increased the number of detectable mutants by approximately 16- to 17-fold over the background mutation frequency arising in the DNA products isolated from reactions performed in the absence of any added mercuric ion (background: 4.4×10^{-4} mutants per 2×10^3 transformants, or 0.02% mutant colonies). Since this fidelity assay system is only able to detect mutations that affect the *lac Zα* gene and the gene makes up only 8.25% of the nucleotide sequence of the pBK-CMV plasmid, and because we only score those mutations which produce a completely inactive β-galactosidase gene, a 16- to 17-fold increase in the mutation frequency represents a significant increase in the total number of mutations produced as a result of carrying out the DNA synthesome-mediated replication assay in the presence of mercuric ion. We also tested higher concentrations of mercuric ion (10–80 μM mercuric ion), for their ability to reduce the *in vitro* DNA synthetic fidelity of the DNA synthesome. We observed mutation frequencies that were approximately 39- to 42-fold higher than background (Fig. 4). The highest concentration of mercuric ion (100 μM) tested in the fidelity assay resulted in a 67-fold increase in replication errors over background levels determined for reactions run in the absence of mercuric ion. The results of our fidelity analysis clearly indicate that the mutation frequency increases as a function of increasing mercuric ion concentration.

We have performed control transformation experiments in order to determine the frequency of mutant transformants that would result from mercuric chloride-treated and untreated pBK-CMV template (unreplicated and non-*Dpn*I digested). We have determined that the frequency and efficiency of transformation with the mercuric chloride treated DNA, as well as the number of mutant colonies produced, do not significantly differ from transformations carried out using the untreated, unreplicated DNA template. Taken together, the results of the strand scission assay (Fig. 1C) and this mercuric chloride-treated DNA transformation control

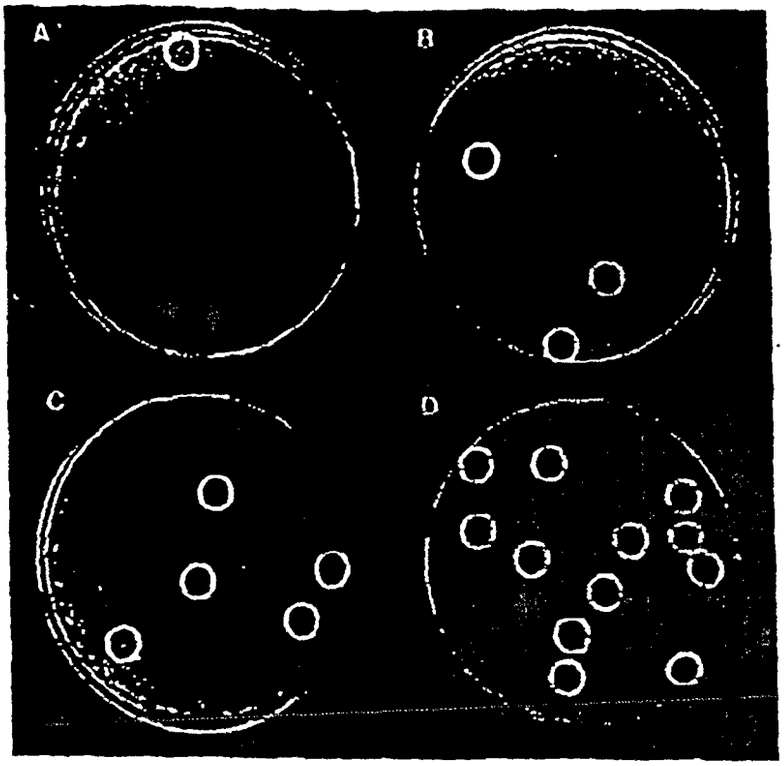

FIG. 3. Bacterial colony formation and detection of colonies expressing a defective β-galactosidase gene. Bacterial host cells were transformed with newly replicated plasmid containing the *lac Za* gene (pBK-CMV) and plated as described under Materials and Methods. The plasmid DNA expressed by the bacterial colonies was isolated from DNA synthesome-mediated *in vitro* DNA replication reactions carried out in the absence (A) or presence (B–D) of mercuric chloride. The following plates are representative of results obtained from the forward mutagenesis assay. (A) (no metal) = 1 mutant per 4.5×10^3 transformants = 0.02% mutant frequency; (B) (2 μM $HgCl_2$) = 3 mutants per 850 transformants = 0.14% mutant frequency; (C) (50 μM $HgCl_2$) = 5 mutants per 700 transformants = 0.86% mutant frequency; (D) (100 μM $HgCl_2$) = 11 mutants per 400 transformants = 1.48% mutant frequency.

suggest that strand scission or other DNA–mercuric ion events do not alone create mutant transformants in our assay system.

DISCUSSION

In this report we have demonstrated that mercuric ion selectively inhibits *in vitro* synthesome-mediated SV40 DNA replication. Our experimental results suggest that the synthesome-mediated DNA replication system may be a useful model for examining the effect of mercuric ion on DNA synthesis. Mercuric ion inhibits *in vitro* DNA replication in a concentration-dependent manner that closely correlates with that observed in intact HeLa cells. In intact cells, DNA synthesis was rapidly and dramatically inhibited in the presence of high concentrations (10–100 μM) of mercuric ion, while lower concentrations of the metal (1–10 μM) were observed to inhibit DNA synthesis to a much lower degree. The inhibition of DNA synthesis observed in intact cells following prolonged exposure to the metal ion could be the result of the interaction of mercuric ion with other cellular processes that support intact cell DNA synthesis. The close correlation between the effects of mercuric ion on intact cell DNA synthesis and synthesome-mediated *in vitro* DNA replication over the time frame of the *in vitro* assays suggests, however, that mercuric ion directly affects the cellular DNA replication machinery.

It has been previously reported that mercuric ion inhibits the mammalian DNA polymerase activity present in whole-cell extracts or in purified enzyme preparations (Williams *et al.*, 1986; Robinson *et al.*, 1984; Rivedal and Sanner, 1981) at higher concentrations of the metal (i.e., greater than 10 μM mercuric ion). Our observations using the isolated DNA synthesome support the conclusions of these researchers, while further clarifying the inhibitory and mutagenic effects of mercuric ion on the intact multiprotein DNA replication machinery of the cell. Mercuric ion significantly inhibits synthesome-associated DNA polymerase activity. The inhi-

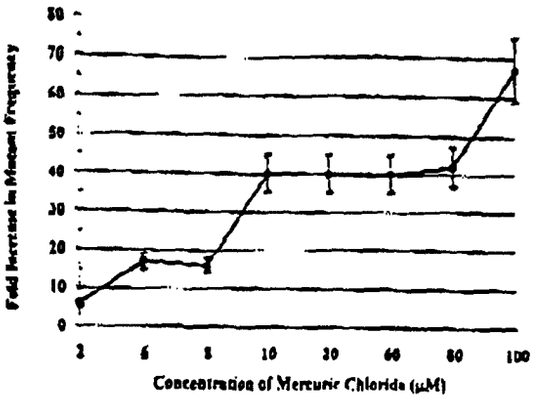

FIG. 4. The effect of a range of mercuric chloride ion concentrations on the frequency of mutations created during DNA synthesome-mediated *in vitro* DNA replication. Bacterial colonies expressing the β-galactosidase gene on newly replicated plasmids isolated from DNA synthesome-mediated *in vitro* DNA replication reactions were scored as described under Materials and Methods. The number of mutations normally produced by the DNA synthesome during the *in vitro* DNA replication reaction was determined by conducting assays in the absence of mercuric ion (4.4×10^4 mutants per 2×10^6 transformants = 0.02% mutants), and the value was used to calculate the fold increase in mutations created by the DNA synthesome during *in vitro* DNA replication reactions carried out in the presence of mercuric chloride. All reactions were normalized to the total number of transformants produced by a 100% transfection efficiency of the replicated DNA from each assay condition.

bition of DNA polymerase activity correlates with the observed effect of mercuric ion on synthesome-mediated *in vitro* DNA replication, as well as the ability of mercuric ion to decrease intact cell DNA synthesis. Our studies, together with the earlier reports of Williams *et al.* (1986), Robinson *et al.* (1984), and Rivedal and Sanner (1981), suggest that mercuric ion (at concentrations above 10 μM) actively inhibits the elongation stage of DNA replication.

Although our data suggest that mercuric ion inhibits DNA synthesis, and the literature reports that mercuric ion is cytotoxic and has diverse effects on cellular function, the concentrations at which these effects are found (greater than 10 μM) are greater than would normally be found physiologically (Iyengar *et al.*, 1987; Nakada and Imura, 1980; Zaichick *et al.*, 1995). Because the literature reports that clinical cases involving mercury exposure are largely due to chronic exposure of the individual to low concentrations of mercuric ion (Zaichick *et al.*, 1995), we also investigated the effects of low concentrations of mercuric ion (2, 4, 8, 10 μM) on DNA replication fidelity. By utilizing the purified, intact multiprotein DNA replication apparatus of the cell (the DNA synthesome) we were able to detect an increase in the number of mutations produced by the synthesome in the presence of low concentrations of mercuric ion. The results presented in this paper suggest that the DNA replication machinery of the cell has a role in mediating the observed mutagenic potential of mercuric ion (Ariza and Williams, 1996; Christie *et al.*, 1984) and perhaps the cytotoxic and carcinogenic effects attributed to chronic exposure of patients to low doses of mercuric ion (Zaichick *et al.*, 1995). Taken together our results suggest that the isolated human cell DNA synthesome may be a novel model system for studies examining how mercuric ion, and potentially other heavy metal ions, affect DNA synthesis.

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REFERENCES

Applegren, N. B., Hickey, R. J., Kleinschmidt, A. M., Zhou, Q. Q., Coll, J., Wills, P., Swaby, R., Wie, Y-T., Quan, J. Y., Lee, M. Y. W. T., and Malkas, L. (1995). Further characterization of the human cell multiprotein DNA replication complex. *J. Cell. Biochem.* 59, 91–97.

Ariza, M. E., and Williams, M. V. (1996). Mutagenesis of AS52 cells by low concentrations of lead (II) and mercury (II). *Environ. Mol. Mutagen.* 27(1), 30–33.

Bell, S. P., Kobayashi, R., and Stillman, B. (1993). Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science* 262, 1844–1848.

Boffetta, P., Merler, E., and Vainio, H. (1993). Carcinogenicity of mercury and mercury compounds. *Scan. J. Work Environ. Health* 9(1), 1–7.

Caldentey, J., Blanco, L., Savilahti, H., Bamford, D. H., and Salas, M. (1992). *In vitro* replication of bacteriophage PRD1 DNA. Metal activation of protein-primed initiation and DNA elongation. *Nucleic Acids Res.* 20, 3971–3976.

Cantoni, O., Christie, N. T., Swann, A., Drath, D. B., and Costa, M. (1984). Mechanism of $HgCl_2$ cytotoxicity in cultured mammalian cells. *Mol. Pharmacol.* 26, 360–368.

Casto, B. C., Myers, J. D., and DiPaolo, J. A. (1979). Enhancement of viral transformation for evaluation of the carcinogenic or mutagenic potential of inorganic metal salts. *Cancer Res.* 39, 193–197.

Christie, N. T., Cantoni, O., Evans, R. M., Meyn, R. E., and Costa, M. (1984). Use of mammalian DNA repair deficient mutants to assess the effects of toxic metal compounds on DNA. *Biochem. Pharmacol.* 33, 1161–1670.

Coll, J. M., Sekowski, J. G. W., Hickey, R. J., Schnaper, L., Wei, Y., Brodie, A., Syvaoja, J., and Malkas, L. H. (1996). The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. *Oncol. Res.* 8, 435–447.

DePamphilis, M., and Bradley, M. (1986). Replication of SV40 and polyoma virus chromosomes. In *The Papovaviridae*, Vol. 1, *The Polyomavirus* (Salzman, N. P., Ed.), pp. 99–246. Plenum Press, New York.

Dermody, J. J., Lawlor, K. O., Du, H., Wojcik, B., Jha, K. K., Malkas, L., Hickey, R., Baril, E. F., and Ozer, H. L. (1988). Polyomavirus DNA synthesis *in vitro*: Studies with CHO, 3T3 and their tsDNA mutants. *Cancer Cells* 6, 95–100.

Goldberg, R. L., Kaplan, S. R., and Fuller, G. C. (1983). Effect of heavy

metals on human rheumatoid synovial cell proliferation and collagen synthesis. *Biochem. Pharmacol.* **321**, 2763–2766.

