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Fidelity of DNA Replication in Normal and Malignant Human Breast Cells

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In order 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 completed a series of experiments utilizing the multiprotein DNA synthesize isolated from malignant (cell culture and human tumors) and non-malignant (cell culture, benign human breast disease tissue, and disease-free human breast tissue) breast cells. The DNA synthesize has been extensively demonstrated to carry out full length DNA replication in vitro, and to accurately depict the DNA replication process as it occurs in the intact cell. By examining the fidelity of the DNA replication process carried out by the DNA synthesize from a number of breast cell types, we have demonstrated for the first time, that the cellular DNA replication machinery of malignant human breast cells is significantly more error-prone than that of non-malignant human breast cells. To begin to explore the potential contribution of DNA mismatch repair processes to the observed decreased fidelity of the malignant breast cell synthesize, we have initiated studies to map the possible interactions of the DNA synthesize with several DNA repair proteins. To date, our data strongly indicate that a number of key DNA repair proteins are directly coupled to the breast cancer cell DNA synthesize.

Breast Cancer, fidelity, DNA replication, multiprotein complex, cell proliferation, DNA repair

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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 (1,2), 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 normal breast cells. Several studies have reported that the activity of DNA β polymerase, an enzyme implicated in gap filling DNA synthesis during DNA repair (18,19) 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, 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 normal and 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 synthesize) from both malignant and non-malignant breast cells and have begun to determine the ability of the DNA synthesize derived 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 synthesize isolated from mammalian cells is fully competent to carry out large T-antigen-dependent DNA synthesis in vitro (27, 28). The DNA synthesize 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 synthesize faithfully mimics the replication process carried out in intact cells. Using this multiprotein DNA replication complex we have carried out
experiments designed to determine whether breast cancer cells exhibit a higher mutation frequency due to a defect in the fidelity of the DNA synthetic process. To date, our data strongly indicate that the DNA synthesize isolated from malignant breast cells (both breast cell culture and surgically resected human breast tumors) carry out DNA replication which is significantly more error-prone than that carried out by the synthesize derived from non-malignant breast cells (both cell culture and non-malignant breast tissue). This highly exciting discovery has resulted in the publication of a rapid communication in the journal Cancer Research (August 1, 1998 issue).

After making the exciting observation that the DNA synthesize derived from malignant human breast cells mediates DNA replication with decreased fidelity, it is important to begin to consider the contribution of the interplay between the DNA replication and the DNA repair processes of the cell to the overall decreased fidelity. Malfunction of either process can result in the accumulation of errors in the DNA, and contribute an increasingly unstable genome. Since even the non-malignant DNA synthetic machinery was observed to incorporate a few errors in the DNA, it seems likely that the DNA repair and the DNA replication machinery work in close physical and temporal synchronicity to ensure that newly replicated DNA is properly proofread after replication. To begin to address this question we have initiated a series of experiments designed identify the structural and functional realtionships of a number of key DNA repair proteins with the breast cell DNA synthesize. To date, these experiments have yielded exciting data which suggest a direct interaction between the DNA synthesize and key DNA mismatch repair proteins. Experiments are ongoing to further characterize the association between the repair and replication proteins associated with the DNA synthesize 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(mcrB-hsdSMR-mrr) 173, endA1, supE44, thi 1, recA1, gyrA96, relA1, lac[F'proAB, lacIqZ (m15, Tn10(tetR)] 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 (34). 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 (34). 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 human breast adenocarcinoma. Cells from the plurale 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. It is maintained in Eagles' medium with non-essential amino acids, 1 mM sodium pyruvate, bovine insulin (10µg/ml), 90%, and fetal bovine serum, 10%.

The non-malignant MCF10A cell line is an immortalized cell line from human fibrocystic breast disease of a 34 year-old Caucasian patient. 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 ATCC.

Fractionation scheme for the isolation of breast cell DNA synthetase.

All breast cells from tissue culture were harvested and proteins fractions were prepared on ice or at 4°C following modified procedures of Malkas et al. (1990) (27) and Coll et al (1996)(36) (see also to figure 1 in the appendix). 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 fraction containing the replication-competent DNA synthesize is 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 Lamoth et al., 1981 (54) and Vishwanatha et al., 1986 (55). One unit of polymerase α activity is equivalent to the incorporation of 1nmol of [3H]-TMP into DNA per hour per mg protein at 37°C. The polymerase δ activity was measured according to procedures previously described in Malkas et al, 1990 (27).