Horwitz, S. B., Change, C. K., and Grollman, A. P. (1971). Studies on camptothecin: Effects on nucleic acid and protein synthesis. *Mol. Pharmacol.* **7**, 632–644.

Hsie, A. W., Johnson, M. P., and Couch, D. B. (1979). Quantitative mammalian cell mutagenesis and a preliminary study of the mutagenic potential of metallic compounds. In *Trace Metals in Health and Disease* (Kharasch, N., Ed.), pp. 5–09. Raven Press, New York.

Hutchinson, F., Macleod, T. M., and Raffle, E. J. (1976). Leukocyte aggregation and lymphocyte transformation induced by mercuric chloride. *Clin. Exp. Immunol.* **2**, 531–533.

Iyengar, G. V., Kollmer, W. E., and Bowen, H. J. M. (1978). *The Elemental Composition of Human Tissue and Body Fluids*, Verlag Chemie, Weinheim.

Leonard, A., Jacquet, P., and Lauwers, R. R. (1983). Mutagenicity and teratogenicity of mercury compounds. *Mutat. Res.* **114**, 1–18.

Lin, S., Hickey, R. J., and Malkas, L. H. (1996). A multiprotein DNA replication complex isolated from human leukemia cells is competent for DNA synthesis. *Leuk. Res.* **19**(9), in press.

Malkas, L. H., Hickey, R. J., Li, C.-J., Pederson, N., and Baril, E. F. (1990). A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*. *Biochemistry* **29**, 6362–6374.

Micklem, G., Rowley, A., Harwood, J., Nasmyth, K., and Diffley, J. F. X. (1993). Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. *Nature* **366**, 87–91.

Murakami, Y., Eki, T., Yamada, M., Prives, C., and Hurwitz, J. (1986). Species-specific *in vitro* synthesis of DNA containing the polyomavirus origin of replication. *Proc. Natl. Acad. Sci. USA* **83**, 6347–6351.

Nakada, S., and Imura, N. (1980). Stimulation of DNA synthesis and pyrimidine deoxyribonucleoside transport systems in mouse glioma and mouse neuroblastoma cells by inorganic mercury. *Toxicol. Appl. Pharmacol.* **53**, 24–28.

Oberly, T. J., Piper, C. E., and McDonald, D. S. (1982). Mutagenicity of metal salts in the 151784 mouse lymphoma assay. *J. Toxicol. Environ. Health* **9**, 367–376.

Rivedal, E., and Sanner, T. (1981). Metal salts as promoters of *in vitro* morphological transformation of hamster embryo cells induced by benzopyrene. *Cancer Res.* **41**, 950–953.

Roberts, J. D., and Kunkel, T. A. (1988). The fidelity of a human cell DNA replication complex. *Proc. Natl. Acad. Sci. USA* **85**, 7064–7068.

Robinson, S. H., Cantoni, O., and Costa, M. (1984). Analysis of metal-induced DNA lesions and DNA repair replication in mammalian cells. *Mutat. Res.* **131**, 173–181.

Sambrook, J., Fritsch, E., and Maniatis, T. (Eds.) (1989). Commonly used techniques in molecular cloning. In *Molecular Cloning: A Laboratory Manual, 2nd edition, pp. E1–F28. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.*

Simianis, V., and Lane, D. P. (1985). An immunoaffinity purification procedure for SV40 large T-antigen. *Virology* **144**, 88–100.

Stacey, N. H., and Kappus, H. (1982). Cellular toxicity and lipid peroxidation in response to mercury. *Toxicol. Appl. Pharmacol.* **63**, 29–35.

Stillman, B. W., and Gluzman, Y. (1985). Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Mol. Cell. Biol.* **5**, 2051–2060.

Svensson, B-G., Nilsson, A., Jonsson, E., Schutz, A., Akesson, B., and Hagmar, L. (1995). Fish consumption and exposure to persistent organochloride compounds, mercury, selenium, and methylamines among Swedish fishermen. *Scand. J. Work Environ. Health* **21**, 96–105.

Umeda, M., Saito, K., Hirose, K., and Saito, M. (1969). Cytotoxic effect of inorganic phenyl, and alkyl mercuric compounds on HeLa cells. *Jpn. J. Exp. Med.* **39**, 47–58.

Umeda, M., and Nishimura, M. (1979). Inducibility of chromosomal aberrations by metal compounds in cultured mammalian cells. *Mutat. Res.* **67**, 221–229.

Williams, M. N., and Crothers, D. M. (1975). The binding kinetics of mercury (II) to polyribonucleotides. *Biochemistry* **14**, 1944–1950.

Williams, M. V., Winters, T., and Waddell, K. S. (1986). In vivo effects of mercury (II) on deoxyuridine triphosphate nucleotidohydrolase, DNA polymerase (α, β), and uracil-DNA glycosylase activities in cultured human cells: Relationship to DNA damage, DNA repair, and cytotoxicity. *Mol. Pharmacol.* **31**, 200–207.

Wu, Y., Hickey, R., Kleinschmitz, A. M., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Quan, J. Y., Lee, M., and Malkas, L. H. (1994). A 17S form of DNA polymerase from mouse cells mediates the *in vitro* replication of polyomavirus DNA. *J. Cell. Biochem.* **54**, 32–46.

Zaichick, V. Y., Tsyb, A. F., and Vtyurin, B. M. (1995). Trace elements and thyroid cancer. *Analyst* **20**, 817–821.

THE HUMAN CELL DNA SYNTHESOME: EVALUATION OF ITS DNA REPLICATION MECHANISM AND THE IDENTIFICATION OF ADDITIONAL COMPONENTS

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Running head: The Human Cell DNA Synthesome

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ABSTRACT

Little definitive information has emerged to sufficiently describe the mechanism by which mammalian chromosomal DNA is replicated. However, it is becoming more apparent that intracellular metabolism does not occur by random collisions between soluble enzymes and substrates but rather is mediated by the action of organized multiprotein complexes. We have previously reported on a replication competent multiprotein form of DNA polymerase, subsequently named synthesome, which supports *in vitro* simian virus 40 (SV40) DNA synthesis. This report describes our most recent efforts toward defining the identity or function of several new components of the human cell DNA synthesome.

The CsCl equilibrium sedimentation data together with the limited susceptibility of the daughter DNA duplexes to digestion by the enzyme *MboI* demonstrate the semi-conservative mechanism of action by the synthesome during *in vitro* DNA synthesis. Based upon results of western blot analyses and strand displacement assays, it was observed that two specific DNA helicases, HDH I and HDH IV co-purify with the synthesome. In addition, it was predicted that the synthesome may be tied to the mismatch repair process and this was demonstrated by immunoblot analysis utilizing the postreplication mismatch repair gene, hMLH1. These results may suggest that repair can occur during replication and that there is some benefit to coordination of progress of the replication fork and removal of lesions. Another protein thought to be involved in active DNA processes such as replication, transcription or repair is Ku. It was demonstrated that the Ku protein does co-purify along with synthesome. It was also demonstrated by immunoblot analysis that DNA polymerase e co-fractionates and thus can be included as a new component of the synthesome. However, DNA polymerase b was not found to co-purify with the mammalian synthesome. In addition, proteins involved with transcription and nucleotide excision repair (NER), such as RNA polymerase I, TFIIH and the excision repair XPA protein, do not co-purify with the synthesome. This suggests that transcription and NER are not an integral part of the synthesome.

Further characterization of the human cell DNA synthesome will increase our understanding of the molecular mechanisms involved in regulating the activity of the DNA replication apparatus and potentially integrating it into the processes of both DNA replication and repair.

The mechanisms by which mammalian chromosomal DNA replication occurs are just beginning to be described and understood. Currently, a number of mammalian proteins have been identified that support *in vitro* DNA synthesis in reconstituted replication systems, and some data exist on the functional association of several of these enzymes and factors (reviewed in Hickey and Malkas, 1997). Concurrently with this work has been a growing body of experimental evidence indicating that these DNA replication proteins and factors are organized into large macromolecular complexes (Malkas et al., 1990a; Applegren et al., 1995; Wu et al., 1994; Coll et al., 1996; Tom et al., 1996; Lin et al., 1997, and reviewed in Mathews and Slabaugh, 1986; Reddy and Fager, 1993; Hickey and Malkas, 1997).

The proteins thus far identified and shown to be required to support animal cell DNA replication using *in vitro* DNA replication systems include: DNA polymerase α-primase complex (Wobbe et al., 1985; Murakami et al., 1986), DNA polymerase δ (So and Downey, 1988; Lee et al., 1989; Weinberg and Kelly, 1989), proliferating cell nuclear antigen (PCNA) (Prelich et al., 1987; Wold et al., 1989; Wobbe et al., 1987), RP-A (Wobbe et al., 1987; Fairman and Stillman, 1988), topoisomerases I and II (Yang et al., 1987), and the RF-C protein complex (Lee et al., 1989; Tsurimoto and Stillman, 1989). These proteins represent the minimal number of factors needed to drive the mammalian DNA replication fork. It is most likely that there are a number of other human cell proteins which are required to: (1) support efficient DNA replication, (2) promote the initiation of human cell DNA synthesis, (3) maintain DNA replication fidelity and DNA repair, (4) permit the attachment of the replication apparatus to the nuclear matrix, and (5) precisely regulate all of these functions of the DNA replication apparatus. In addition, the functional organization of the components of the DNA replication apparatus has not been adequately defined, nor has the mechanism by which these proteins act in concert with one another to efficiently replicate DNA.

The first report of a successful isolation of a fully functional multiprotein form of DNA polymerase α from human (HeLa) cell extracts was described by Malkas et al. (1990b). This multiprotein form of DNA polymerase was shown to be fully competent for supporting origin-specific and large T-antigen dependent SV40 DNA replication *in vitro* (Malkas et al, 1990b). This replication-competent multiprotein form of DNA polymerase was isolated using several biochemical steps of purification that included subcellular fractionation, polyethylene glycol precipitation, discontinuous gradient centrifugation, Q-Sepharose (Pharmacia)

chromatography, and velocity density gradient centrifugation. The proteins initially found to co-purify with the human cell multiprotein form of DNA polymerase included: DNA polymerase α, DNA primase, topoisomerase I and PCNA (Malkas et al., 1990b). Additional enzymes and factors were subsequently found to co-purify with this complex and include DNA polymerase δ, DNA ligase I, topoisomerase II, RF-C, RP-A and a DNA helicase activity (Applegren et al., 1995). Poly(ADP-ribose) polymerase (PARP) has also been identified as a part of the complex (Simbulan-Rosenthall et al., 1996). A similar replication-competent multiprotein form of DNA polymerase was also isolated from mouse cells and was demonstrated to support *in vitro* polyomavirus DNA synthesis (Wu et al., 1994). A model was proposed to represent the structure of the Multiprotein DNA Replication Complex (MRC) which was isolated from both human and murine cells. This model is based upon the observed fractionation, chromatographic, and sedimentation profiles of the individual proteins found to co-purify with the complex (Wu et al., 1994; Applegren et al., 1995; reviewed in Hickey and Malkas, 1997). We recently renamed the replication complex designating it the DNA synthesome (Coll et al., 1996; Lin et al., 1997; Hickey and Malkas, 1997). This was done to differentiate the mammalian cell derived complex from the previously proposed elongation model, the replisome, (reviewed in Kornberg and Baker, 1992) which describes the putative superassembly of replication factors at the prokaryotic replication fork.

Two of the goals of this laboratory are to identify all of the protein components of the human cell DNA synthesome, and to then describe how these proteins act in concert to mediate DNA replication. To date, the identity and function of approximately half of these proteins have been determined. In this report we describe our most recent efforts toward defining the identity and function of several new components of the human cell DNA synthesome. We also report here for the first time results of experiments evaluating the fidelity of the replication mediated by the synthesome, and further verify the semi-conservative mechanism of action by the synthesome during *in vitro* DNA synthesis.

Experimental Procedures

Cell Culture and Harvest

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

DNA Synthesome Preparation

The DNA synthesome was isolated from HeLa cells using previously published procedures (Malkas et al., 1990b; Applegren et al., 1995) and outlined in Fig. 1.

DNA Helicase Assay

DNA helicase activity was assayed according to published procedures (Bachur et al., 1992; Wu et al., 1994; Applegren et al., 1995). Human DNA helicase antibodies HDH I and HDH IV (Tuteja et al., 1990-1991) and rabbit pre-immune sera, purified DNA polymerase α or SV40 large T-antigen antibodies were pre-incubated for 1 hour at 4°C before addition to the helicase assay.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Thirty micrograms of each of the various described protein fractions were loaded into each lane of the gels, and after their resolution by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) or non-denaturing gel electrophoresis (Native-PAGE) (Tom, et al., 1996) the resolved proteins were electrophoretically transferred to nitrocellulose. Immunodetection was carried out using a light-enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions (Amersham, Arlington Heights, IL). The anti-DNA polymerase ε antibody was used at a 1:1000 dilution. The anti-polymerase β antibody was used at a dilution of 1:1500. HDH I and IV antibodies were used at dilutions of 1:1000. The antibodies anti-Ku (Sigma), anti-hMLH1 antibody (Santa Cruz Biotechnology), anti-XPA antibody (Santa Cruz Biotechnology), anti-RNA Polymerase II antibody (Santa Cruz Biotechnology), anti-TFIIH (Santa Cruz Biotechnology), anti-Annexin I and anti-Annexin II (Transduction Laboratories) were all used at working dilutions of 1:1000. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots to identify

the bands reactive with primary antibodies. Pre-stained SDS-PAGE molecular size markers were obtained from the Amersham Corporation (Arlington Heights, IL).

Purification of SV40 Large T-Antigen

SV40 large T-antigen was purified from human 293 cells which had been infected with the recombinant adenovirus vector Ad-SVR284 via immuno-affinity chromatography of the clarified cell lysate using an immobilized protein A matrix to which was covalently coupled a monoclonal antibody derived from the hybridoma Pab 419. This monoclonal antibody is reactive against the SV40 large T-antigen (Simanis and Lane, 1985).

***In Vitro* SV40 DNA Replication Assay**

Assay reaction mixtures (25 µl) contained 80 mM Tris-HCl, pH 7.5; 7mM $MgCl_2$; 1 mM DTT; 3-20 µg of protein fraction; 0.5-1.0 µg of purified SV40 large T-antigen; 50 ng of plasmid $pSV0^+$ containing an insert of SV40 replication-origin DNA sequences (Stillman et al., 1985); 100 µM each dTTP, dATP, dGTP; 200 µM each rCTP, rGTP, UTP; 4 mM ATP; 25 µM [^{32}P]dCTP; 40 mM creatine phosphate; 1 µg of creatine kinase. The requirements for each of these components have been established and published previously (Applegren et al, 1995). The standard reaction was incubated for 2 hours at 35°C or for the length of time indicated in the Results. The replication assay reaction products were processed using DE81 filter binding to quantify the amount of radiolabel incorporated into the replication products (Sambrook et al., 1989). One unit of SV40 replication activity is equal to the incorporation of 1 pmol of dNMP into SV40 DNA per 2 hours under the standard assay condition.

Analyses of DNA Synthesome Replication Products

(a) Gel Assays

The DNA products of DNA synthesome-driven replication reactions were precipitated by the addition of ammonium acetate to 2.5M, followed by 2.5 volumes of ethanol. The precipitation was carried out at -20°C, overnight. The precipitated DNA was pelleted by centrifugation in an Eppendorf centrifuge for 30 minutes at 4°C, and the pellet DNA was then washed with an ice-cold solution of 70% ethanol. The DNA was dried in a Speed-Vac, resuspended in 50 µl of TE (10 mM Tris HCl pH 7.5, 0.1 mM EDTA), and digested for 8 hours with 5 units each of the endonucleases *MboI* and *SalI*. The resulting DNA fragments were then resolved by

electrophoresis at 2.5 V/cm for 16 hours through a neutral 1% agarose gel containing 1X TBE (i.e., 89 mM Tris-borate, 1 mM EDTA). After electrophoresis, the gels were dried and exposed to XAR5 (Kodak) film.

(b) CsCl density gradient analysis

For the analysis of synthesome DNA products by CsCl density gradient sedimentation, the *in vitro* DNA replication reactions were performed as described above except that 5-bromodeoxyuridine triphosphate (BrdUTP) (Sigma) was substituted for dTTP. The BrdUTP was added fresh at the initiation of the replication reaction to a final concentration of 100 μM. For the preparation of non-BrdUTP-substituted DNA replication products in these studies, parallel *in vitro* DNA replication reactions were performed using dTTP. After incubation of the replication reaction, the DNA products were phenol-extracted and precipitated twice in ethanol. The DNA products were then subjected to gel filtration chromatography using a 1 ml column of TE-400 Sephadex (Clontech Laboratories, Inc.) to remove unincorporated nucleotides. The DNA products were precipitated in ethanol, and linearized by digestion with *SalI* (NEB) for two hours at 37°C. The DNA products were subsequently analyzed by CsCl equilibrium centrifugation under neutral conditions (Epner et al., 1981). The DNA products were loaded directly onto the gradients with an initial refractive index of 1.403. The CsCl gradients were subjected to centrifugation in a Beckman NVT90 rotor for 48 hours at 55,000 rpm. The gradient was divided into 30 fractions beginning at the top. The CsCl density for each gradient fraction was calculated from its refractive index as measured on a Bausch and Lombe refractometer. Aliquots of 60 μl were taken from each CsCl gradient fraction and subjected to Cerenkov counting using a liquid scintillation counter.

Forward Mutagenesis Assay: Transfection and Plating.

To measure the spontaneous mutation frequency created during DNA synthesome mediated *in vitro* replication a fidelity assay described by Sekowski et al. (1997), was used. Because the background mutation frequency for a *lacZα* forward mutation assay employing M13 phage DNA is typically 2-5 x 10^{-4} we have chosen a plasmid DNA template, (i.e., pBK-CMV), for which we have measured a spontaneous mutation frequency that is less than 1 per 5.0 x 10^8 colonies examined (Sekowski et al., 1997).

The *in vitro* replicated pBK-CMV DNA template was used to transfect the *E. coli* host XL1-Blue MRF' following digestion of the parental template DNA with *DpnI*. Electrocompetent bacteria (XL1-Blue MRF') (60ul) were mixed with approximately 100-200 ng of the newly replicated pBK-CMV DNA (10ul) isolated from the *in*

vitro DNA replication reaction, incubated on ice for 10 minutes, and subjected to electroporation using a BioRad electroporator. The electroporation was adjusted to 1.4 kV, 25 uF, 200 Ohms. Immediately following electroporation, 1 ml of chilled sterile SOC buffer (20mM glucose in LB media) was added to the cuvette. The electroporated mixture was then incubated in a rotary shaker (250rpm) at 37°C for 1 hour. An amount of the culture sufficient to yield 1000-2000 bacterial colonies per plate was plated onto 20 ml LB agar containing 0.5 mg/ml kanamycin, 25 μg/ml IPTG and 25 μg/ml X-gal. These plating conditions allow the *E. coli* containing wild-type plasmid to express an intense blue phenotype. These plating conditions facilitate the visualization of various mutant phenotypes. The color of the mutant colonies range from white to an intermediate shade of blue, while the wild type colonies are a deep blue-green color.

Scoring of Mutants.

The inactivation of the α-complementation gene (the product of which is the catalytic subunit of β-galactosidase) due to a mutation in the *lacZα* gene contained in pBK-CMV yields a variety of mutant phenotypes when expressed in *E. Coli* strain XL1 Blue MRF'. The mutant phenotypes were scored on agar plates incubated approximately 12-15 hours at 37°C. Using the plating conditions described above, expression of the wild-type pBK-CMV DNA in the *E. Coli* host generates a dark blue bacterial colony. Bacterial colonies expressing the mutated pBK-CMV plasmid express an altered β-galactosidase gene which can be distinguished from those colonies expressing the wild type plasmid by their light blue to yellowish white color phenotypes. In order to reproducibly and precisely score the variable mutant phenotypes, only the pure white colonies (i.e. colonies lacking any trace of blue or green color) were scored as mutant. The light blue colonies comprise 25% (+/- 2%) of the total number of colonies.

Results

Human cell DNA synthesome-driven *in vitro* DNA replication is semi-conservative and does not readily re-initiate.

It was previously demonstrated by Malkas et al. (1990b) that the majority of the SV40 replication products synthesized by the DNA synthesome are resistant to digestion by the endonuclease *DpnI*. This result suggested that the synthesome mediates DNA replication by a semi-conservative replication mechanism.

To further verify that the replication products synthesized by the human cell DNA synthesome were the result of semi-conservative DNA replication, *in vitro* DNA replication reactions were performed as described in the Methods except that BrdUTP was substituted in place of dTTP. The replication products were analyzed by isopycnic centrifugation as described in the Methods. Figure 2 shows a typical sedimentation profile for the replication products. It was observed that the majority of the products of the *in vitro* DNA replication reaction sediment to the CsCl density expected for DNA molecules in which only one strand of the daughter DNA duplex has been fully substituted with BrdUMP, (designated the HL (heavy-light) DNA duplex). As expected, all of the DNA synthesome replication products synthesized in the presence of dTMP sedimented as LL (light-light) DNA duplexes. The minimal level of DNA synthesome replication products containing BrdUMP incorporated into both strands of the daughter DNA duplex (i. e., - a heavy-heavy (HH) DNA duplex) suggests that the *in vitro* initiation of DNA synthesome-mediated initiation of replication of the $pSVO^+$ plasmid DNA occurs only once, with very few additional rounds of replication taking place.

The premise that DNA synthesome-mediated *in vitro* DNA synthesis does not readily support the re-initiation of replication in new daughter DNA molecules is supported by studies examining the sensitivity of the DNA products to the endonuclease *MboI*. The restriction enzyme *MboI* cleaves at an unmethylated recognition sequence but not the fully methylated or hemi-methylated recognition sequence. The input parental plasmid DNA used in our *in vitro* DNA replication reaction is fully methylated (i.e., methylated on both DNA strands of the double helix) by the bacterial methylase which is present in the bacteria used to amplify the plasmid DNA. Since human cells lack an equivalent activity, one round of semi-conservative DNA synthesis will yield a hemi-methylated daughter DNA molecule. Additional rounds of DNA replication will yield daughter DNA molecules

which are unmethylated on both strands of the DNA duplex. In these studies the treatment of the products of the synthesome-driven *in vitro* DNA replication reaction with *MboI* demonstrates that only a minor fraction (i.e., <20%) of the newly synthesized DNA product was re-replicated, yielding *MboI* sensitive fragments (Fig. 3). The fraction of the DNA replication products that were cleaved by this enzyme yielded fragments that were heterogeneous in size and these fragments migrated as a background haze. The band intensities of the daughter DNA molecules replicated only once were compared by scanning densitometry. The *MboI* sensitive products comprised approximately 19% of the newly replicated DNA. Since the majority of the products formed during the *in vitro* DNA replication reaction were resistant to digestion by *MboI*, our results indicate that the DNA synthesome initiates essentially only one round of DNA replication *in vitro*.

Fidelity of HeLa Cell DNA Synthesome DNA Replication.

A forward mutagenesis assay (Sekowski et al. 1997) was utilized to test the fidelity of the DNA replication reaction mediated by the HeLa cell DNA synthesome. We observed that the DNA synthesome mediated *in vitro* DNA replication reaction had an intermediate level of fidelity between that exhibited by purified human DNA polymerases and that exhibited by whole human cell extracts. Using the HeLa cell DNA synthesome, we report that $4.5 \times 10^{-3} = 0.02\%$ (# mutant (white) colonies/total transformed colonies) expressed detectable mutations in the replicated (*DpnI* resistant) template DNA (Table 1).

The DNA synthesome contains two different helicase activities.

Previously, DNA helicase activity was found to co-purify with the replication-competent human cell DNA synthesome (Applegren et al., 1995), as well as that isolated from mouse cells (Wu et al., 1994). We now report that the DNA synthesome helicase activity is composed of at least two different DNA helicases. These helicases are the previously described HDH I and HDH IV (Tuteja et al., 1990-1991). The identity of the synthesome DNA helicases was determined by both Western blot and enzymatic activity analyses.

Immunoblot analysis was performed on different HeLa cell protein fractions derived during the isolation of the DNA synthesome using antibodies directed against human DNA helicases (HDH) I and IV (Fig. 4). It was observed that these specific human helicases co-purify with the synthesome-enriched protein fractions. Both helicases were found to be contained in the P-4, Q-Sepharose peak (QS) and the sucrose gradient peak (SG) fractions (Fig. 1). It has been previously shown that the synthesome is enriched in these protein fractions (Malkas

et al., 1990b; Applegren et al., 1995; Tom et al., to be published elsewhere). Although these helicases can also be found in the replication poor S-4 fraction (Malkas et al., 1990b), they were observed to exclusively co-purify with the DNA synthesome following Q-Sepharose column chromatography and sucrose gradient sedimentation. These helicase proteins were not detectable in the combined Q-Sepharose flow-through and wash fractions.