**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 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 figure 6 been made to convert the percentage mutant colonies to a number able to reflect the average number of nucleotide errors in the replicated plasmid (39).

**Determination of the types of nucleotide errors in the DNA replicated in vitro.** Two flanking primers of 25 bp each 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.

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting Analyses**

Twenty micrograms of sucrose gradient fractions were loaded per lane of the gels, and after resolution by SDS-PAGE (Laemmli, 1970) the resolved proteins were electrophoretically 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, hPMS1, hPMS2, and hMSH6 (GTBP) were used at a dilution of 1μg/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-Ku80 was used at a dilution of 1:1000. The anti-PCNA (Oncogene Science) was used at a dilution of 1:1000, while the anti-polo, a gift from Matritech, Inc., was used at a dilution of 1:50. The anti-XPA (Santa Cruz Biotechnology) and anti-RNA polymerase (Santa Cruz Biotechnology) antibodies were both used at concentration of 1:1000. 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-32P]dTTP (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 sucrose gradient purified DNA synthesome was incubated with 1.9fmol 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. 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-immunoprecipitation Analyses**

One hundred micrograms of the sucrose gradient (peak activity) fraction was subjected to co-immunoprecipitations according to a modified procedure of the protocol described in Coll et.al. (60). 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), polymerase δ 20μg/reaction. Eighty 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 4,000 rpm for 5 minutes at 4°C, and the supernatant and pellet fractions were resolved on a 8% or 12% SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with antibodies directed against PCNA, polymerase δ, polymerase α, hMSH2, hMLH1, hPMS1, hPMS2, GTBP (hMSH6), MYH, and Ku 80 as described above.
Results

I. Results of Task 1: Preparation of the replication template DNA, months 1-12.
Since submitting the original proposal, in which we described 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 $2 \times 10^{-4}$. In order to minimize the background mutation frequency rate we will use the pBK-CMV plasmid (from Stratagene), for which the detectable mutation rate has been observed to be less than $1 \times 10^{10}$ 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, 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 (also from 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) (35,36), from mouse mammary cells (FM3A) (28) and more 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). Additionally we have successfully isolated the DNA synthesome from malignant and non-malignant breast tissue as well as from normal human breast reduction tissue and normal primary breast cell culture.

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 (36, 39), 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 I (A-1), and poly(ADP)-ribose polymerase (PARP) (28,35,36). 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 1-24.

DNA replication products have been isolated and purified from Hs578Bst and Hs578T, MCF10A, MCF7, and breast tissue DNA synthesome-mediated in vitro DNA replication assays. The purified DNA product has 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 all four cell type DNA synthesomes are capable of producing a full length DNA replication product. 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. Interestingly, the fold T-antigen dependent replication activity carried out by the DNA synthesome derived from malignant breast cells is not significantly different than that carried out by the DNA synthesome isolated non-malignant breast cells. (see figure 5 in the appendix)(39).

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

The XL1-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%). Using a derivation based in the work of Roberts and Kunkel (see figure 6 in the appendix) the number of mutant colonies per total number of colonies was converted to a number estimating the mutant frequency produced by the DNA synthesome (see figure 6 in the appendix).

Three separate experiments have been completed for each type of breast cell DNA synthesome, under our empirically determined optimal conditions, all of which suggest that the frequency of replication errors created by the malignant breast cell DNA synthesome (from either malignant breast cell culture or from malignant breast tumors) is significantly higher than that created by the DNA synthesome derived from non-malignant breast cells (including non-malignant breast cell culture, non-malignant breast biopsy tissue, benign breast disease biopsy tissue, normal breast reduction tissue, and normal primary breast cell culture). For example, the DNA synthesome derived from the Hs578T (malignant) breast cell line demonstrated a mutation frequency of approximately $8.65 \times 10^{-5}$ nucleotides, a mutation frequency 5.7 fold higher than that created by its genetically matched counterpart Hs578Bst (Figure 6 in the appendix)(39).
The DNA synthetosome derived from the malignant breast tumors also displayed elevated error-frequencies (1.92-3.72 fold). The less dramatic fold difference (than the cell culture results) as well as the high individual variability between samples is due to: 1) the intercellular heterogeneity of the resected tumor samples, and 2) the individual variability between the genetically matched human tumor samples.