Helicase activity analysis of the DNA synthesome using the antibodies directed against HDH I and HDH IV was performed (Fig. 5). Using a strand displacement assay (Methods), it was observed that both of the helicase antibodies were able to inhibit a significant amount of the endogenous synthesome helicase activity (Fig. 5). However, antibodies derived from antigen naïve rabbits, or antibodies direct against purified DNA polymerase α or the SV40 large T-antigen were found to have no detectable inhibitory affect on the synthesome helicase activity (Fig. 5).

Several proteins that play a role in DNA repair co-purify with the human cell DNA synthesome.

Several proteins found to co-purify with the replication-competent DNA synthesome have been previously described to be involved in repair. There is an extensive literature reporting that DNA polymerases α and δ, RP-A, PCNA, RF-C, PARP and DNA ligase I participate in certain types of human cell DNA repair (Coverley et al., 1991, 1992; Dresler and Frattini, 1986; Podust et al., 1992; Shivji et al., 1995; Wang, 1991; Zeng et al., 1994). To begin to explore the possible link between DNA synthesome mediated DNA replication and the DNA repair process in human cells, we performed studies to determine whether several other DNA repair associated proteins co-purified with the human cell DNA synthesome.

The mismatch repair protein, hMLH1, was found to co-purify with the replication-competent DNA synthesome (Fig. 6, panel B). SDS-PAGE and immunoblot analysis using an antibody directed against hMLH1 were performed on protein fractions derived during the course of synthesome isolation. The hMLH1 protein was observed to co-purify with the replication-competent synthesome following chromatography on Q-Sepharose (QS peak) and sedimentation through sucrose density gradients (SG fraction) (Fig. 6, panel B).

The DNA binding protein Ku along with DNA-Protein Kinase (DNA-PK) has been suggested to have a role in the detection and/or repair of DNA damage. The Ku protein has also been implicated in many other processes such as cell signaling (Prabhakar et al., 1990) and DNA replication (Stuvier et al., 1990). To evaluate whether the Ku protein co-purified with the human cell DNA synthesome, immunoblot analyses of the protein

fractions derived in the course of the synthesome isolation were performed (Fig. 6, panel A). Ku protein was found to exclusively co-purify with DNA synthesome enriched protein fractions (i.e., P-4, QS peak and SG) (Fig. 6, panel A). Little detectable Ku protein was found in the synthesome poor S-4 and QS flow-through and wash fractions.

Similar to our observations with the hMLH1 and Ku proteins we have found that DNA polymerase ε co-purifies with the HeLa cell DNA synthesome (Fig. 6, panel C). DNA polymerase ε has also been implicated in DNA repair (Coverley et al., 1992; Nishida et al., 1988). Immunoblot analyses of the P-4, QS peak, S-4, QS flow through and SG peak fractions were performed using an antibody prepared against DNA polymerase ε (Syvaoja et al., 1990). DNA polymerase ε was found to exclusively co-purify with the replication competent DNA synthesome in the P-4, QS peak and SG peak fractions (Fig. 6, panel C). The DNA polymerase ε polypeptide was not detectable in the replication poor S-4 and Q-Sepharose flow-through fractions. These results with DNA polymerase ε are similar to those most recently reported for the purification of the synthesome from human breast cell lines and breast tissue (Coll et al., 1996).

When the protein fractions derived during the course of purification of the HeLa cell DNA synthesome were examined for the presence of two other proteins involved in DNA repair, (i.e., DNA polymerase β and the xeroderma pigmentosum group A protein (XPA)), it was observed that these proteins were not associated with the DNA synthesome and purified away from the DNA synthesome (Fig. 6, panels D and E). Monoclonal antibody directed against DNA polymerase β (Jenkins et al., 1992) was used to probe the human cell homogenate, NE-S-3, PEG NE-S-3, P-4, QS peak, S-4, QS Flow Through and SG peak fractions. The results of our immunoblot analysis indicate that DNA polymerase β did not co-purify with the synthesome (Fig. 6, panel D). DNA polymerase β was found in the homogenate and NE-S-3 fraction (Fig. 6, panel D). However, DNA polymerase β is not observed in the PEG NE/S-3, S-4, P-4, QS peak or SG fractions. These results indicate that the DNA polymerase β observed in the whole cell homogenate partitions to the NE-S-3 step of the fractionation scheme (Fig. 1) but is resolved from the DNA synthesome following precipitation of the NE-S-3 fraction with polyethylene glycol.

Similarly, immunoblot analysis using antibody directed against the human XPA protein (Oncogen sciences), which is a protein required during the incision step of nucleotide excision repair process (NER) (Li et

al., 1995) revealed that the XPA protein partitions with the homogenate, NE-S-3, P-4, and S-4 fractions but is resolved from the synthesome during ion exchange chromatography over Q-Sepharose (Fig. 6, panel E).

The Mammalian Cell RNA Transcription Apparatus Does Not Co-purify with the DNA Synthesome.

It has been suggested in the literature that the mammalian cell RNA transcription and DNA replication machinery may interact in some manner with the DNA replication apparatus (Echols, 1986; Sancar, 1995). To determine whether polypeptides responsible for mediating RNA transcription co-purified with the human DNA synthesome, we analyzed the protein fractions derived throughout the course of the synthesome purification for the presence of the RNA polymerase II holoenzyme (Barberis et al., 1995; reviewed by Carey, 1995). Utilizing an antibody directed against RNA polymerase II (Santa Cruz Biotechnology) the homogenate, NE-S-3, P-4, QS Peak, QS Flow-Through and SG Peak protein fractions (Fig. 1) were probed for the RNA polymerase II holoenzyme (Fig. 8). RNA polymerase II was not observed to co-purify with the DNA synthesome but readily partitioned away from the synthesome (Fig. 8) during the initial differential centrifugation steps used in the replication complex purification protocol.

Similar to observations regarding the RNA polymerase II holoenzyme, we also observed that the transcription factor TFIIH does not co-purify with the DNA synthesome. TFIIH is a multiprotein complex composed of at least five polypeptides. It is capable of phosphorylating RNA polymerase II and is involved in the activation of the pre-initiation complex for transcription (Zawel and Reinberg, 1995). This protein has also been shown to participate in the nucleotide excision repair process in human cells (Schaeffer et al., 1993). An antibody directed against the TFIIH protein (Santa Cruz Biotechnology) was used to probe the protein fractions isolated during the purification of the DNA synthesome. As observed with RNA polymerase II holoenzyme, TFIIH was found to purify away from the protein fractions containing the DNA synthesome during the initial differential centrifugation steps of the purification protocol, (data not shown).

Annexins Do Not Co-Purify with the DNA Synthesome.

Previous reports in the literature abound concerning proteins and factors that co-purify with putative high molecular weight replication complexes. Immunoblot analyses were initiated to determine if two members of a family of phospholipid and actin binding proteins, Annexin I and II, are able to co-fractionate with the DNA synthesome. These proteins have been implicated to have a role during in differentiation, cell proliferation and

carcinogenesis (Gupta et al., 1984; Schlaepfer and Haigler, 1990; Frohlich et al., 1990), and they have been reported to co-purify with the multiprotein DNA polymerase isolated from HeLa cells and affect its activity, (Kumble and Vishwanatha, 1991). Antibodies directed against both Annexin I and II (Transduction Laboratories) were used to probe the protein fractions derived during the course of DNA synthesome purification. Annexin I and II polypeptides were observed in the homogenate fraction but were absent in the replication competent QS peak and SG peak fractions containing the DNA synthesome, (Fig. 8).

Discussion

The DNA Synthesome Mediates Semi-conservative DNA Replication

Mammalian cell proliferation is a highly regulated process with multiple levels of control that affect a variety of essential cell functions. With the advent of the SV40 *in vitro* DNA replication system (Li and Kelly, 1984) there is now an accumulation of evidence regarding the existence of functional multiprotein complexes for DNA synthesis in eukaryotes (reviewed in Hickey and Malkas, 1997). Studies were conducted utilizing the DNA synthesome-driven SV40 *in vitro* replication system in order to determine if the DNA molecules containing the SV40 origin of replication support the semi-conservative synthesis of complete daughter DNA strands.

The semi-conservative replication of SV40 DNA templates *in vitro* are expected to yield hemi-methylated or unmethylated DNA molecules that are resistant to *DpnI* digestion (Vovis and Lacks, 1977). It was previously demonstrated that the DNA replication products formed during DNA synthesome mediated *in vitro* DNA replication are *DpnI* resistant. This indicates that he DNA template used in the *in vitro* assay had undergone at least one round of semi-conservative replication (Malkas et al., 1990b). To determine whether the DNA template and replication assay products could support multiple rounds of replication we digested the SV40 DNA replication products with the restriction endonuclease *MboI*. This enzyme cleaves only fully unmethylated DNA. Because the template DNA used in the assay is fully methylated, only molecules having undergone two rounds of replication will be digested by *MboI*. Similar to reports by others, only a minor fraction of the newly replicated DNA molecules supported a second round of replication. This result indicated that only a small number of the daughter DNA duplexes underwent two rounds of replication.

To rule out the possibility that *in vitro* DNA synthesis mediated by the DNA synthesome represented repair synthesis at nicks or gaps in the parental DNA template molecule, DNA replication reactions were performed containing 5-BrdUTP in place of dTTP. The DNA products were then analyzed by CsCl equilibrium centrifugation. If the BrdUMP incorporation was due to repair of the input parental DNA, the amount incorporated into the DNA molecules would not be sufficient to cause a specific and significant density shift (Meselson et al., 1958). Under neutral conditions, essentially all of the newly synthesized daughter DNA molecules in our study were observed to have a density expected for that of a semi-conservatively replicated DNA in which the daughter strand incorporated BrdUMP. In addition, the absence of detectable amounts of BrdUMP substituted into both strands of the daughter DNA duplexes suggests that the majority of DNA molecules only undergo a single round of DNA replication in the synthesome-driven system. These results reflect those found for *in vitro* DNA replication in crude extracts, namely that only a small proportion of the parental DNA template molecules actually re-initiate DNA replication *in vitro* (Stillman and Gluzman, 1985; Li and Kelly, 1984). These CsCl equilibrium sedimentation data together with the limited susceptibility of the daughter DNA duplexes to digestion by the enzyme *MboI* indicate that the DNA products formed by the DNA synthesome are the result of semi-conservative synthesis.

The Fidelity of Synthesome-mediated *In Vitro* DNA Replication.

The close relationship of the two cellular processes of DNA replication and DNA repair is illustrated by the recognition and/or maintenance of a subset of proteins utilized by both processes. These proteins include DNA polymerases α and δ, PARP, DNA ligase I, RP-A, PCNA, RFC and DNA helicase (Coverley et al., 1991, 1992; Dresler and Frattini, 1986; Podust et al., 1992; Shivji et al., 1995; Wang, 1991; Zeng et al., 1994). The observations of these individuals suggest that there may be a direct link between the apparatus that controls and carries-out DNA replication and the apparatus that mediates DNA repair. The high degree with which these proteins have been conserved across evolutionary lines suggests that the two processes may work in concert to maintain the fidelity of the genome and regulate the mutation frequency at a level required to sustain or promote evolution. The high degree of accuracy of the DNA replication process must occur through the active participation of the DNA replication enzymes (polymerases) and/or other cellular factors (DNA repair enzymes) to either enhance base selection or remove errors before they become fixed in the genome.

Our forward mutagenesis assay was utilized to test the fidelity of DNA replication mediated by the HeLa DNA synthesome. The data demonstrated an intermediate level of fidelity when compared to studies using purified human DNA polymerase-primase or HeLa whole cell extracts Roberts and Kunkel, (1988). The fidelity of the DNA synthesome-mediated *in vitro* DNA replication process may be directly compared to the polymerase-primase and HeLa cell extract fidelity results of Roberts and Kunkel (1988) by using the equation described in Table 1. In contrast to Kunkel's fidelity assay in crude cell extracts, our forward mutagenesis assay using the DNA synthesome is capable of providing a measurement of the fidelity of DNA replication in the absence of competing biological processes. Therefore, we believe our forward mutagenesis assay may provide a more accurate estimate of DNA replication fidelity.

It has been suggested that the fidelity of cellular DNA replication process is approximately three orders of magnitude higher than the fidelity of DNA synthesis mediated by purified DNA polymerase enzymes. This increase in fidelity has been attributed to post-replication mismatch repair of the replicated DNA (Modrich, 1991). Because the human cell DNA synthesome has demonstrated a higher level of replication fidelity than that reported for isolated DNA polymerases, it appears that the synthesome may be capable of carrying out critical proofreading or DNA mismatch repair functions. While the DNA replication fidelity of the human cell extracts appears to be higher than was found for the isolated DNA synthesome, the whole cell extract potentially contains many accessory proteins or factors that may alter the post replication repair process. Our observations and those reported by Kunkel's group reinforce the idea that the maintenance of high fidelity DNA synthesis and repair requires at least some of the proteins used during the DNA replication process.

Several New Components of the DNA Synthesome Are Identified.

As an initial step toward determining whether a known DNA mismatch repair protein may be associated with the DNA synthesome, we chose to study the hMLH1 protein (Modrich, 1989,1991). The hMLH1 protein is the human homolog of the bacterial mutL protein, a member of the highly conserved family of post-replication mismatch repair proteins. Immunoblot analyses demonstrated that hMLH1 co-fractionates with other proteins composing the core of the DNA synthesome. This co-fractionation may be indicative of a direct interaction between the replication protein components of this replication complex which are physically associated with the

newly synthesized DNA strand and the DNA mismatch repair process. Therefore, the DNA synthesis and mismatch repair proteins may work in concert to maintain the high fidelity of the DNA replication process. Further studies are underway to identify the presence of other repair proteins which may be associated with the DNA synthesome and to examine the role that the synthesome may play in the mismatch repair process.

The synthesis of daughter DNA, as well as the repair, transcription and recombination processes, require a transient unwinding of the DNA double helix. The DNA helicases are responsible for unwinding the DNA helix during the replication and repair of DNA, and during the transcription process helicases catalyze DNA unwinding and translocation by coupling the energy derived from hydrolysis of nucleoside 5'-triphosphates (NTP or dNTP) to the processivity of the helicase enzyme, (Geider and Hoffmann, 1981). This analysis has identified several additional components of the DNA synthesome and two of these are specific DNA helicases (HDH I and IV). Tuteja et al. (1990-1991) have reported the existence and purification of five different HeLa cell DNA helicases. Two of these purified enzymes isolated from nuclear extracts have been designated human DNA helicases HDH I and IV (Tuteja et al., 1990-1991). HDH I is a 65 kDa protein that exhibits a 3' to 5' directional unwinding polarity, while HDHIV is a 100 kDa protein that exhibits a 5' to 3' polarity. We suggest that these synthesome associated DNA helicases may promote the melting of parental DNA strands during the DNA replication reaction, and permit access of the elongation components of the synthesome to the DNA. Based upon the results of our Western blot and antibody neutralization analyses, the model proposed for the human cell DNA synthesome (Wu et al., 1994; Applegren et al., 1995) can now be extended to include these two DNA helicase activities.

We are particularly interested in identifying human cell DNA helicases that are involved in origin activation and possibly performing functions analogous to those of the helicase associated with the large T-antigens of SV40 and polyomavirus (Stahl and Knippers, 1987; Goetz et al., 1988; and Seki et al., 1990). More in depth studies on the roles that HDH I and IV play in DNA synthesome-mediated DNA replication, are underway to establish whether these enzymes, are involved in: 1) initiating replication at the origin, and/or 2) mediating replication fork movement.

Previously, DNA polymerases α and δ were found to co-purify as integral components of this human cell DNA synthesome (Malkas et al., 1990b; Applegren et al., 1995). In continuing to identify the components of the DNA synthesome we examined the various protein fractions isolated during the purification of the synthesome for

the presence of other DNA polymerases. Our work demonstrates for the first time that polymerase ε also co-fractionates with the other core components of the human cell DNA synthesome, and thus can be included as a newly recognized component of the synthesome. In addition, this laboratory has confirmed the direct interaction between DNA polymerase ε and the DNA synthesome by co-immuno-precipitation studies (Coll, et al., to be published elsewhere). DNA polymerase ε appears to have functional roles in both DNA replication and/or in a repair process closely associated with replication (Shivji et al., 1995) but further studies are required to determine this enzyme's primary role.

It was also demonstrated that DNA polymerase β does not co-purify with the human DNA synthesome. It has been demonstrated that model DNA replication systems that use small double stranded viral DNA molecules, such as the SV40 system, do not require DNA polymerase β for activity because the systems can be reconstituted with purified proteins in its absence (van der Vliet, 1990). Our immunoblot analyses are in agreement with this observation, as they indicated that DNA polymerase β is not required for efficient *in vitro* DNA replication activity mediated by the DNA synthesome.

The requirements for human cell synthesome mediated *in vitro* DNA replication reaction have been previously published (Applegren, et al., 1995). It was demonstrated that the *in vitro* DNA replication reaction was resistant to the inhibitory effect of dideoxynucleotide triphosphates. Both DNA polymerases α and δ have been shown to be resistant to dideoxynucleotide triphosphates and DNA polymerase β is strongly inhibited by ddTTP (Kornberg and Baker, 1992). This can be seen as another line of evidence that DNA polymerase β is not a functional component in the DNA synthesome, and is not involved in the replication process.

In this report, several other proteins related to the DNA repair and DNA replication processes that co-purify with the DNA synthesome were identified. Another protein thought to be involved in the DNA synthetic processes is Ku. The Ku protein serves as a regulatory or targeting subunit for the DNA-Protein Kinase which phosphorylates several nuclear proteins *in vitro* (Anderson, 1993). Because several of the protein substrates for DNA-PK are involved in DNA replication, (i.e., topoisomerases I and II, RF-C and SV40 large T-antigen) it was decided to examine whether the Ku protein was a component of the DNA synthesome. We have shown that the Ku protein is present in the highly purified DNA synthesome protein fraction. This observation suggests that the DNA synthesome may participate in some form of DNA repair which involves the Ku protein.

Annexin I and II have been implicated in a variety of cellular processes such as differentiation, cell proliferation and carcinogenesis (Gupta et al., 1984; Schlaepfer and Haigler, 1990; Frolich et al., 1990). Although the conservation of Annexins through evolution argues strongly for an important physiological role for these proteins, the precise role of these proteins remains confusing and controversial. Because reports in the literature have described the Annexins as co-purifying with multiprotein forms of DNA polymerase (Kumble and Vishwanatha, 1991), we chose to examine the DNA synthesome for the presence of these proteins. Neither of the Annexins were not found to co-purify with the replication-competent synthesome, and are not required for synthesome-mediated DNA replication. We therefore conclude that Annexins I and II are unlikely to be integral components of the human cell DNA replication apparatus.

Nucleotide excision repair is a process involving: (1) the recognition of a lesion, (2) incision of the altered DNA strand, (3) excision of the damaged nucleotides along with a portion of the strand surrounding the lesion, (4) repair of the gap created in the DNA during excision, and (5) ligation of the newly synthesized patch into the adjacent DNA strands. This process requires the concerted activity of several proteins which mediate each of these steps. DNA sequence dependent events such as transcription, replication, or site-specific recombination are therefore unlikely to be directed by the binding of a single protein to a single DNA site (Echols, H., 1986). On the contrary, these complex processes involve multiple protein-DNA and protein-protein interactions (Sancar, A., 1995). Such macromolecular interactions are thought to regulate the assembly of DNA-bound proteins to generate highly organized nucleoprotein structures with a high specificity of protein-DNA recognition. As analysis of the NER process continues, an increasing number of protein-protein interactions have been identified to be involved. However, it remains to be elucidated whether these interactions occur sequentially or result in progressively more complex structures at sites of damage. Most of the gene products used in NER appear to participate in the early steps of this repair process. Our analysis of the DNA synthesome has demonstrated that several proteins required for transcription and NER do not co-purify with the synthesome. These include the transcription proteins, RNA polymerase II, TFIIH, and the excision repair XPA protein. These data suggest that NER components are not an integral part of the synthesome.

Some of the transcription elongation factors are thought to couple transcription to repair and later clear a path for the replication machinery. It is conceivable that the NER machinery responsible for incision/excision of

damaged DNA could be juxtaposed to the DNA synthetic machinery. The NER machinery could then recruit the DNA synthesome for completing the synthesis and ligation steps of the repair process. This would allow for an interconnection between NER and DNA replication processes previously thought to function more or less independently.

Proteins involved in DNA repair, DNA replication, transcription, recombination or cell cycle progression are all part of a complex network of overlapping biochemical reactions and pathways that function to maintain cellular homeostasis. Further characterization of the human cell DNA synthesome will increase our understanding of the molecular mechanisms involved in regulating the activity of the DNA replication apparatus and potentially integrating it into the processes of both DNA replication and repair.

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REFERENCES

Anderson, C.W. (1993) DNA damage and the DNA-activated protein kinase, Trends in Biochem. Sci., 18: 433-437.

Applegren, N., Hickey, R.J., Kleinschmidt, A., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Quan, J.Y., Lee, M.Y.W.T., and Malkas, L.H. (1995) Further Characterization of the Human Cell Multiprotein DNA Replication Complex, J. Cell. Biochem., 59: 91-107.

Bachur, N., Yu, F., Johnson, R., Hickey, R., Wu, Y. and Malkas, L., (1992) Helicase Inhibition By Anthracycline Anticancer Agents, Mol. Pharmacol., 41: 993-998.

Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G., and Ptashne, M. (1995) Contact with a component of the polymerase II holoenzyme suffices for gene activation, Cell, 81: 359-368.

Carey, M.F. (1995) A holistic view of the complex, Curr. Biol., 9: 1003-1005.

Coll, J., Sekowski, J., Hickey, R.J., Schnaper, L., Yue, W., Brodie, A., and Malkas, L.H. (1996) The human breast cell DNA synthesome: Its purificaiton from tumor tissue and cell culture, Oncology Research, 8: 435-447.

Coverley, D., Kenny, M.K., Munn, M., Rupp, W.D., Lane, D.P. and Wood, R.D. (1991) Requirement for the replication protein SSB in human DNA excision repair. Nature, 349: 538-541.

Coverley, D., Kenny, M.K., Lane, D.P. and Wood, R.D. (1992) A role for the human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair. Nucl. Acids Res., 20: 3873.

Dresler, S.L. and Frattini, M.K. (1986) DNA replication and UV-induced DNA repair synthesis in human fibroblasts are much less sensitive than DNA polymerase alpha to inhibition by butylphenyl-deoxyguanosine triphosphate. Nucleic Acids Res., 14: 7093-7102.

Echols, H (1986) Multiple DNA-protein interactions governing high-precision DNA transactions. Science 233, 1050-1055.

Epner, E., Rifkind, R.A. and Marks, P.A. (1981) Replication of α and β globin DNA sequences occurs during early S phase in murine erythroleukemia cells, Proc. Natl. Acad. Sci. USA, 78: 3058-3062.

Fairman MP, Stillman B. (1988) Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J.; 7:1211-1218.

Frohlich, M., Motte, P., Galvin, K., Takahashi, H., Wands, J. and Ozturk, M. (1990) Enhanced expression of the protein kinase substrate p36 in human hepatocellular carcinoma, Molec. cell. Biol., 10: 3216-3223.

Geider, K, and Hoffmann-Berling, H. (1981) Proteins controlling the helical structure of DNA. Ann. Review of Biochem., 50: 233-260.

Goetz, G.S., Dean, F.B, Hurwitz, J. and Matson, S.W. (1988) The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem., 263: 383-392.

Gupta, C., Katsumata, M., Goldman, A., Herold, R. and Piddington, R. (1984) Glucocorticoid-induced phospholipase A2-inhibitory proteins mediate glucocorticoid teratogenicity in vitro, Proc. Natl. Acad. Sci. USA, 81: 1140-1143.

Hickey, R.J. and Malkas, L.H. (1996) Mammalian Cell DNA Replication, Critical rev. in Eukaryotic Gene Exp. eds. Stein G., Stein, J., and Lian, J. (in press).

Jenkins, T.M., Saxena, J.K., Kumar, A., Wilson, S.H. and Ackerman, E.J. (1992) DNA polymerase β and DNA synthesis in *Xenopus* Oocytes and in a nuclear extract. Science, 258: 475-478.

Kornberg, A. and Baker, T.A. (1992) DNA Replicaton, second edition, W.H. Freeman and Co. 1992, New York.