A secondary and totally unexpected observation was also made in this study. The DNA synthetosome derived from samples of benign breast disease demonstrated a slightly higher DNA replication fidelity (lower mutation frequency) \((1.7 \times 10^{-4} \text{ to } 4.4 \times 10^{-4} \text{ nucleotides})\) than that derived from the samples of normal breast reduction tissue and normal primary breast cell culture (mutation frequency of \(6.4 \times 10^{-4} \text{ to } 8.7 \times 10^{-4} \text{ nucleotides})\). Perhaps the higher DNA replication fidelity of the DNA synthetosome from these benign breast disease samples has permitted the cells to proliferate in a hyperplastic manner with out developing a malignant phenotype. Experiments to more clearly define this observation are ongoing.

Mutant and wildtype colonies were collected from all of the DNA replication fidelity experiments and were 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°C 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 three to four mutant and three to four normal plasmid extracted from bacteria transfected with the DNA pBK-CMV replicated by the each of the types of DNA synthetosome examined. We have utilized the automated sequencing (UMAB sequencing facility) to sequence the β-galactosidase gene (Lac Zα). Using flanking primers (27 bp each), we were able to identify the types of errors which occur as a result of the DNA synthetosome-mediated DNA replication. Interestingly, while the total number of errors created by the malignant and non-malignant breast cell DNA synthetosomes differed, the frequency of the types of errors did not significantly differ (see figure 7 in the appendix)(39).

VI. Results of additional experiments: Months 18-48.

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

As shown in figure 9 in the appendix the DNA repair proteins hMSH2, hMLH1, hPMS1, hPMS2, GTBP (hMSH6), MYH, and Ku80 are detected exclusively in the sucrose gradient fractions #4-6. Excitingly, 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 (figure 8 in the appendix).

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. Thus, DNA repair proteins directly associated with the DNA synthesome may make a significant contribution toward the overall fidelity of the DNA replication machinery.

Co-immunoprecipitation Analyses

Co-immunoprecipitation analyses were able to identify the close physical interaction of various DNA replication and DNA repair proteins with those proteins directly bound by the antibodies directed against DNA polymerase δ (figure 10 A-I), PCNA (figure 11 A-J), and hMSH2 (figure 12 A-F)(58). The results of the co-immunoprecipitation reactions are summarized in the table in figure 13 in the appendix. Briefly, precipitating antibodies directed against PCNA and hMSH2 were able to pull down from the purified DNA synthesome the DNA polymerases α and δ, as well as PCNA, hMSH2, hMSH6, hMLH1, hPMS1, hPMS2, Ku80 and MYH. Precipitating antibody DNA polymerase δ was able to pull down all of the DNA polymerases, and all of the DNA repair proteins mentioned above with the exception of the repair protein hMSH2 (58).

Specific Binding to Mispaired and IDL containing DNA templates

As shown in figure 14A-D in the appendix, reactions which allowed the human breast cancer cell DNA synthesome (MCF7) to bind a radiolabelled DNA template containing either an insertion-deletion loop of 2 nucleotides (fig. A), an insertion-loop of 4 nucleotides (fig. B), a G/T mismatch (fig. C), or an 8-oxo-guanine mispair (i.e. A/GO) (fig. D) had high specific binding activity as they were not able to be competed away by high concentrations (up to 900 fold) of unlabeled homopolymer DNA (containing no errors) (see figure 15 in the appendix). 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 (any of the four types of mismatches examined)(58). Studies comparing the relative binding affinities of the malignant and non-malignant breast cell DNA synthesome for the G/T, IDL₂ and IDL₄ are ongoing.

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 $2.5 \times 10^{-4}$. 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 $1 \times 10^{-10}$ (Sekowski and Hickey, 1997, unpublished data).

While this aspect of the project was being developed, our laboratory group isolated the multiprotein DNA replication complex 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 lines suggests a direct 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 observed the level of DNA replication to be between 5-9 times higher in the replication reactions mediated by the breast cancer cell DNA synthesome than in those reactions mediated by the non-malignant breast cell DNA synthesome. The apparent increased rate of replication carried out by the breast cancer cell DNA synthesome correlates with increased (3-5.5 times) frequency of mutant (white) colonies observed in the bacteria transfected with the plasmid replicated by the breast cancer cell DNA synthesome than in the bacteria transfected with plasmid replicated by non-malignant breast cell DNA synthesome.