Kumble, K.D. and Vishwanatha, J.K. (1991) Immunoelectron microscopic analysis of the intracellular distribution of primer recognition proteins, annexin 2 and phosphoglycerate kinase, in normal and transformed cells, J. Cell Sci., 99: 751-758.

Kunkel, T.A. (1985) The mutational specifications of DNA polymerase beta during in vitro DNA synthesis. J. Biol. Chem., 260: 5787-5796.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature 227:680-685.

Lee S-H, Eki T, Hurwitz J. (1989) Synthesis of DNA containing the simian virus 40 origin of replication by the combined action of DNA polymerases alpha and delta. Proc. Natl. Acad. Sci. USA; 86:7361-7365.

Li, J., and Kelly, T. (1984) Simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA 81: 6973-6977.

Li, L., Peterson, C.A., Lu, X., and Legerski, R.J., (1995) Mutations in XPA that prevent association with ERCC1 are defective in nucleotide excision repair, Mol. Cell. Biol., 15: 1993-1998.

Lin, S., Hickey, R.J. and Malkas, L.H. (1997) The isolation of a DNA synthesome from human leukemia cells. Leukemia Research (in press).

Malkas, L.H., Hickey, R.J. and Baril, E.F. (1990a) Multienzyme complexes for DNA synthesis in eukaryotes: P-4 revisited, In: The Eukaryotic Nucleus, Molecular Biochemistry and Macromolecular Assemblies, Vol. 2, Strauss, P.R. and Wilson, S.H. (Eds), Telford Press, New Jersey, pp. 45-68.

Malkas, L.H., Hickey, R.J., Li, C., Pedersen, N. and Baril, E.F. (1990b) A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*, Biochemistry, 29: 6362-6374.

Mathews, C.K., and Slabaugh, M.B. (1986) Eukaryotic DNA metabolism. Are deoxynucleotides channeled to replication sites?, Exp. Cell Res., 162: 285-295.

Meselson, M. and Stahl, F.W. (1958) The replication of DNA in Escherichia coli. Proc. Natl. Acad. Sci. USA, 44: 671-682.

Meyers, M., Theodosiou, M., Acharya, S., Odegaard, E., Wilson, T., Lewis, J.E., Davis, T.W., Wilson-VanPatten, C., Fishel, R., and Boothman, D. (1997) Cell cycle regulation of the human DNA mismatch repair genes hMSH2, hMLH, and hPMS2. Cancer Res., 57: 206-208.

Modrich, P. (1989) Methyl-directed DNA mismatch correction. J. Biol. Chem., 264: 6597-6600.

Modrich, P. (1991) Mechanisms and biological effects of mismatch repair. A. Rev. Genet., 25: 229-253.

Murakami, Y., Eki, T., Yamada, M., Prives, C., and Hurwitz, J. (1986): Species-specific *in vitro* synthesis of DNA containing the polyomavirus origin of replication. Proc. Natl. Acad. Sci. USA 83: 6347-6351.

Nishida, C., Reinhard, P., Linn, S. (1988) DNA repair synthesos in human fibroblasts requires DNA polymerase delta, J. Biol. Chem., 263: 501-510.

Podust, V.N., Georgaki, A., Strack, B., and Hubscher, U. (1992) Calf thymus RF-C as an essential component for DNA polymerase delta and epsilon holoenzymes function. Nucleic Acids Res. 20, 4159.

Prabhakar, B.S., Allaway, G.P., Srinivasappa, J., Notkins, A.L. (1990) Cell-surface expression of the 70 kD component of Ku, a DNA-binding nuclear autoantigen, J. Clin. Invest., 86: 1301-1305.

Prelich, G., Tan, C.K., Kostura, M., Mathews, MB., So, A.G., Downey, K.M. and Stillman, B. (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxilliary protein, Nature, 326: 517-520.

Reddy, G., and Fager, R. (1993) Replitase: A complex integrating dNTP synthesis and DNA replication, Crit. Rev. Eukaryotic Gene Exp. 3: 255-277.

Roberts, J.D. and Kunkel, T.A. (1988) Fidelity of a human cell DNA replication complex. Proc. Nat'l. Acad. Sci. USA, 85: 7064-7068.

Sancar, A. (1995) DNA repair in humans, Annu. Rev. Genetics, 29: 69-105.

Schaeffer, L. Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H.J., Chambon, P., and Egly, J. (1993) DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor, Science, 260: 58-63.

Schlaepfer, D.D. and Haigler, H.T. (1990) Expression of annexins as a function of cellular growth state, J. Cell Biol., 111: 229-238.

Seki, M., Enomoto, T., Eki, T., Miyajima, A., Murakami, Y., Hanaoka, F., and Ui, M. (1990) DNA helicase and nucleoside-5'-triphosphatase activities of polyoma virus large tumor antigen, Biochem., 29: 1003-1009.

Sekowski, J.W., Malkas, L.H., Wei, Y., and Hickey, R.J. (1997) Mercuric ion inhibits the activity and fidelity of the human cell DNA synthesome. J. Toxicology and Applied Pharm., 145: 268-276.

Shivji, M.K.K., Podust, V.N., Hubscher, U., and Wood, R.D. (1995) DNA repair defect in xeroderma pigmentosum group C and complementing factor from HeLa cells. Biochemistry 34, 5011-5017.

Simanis, V., and Lane, D.P., (1985) An immunoaffinity purification procedure for SV40 large T antigen, Virology, 144: 88-100.

So, A.G. and Downey, K.M. (1988) Mammalian DNA polymerases α and δ: Current status in DNA replication, Biochemistry, 27: 4591-4595.
Stahl, H. and Knippers, R. (1987) The simian virus 40 large tumor antigen. Biochimica et Biophysica Acta, 910: 1-10.

Stillman, B., and Gluzman, Y., (1985) Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells, Mol. Cell. Biol., 5: 2051-2060.

Stuvier, M.H., Coenhaerts, F.E.J., van der Vliet, P.C., (1990) The autoantigen Ku is indistinguishable from NF IV, a protein forming multimeric protein-DNA complexes, J. Exp. Med., 172: 1049-1054.

Syvaoja, J., Suomensaari, S., Nishida, C., Goldsmith, J.S., Chui, G.S.J., Jain, S. and Linn, S. (1990) DNA polymerase α, δ, and ε: Three distinct enzymes from HeLa cells, Proc. Natl. Acad. Sci. USA, 87: 6664-6668.

Tom, T.D., Malkas, L.H., and Hickey, R.J. (1996) Identification of multiprotein complexes containing DNA replicaiton factors by native immunoblotting of HeLa cell protein preparations with T-antigen dependent SV40 DNA replication activity, J. Cell. Biochem., 63: 259-267.

Tsurimoto, T., and Stillman, B., (1989) Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, alpha and delta, EMBO J., 8: 3883-3889.

Tuteja, N., Tuteja, R., Rahman, K., Kang, L.Y., and Falaschi, A. (1990) A DNA helicase from human cells, Nucleic Acids Res., 18: 6785-6792.

Tuteja, N., Rahman, K., Tuteja, R., and Falaschi, A. (1991) DNA helicase IV from HeLa cells, Nucleic Acids Res., 19: 3613-3618.

Tuteja, N., Rahman, K., Tuteja, R., Ochem, A., Skopac, D., and Falaschi, A. (1992) DNA helicase III from HeLa cells: an enzyme that acts preferentially on partially unwound DNA duplexes, Nucleic Acids Res., 20: 5329-5337.

Tuteja, N., Rahman, K., Tuteja, R., and Falaschi, A., (1993) Human DNA helicase V, a novel DNA unwinding enzyme from HeLa cells, Nucleic Acids Res., 21: 2323-2329.

Tuteja, N., Tuteja, R., Ochem, A., Taneja, P, Huang, N-W., Simoncsits, A., Susic, S., Rahman, K., Marusic, L., Chem, J., Zhang, J., Wang, S., Pongor, S., and Falaschi, A., (1994) Human DNA helicase II: a novel DNA unwinding enzyme identified as the Ku autoantigen, EMBO J., 13: 4991-5001.

van der Vliet, P.C. (1990) in The Eukaryotic Nucleus. Molecular Structure and Macromolecular Assembly, P.R. Strauss and S.H. Wilson, Eds. (Telford Caldwell, NJ, vol. 1, pp. 1-31.

Vovis, G.F., and Lacks, kS. (1977) Complementary action of restriction enzymes endo R-DpnI and Endo R-DpnII on bacteriophage f1 DNA. J. Mol. Biol., 115: 525-538.

Wang, T.S.F. (1991) Eukaryotic DNA polymerases, Annu. Rev. Biochem., 60: 513-552.

Weinberg, D.H., and Kelly, T.J. (1989) Requirement for two DNA polymerases in the replication of simian virus 40 DNA *in vitro*, Proc. Nat'l. Acad. Sci. USA, 86: 9742-9746.

Wobbe, C.R., Dean, F., Weissbach, L. and Hurwitz, J. (1985) *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site, Proc. Nat'l. Acad. Sci. USA, 82: 5710-5714.

Wobbe, C.R., Weissbach, L., Borowiec, J.A., Dean, F.B., Murakami, Y., Bullock, P., and Hurwitz, J., (1987) Replication of simian virus 40 origin-containing DNA *in vitro* with purified proteins, Proc. Nat'l. Acad. Sci. USA, 84: 1834-1838.

Wold, M.S., Weinberg, D.H., Virshup, D.M., Li, J.J. and Kelly, T.J. (1989): Identification of cellular proteins required for simian virus 40 DNA replication. J. Biol. Chem. 264: 2801-2809.

Wu, Y., Hickey, R., Lawlor, K., Wills, P., Yu, F., Ozer, H., Starr, R., Quan, J.Y., Lee, M., and Malkas, L., (1994) A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication *in vitro*, *J. Cell.* Biochem., 54: 32-46.

Yang, L., Wold, M.S., Li, J.J., Kelly, T.J. and Liu, L.F. (1987): Roles of DNA topoisomerases in simian virus 40 DNA replication. Proc. Natl. Acad. Sci. *USA* 84: 950-954.

Zawel, L. and Reinberg, D. (1995) Common themes in assembly and function of eukaryotic transcription complexes. Annu. Rev. Biochem., 64: 533-561.

Zeng, X.R., Hao, H., Jiang, Y., and Lee, M.Y., (1994) Regulation of human DNA polymerase delta during the cell cycle, J. Biol. Chem., 269: 24027-24033.

Table 1. Relative rate of HeLa cell DNA synthesome mediated DNA replication fidelity errors.

Purified* Polymerase-Primase Complex	DNA Synthesome	Cell Extract
2.0×10^{-4}	2.4×10^{-5}	6.0×10^{-6}

*Taken from Roberts and Kunkel, 1988.
#Results of our DNA synthesome-mediated replication fidelity assay re-calculated using Kunkel's equation:

A. Number of mutant colonies/total number of transformed colonies = 4.5×10^{-3}

B. Minus background mutation rate (spontaneous mutation rate)(0: 8×10^{4} colonies) = 0

C. Divide by 0.5 (the approximate probability of expressing an error) = 9.0×10^{-3}

D. Divide by number of sites in target gene (373 bp) = 2.4×10^{-5} or 2.5 per 10,000 nucleotides incorporated.

Figure 1. The flow diagram of the isolation scheme used to purify the human cell synthesome (see Methods).

Figure 2. Analysis of products of SV40 *in vitro* DNA replication by CsCl density gradient sedimentation. The *in vitro* DNA replication reaction was performed as described in Materials and Methods. As described in the text, dTTP in the replication reaction was replaced by BrdUTP. The incorporation of [^{32}P]dCMP into acid-insoluble material was measured. The densities of aliquots were measured directly with a refractometer and the positions of the migration of light DNA (LL; 1.69 g/ml) by the non-substituted products and heavy light DNA (HL; 1.74 g/ml) by the BrdUMP substituted products were determined.

Figure 3. *MboI* restriction analysis of pSVO^{+} DNA after replication by the synthesome. More than 80% of the newly synthesized DNA is resistant to *MboI* digestion. The arrow on the left indicates the position of linear DNA insensitive to *MboI* digestion.

Figure 4. Immunoblot analyses for the presence of human DNA helicases I (65 kDa.) and IV (100 kDa.) in P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through, and Sucrose gradient peak fractions. Thirty micrograms of each protein fraction were resolved on 8% polyacrylamide gels. The proteins were then transferred to nitrocellulose filter membranes. The polypeptides human DNA helicase I and IV were visualized by sequentially incubating the membranes with the appropriate primary antibodies followed by anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (see Methods). Light enhanced chemiluminescence detection of the immobilized protein was accomplished using the ECL Western blotting detection system (Amersham). The resolution profile of the ECL protein molecular weight markers is designated by an M.

Figure 5. DNA helicase activity. The Q-Sepharose peak fraction (1ug) was preincubated with increasing amounts of each helicase antibody (0.25ul, 0.5ul and 1ul) or with rabbit pre-immune serum, or antibody directed against DNA polymerase α or SV40 large T-antigen (0.25ul, 0.5ul, 1ul and 2ul) as described in the text for 1 hour at 4°C. These preincubation mixes were then added to the helicase assay and incubated for 30 minutes at 37°C. The DNA bands observed in the gel lanes migrate through a 12% polyacrylamide gel at the position expected for a single-stranded M13 DNA to which a ^{32}P-radiolabeled 17-mer oligonucleotide has been hybridized, or to the position expected for an unwound ^{32}P-radiolabeled 17-mer. Control lanes do not have Q-Sepharose peak protein loaded (NP and ^-heat denatured).

Figure 6. Immunoblot analyses to test for the presence of various proteins in the DNA synthesome. The cell homogenate, NE+S-3, P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through and Sucrose gradient peak fractions were used in these analyses. Thirty micrograms of each protein fraction were size fractionated on 8% polyacrylamide gels, transferred to nitrocellulose filter membranes and visualized using the ECL Western blotting detection system (Amersham). Shown are the 86 kDa. Ku, 90 kDa. HMLH1, 200 kDa. DNA polymerase ε, 38 kDa. DNA polymerase β, and 33 kDa. XPA polypeptides (see Methods). The arrow denotes the hMLH1 protein band.

Figure 7. Immunoblot analyses for the presence of RNA polymerase II protein (215 kDa.) with the synthesome. The cell homogenate, NE+S-3, P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through and Sucrose gradient peak fractions were used in these analyses. Thirty micrograms of each protein fraction were size fractionated and visualized using the ECL Western blotting detection system (Amersham).

Figure 8. Immunoblot analyses to test for the presence of Annexin I protein (38 kDa.) and Annexin II protein (36 kDa.) in the synthesome. The cell homogenate, Q-Sepharose peak and Sucrose gradient peak fractions were used in these analyses. Thirty micrograms of each protein fraction were resolved and light enhanced chemiluminescence detection of the immobilized protein was accomplished using the ECL Western blotting detection system (Amersham).

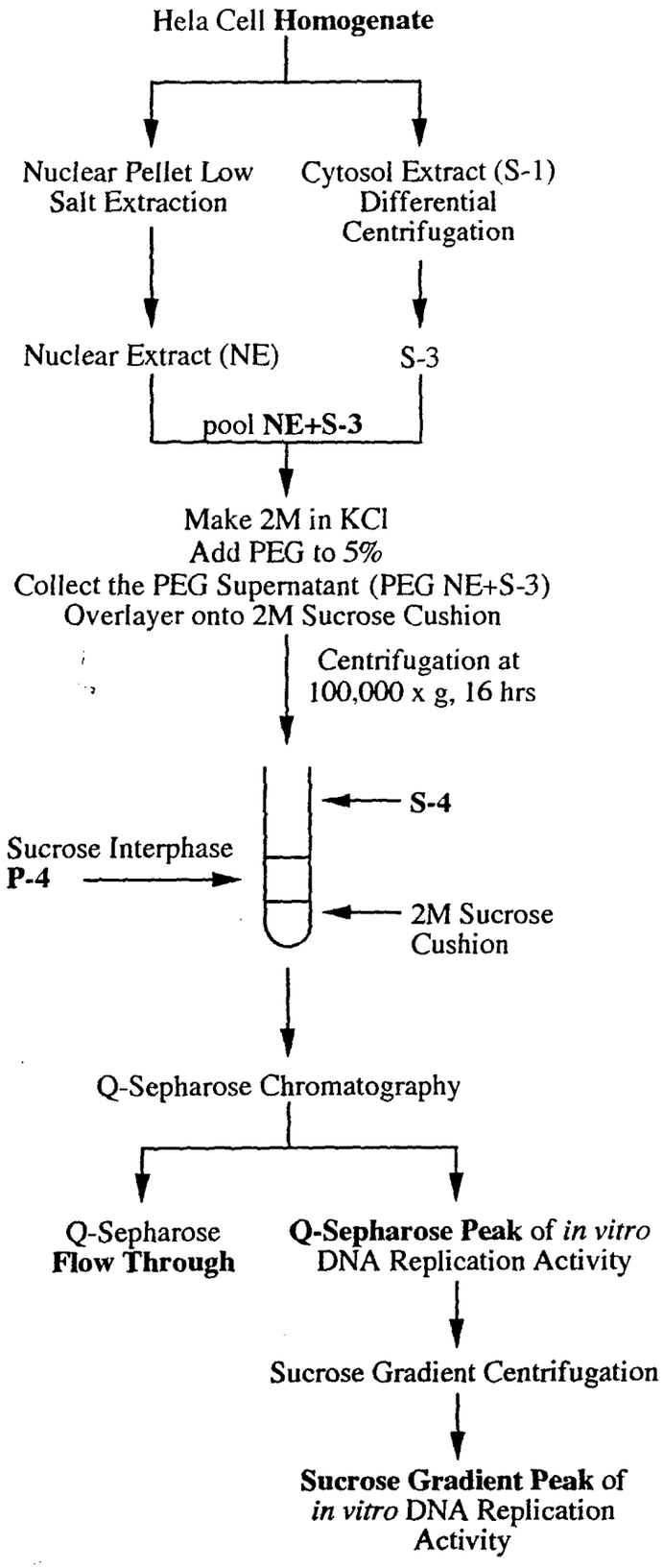
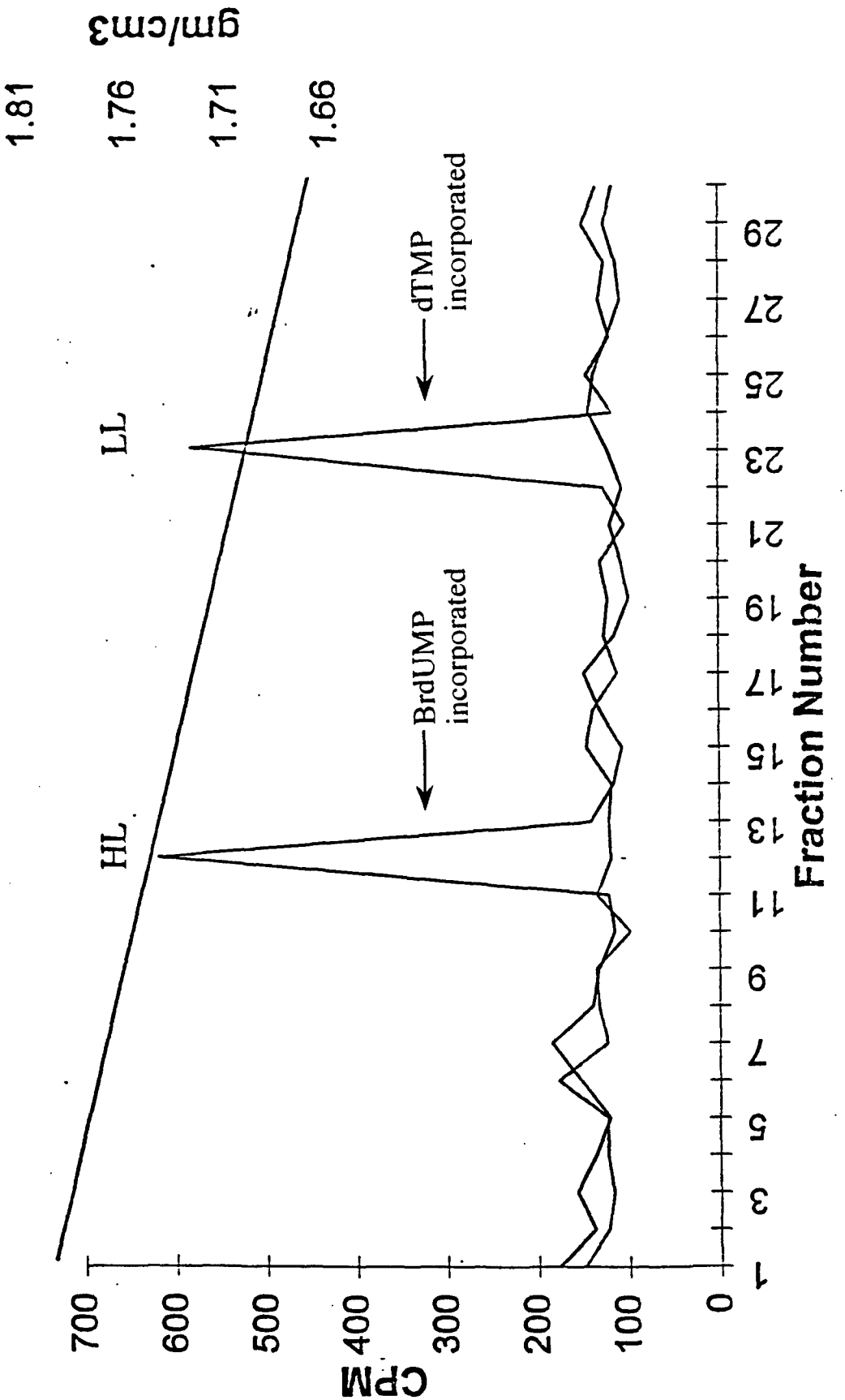
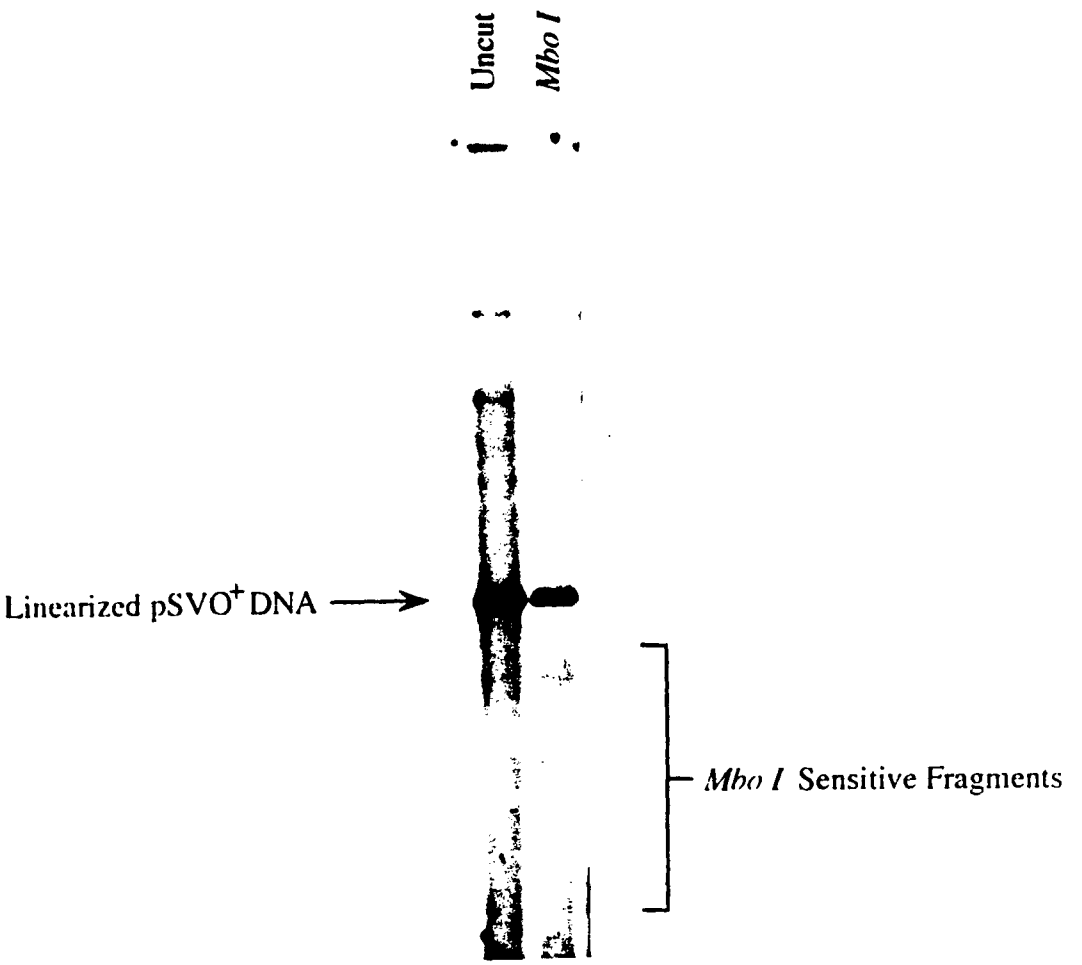

Hela Cell **Homogenate**
Nuclear Pellet Low Salt Extraction
Cytosol Extract (S-1) Differential Centrifugation
Nuclear Extract (NE)
S-3
pool **NE+S-3**
Make 2M in KCl
Add PEG to 5%
Collect the PEG Supernatant (PEG NE+S-3)
Overlayer onto 2M Sucrose Cushion
Centrifugation at 100,000 x g, 16 hrs
S-4
Sucrose Interphase **P-4**
2M Sucrose Cushion
Q-Sepharose Chromatography
Q-Sepharose **Flow Through**
Q-Sepharose Peak of *in vitro* DNA Replication Activity
Sucrose Gradient Centrifugation
Sucrose Gradient Peak of *in vitro* DNA Replication Activity

Figure 2


gm/cm3
1.81
1.76
1.71
1.66
HL
LL
BrdUMP incorporated
dTMP incorporated
CPM
700
600
500
400
300
200
100
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
Fraction Number

A Semiconservative Mechanism of DNA Replication
Sensitivity to *Mbo I* Digestion



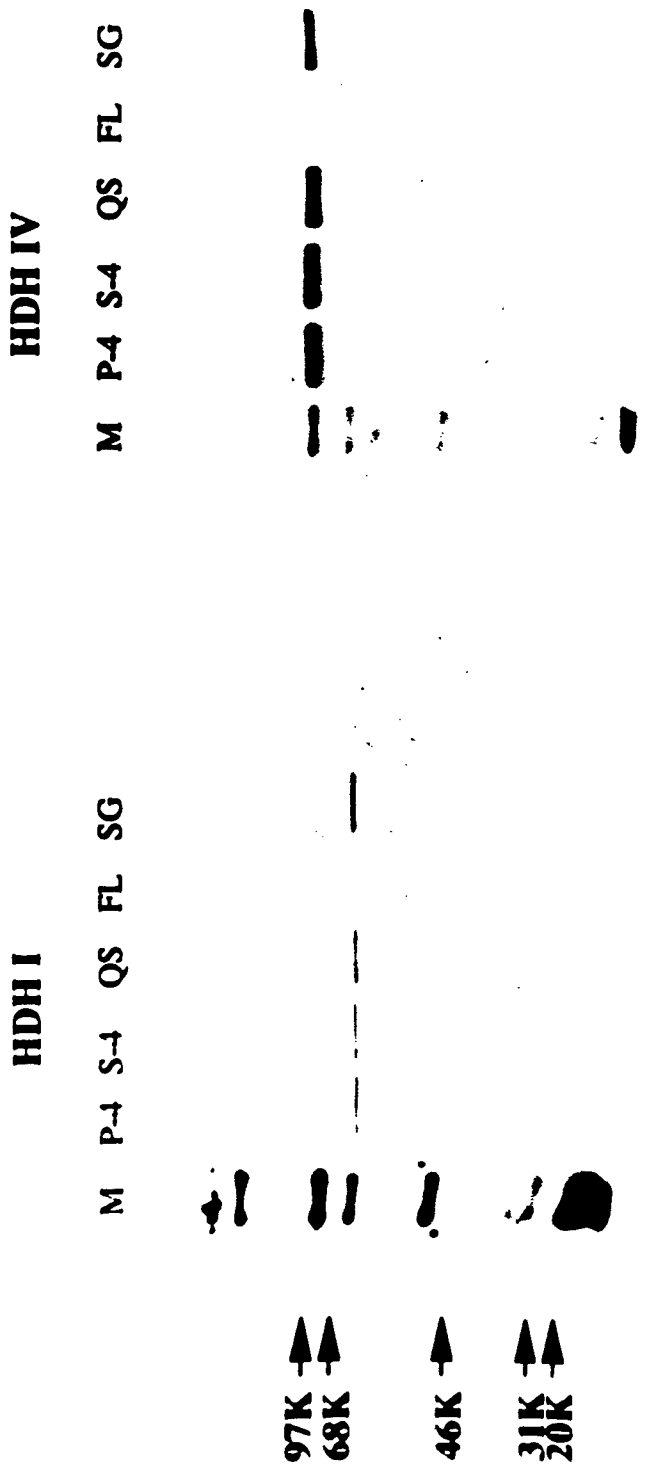

HDH IV
M
P-4
S-4
QS
FL
SG
HDH I
M
P-4
S-4
QS
FL
SG
97K
68K
46K
31K
20K

Figure 5

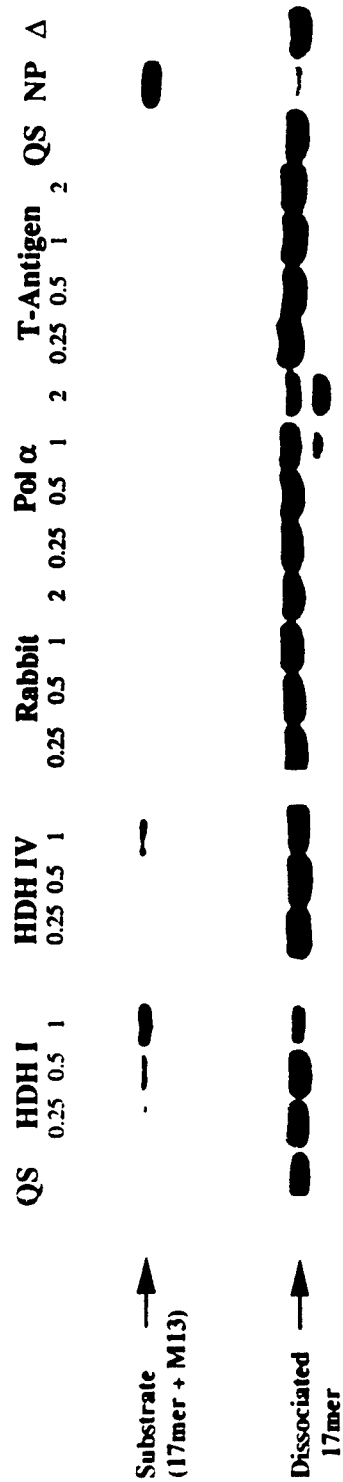

QS
HDH I
0.25 0.5 1
HDH IV
0.25 0.5 1
Rabbit
0.25 0.5 1 2
Pol α
0.25 0.5 1 2
T-Antigen
0.25 0.5 1 2
QS
NP Δ
Substrate
(17mer + M13)
Dissociated
17mer

Figure 6

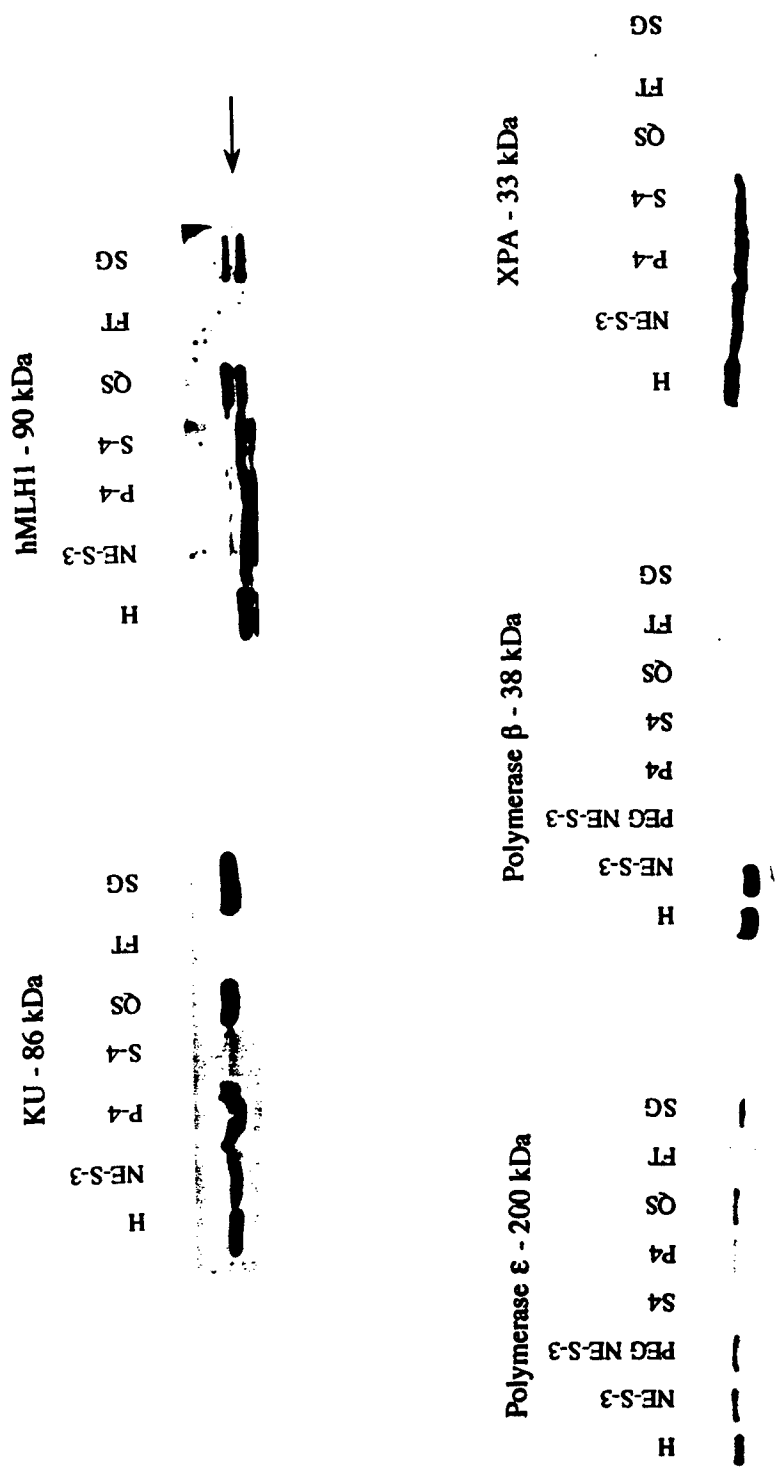

hMLH1 - 90 kDa
KU - 86 kDa
XPA - 33 kDa
Polymerase β - 38 kDa
Polymerase ε - 200 kDa
H
NE-S-3
P-4
S-4
QS
FT
SG

Figure 7

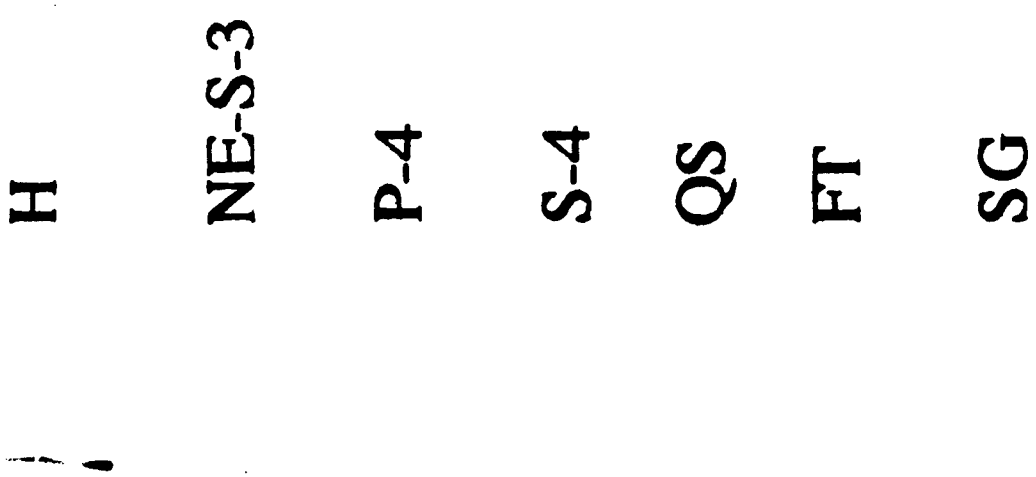


Figure 8