We have also examined 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 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. Specifically, the DNA synthesome from the cell lines appears to serve as
good representative model for the DNA synthesome isolated from genetically matched malignant and non-malignant human breast tissue.

An unexpected observation made in this study was that the DNA synthesome derived from samples of benign breast disease demonstrated a slightly higher DNA replication fidelity (lower mutation frequency) (1.7 x 10^{-4} to 4.4 x 10^{-4} nucleotides) than that derived from the samples of normal breast reduction tissue and normal primary breast cell culture (mutation frequency of 6.4 x 10^{-4} to 8.7 x 10^{-4} nucleotides). Perhaps the higher DNA replication fidelity of the DNA synthesome from these benign breast disease samples has permitted the cells to proliferate in a hyperplastic manner with out developing a malignant phenotype. Experiments to more clearly define this observation are ongoing.

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 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.

As a direct result of the discoveries made during the course of this project, experiments designed to address the question of altered components in the malignant breast cell DNA synthesome have been designed and initiated in our laboratory. In fact a very exciting discovery has been made by my colleague and fellow pre-doctoral U.S. Army breast cancer fellowship recipient, Dr. Pamela Bechtle. She has discovered a unique form of the protein PCNA found exclusively in malignant human breast cells. This altered form of PCNA was not detectable in any of the non-malignant breast cells examined. Interestingly, the altered isoelectric point observed of the protein is not due to any mutational change in the PCNA gene, but is due to a change in the post-translational profile of the protein. The results of her work will be published (57) back to back with the article describing my pre-doctoral work (39) in the August 1 issue of the journal Cancer Research.

To date, the results from my studies 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


Figure 1

Normal and Malignant Breast Cell Homogenates

Nuclear Pellet
- Low salt extraction
  - Nuclear Extract (NE)
  - Pool NE-S-3

Make 2M in KCl
Add PEG to 5%
Collect the PEG Supernatant (PEG NE/S-3)
Overlay onto 2M sucrose cushion
Centrifugation at 100,000 x g, 16 hrs

Sucrose Interphase (P-4)

Q-Sepharose

Sucrose Gradient

Cytosol Extract (S-1)
- Differential centrifugation
  - S-3
FIGURE 2

Termination and Methylation Components

Topo II

DTM

hMLH1
hMSH 2
hMSH 6
hpMS1

Mismatch Repair Components

hPMS2
hMYH
hMSH 3

Replication Components

PARP
DNA ligase I
Ku80
Primase
RFC
Pol α
Pol ε
Pol δ
PCNA
Helicases I and IV

Initiation Components

Topo I

RPA

3' 5'

3' 5'
### FIGURE 5

<table>
<thead>
<tr>
<th>Source of DNA synthesis</th>
<th>Units of T-antigen&lt;sup&gt;d&lt;/sup&gt; dependent DNA replication activity&lt;sup&gt;e&lt;/sup&gt; (x10^-2)</th>
<th>Fold T-antigen&lt;sup&gt;d&lt;/sup&gt; dependent replication activity&lt;sup&gt;f&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>MALIGNANT human breast cells</td>
<td></td>
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<tr>
<td>a tumor A (IDC)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5</td>
<td>0.8</td>
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<td>a tumor B (IDC)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1.3</td>
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<tr>
<td>a tumor C (ILC)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.5</td>
<td>2.0</td>
</tr>
<tr>
<td>a tumor D (ILC)&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1.3</td>
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<td>NON-MALIGNANT human breast cells</td>
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<td>a tissue A</td>
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<td>1.0</td>
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<td>a tissue B</td>
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<td>a tissue C</td>
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<td>a tissue D</td>
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</tr>
<tr>
<td>average</td>
<td>11.7</td>
<td>1.0</td>
</tr>
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</table>

**Legend for Table**

<sup>a</sup> Surgically resected female human breast tissue. Genetically matched samples are denoted by corresponding alphanumerical designations (tumor A, tissue A, etc.) Factors such as stage of malignancy, genetics, race, age, were double blind during data collection.

<sup>b</sup> IDC= Infiltrating ductal carcinoma as determined by pathological diagnosis of tumor tissue.

<sup>c</sup> ILC= Infiltrating lobular carcinoma determined as above.

<sup>d</sup> T-Ag = SV40 large T-antigen

<sup>e</sup> In vitro DNA replication activity assays were performed as described previously. Units represent the amount of T-antigen dependent DNA replication (+(+)T-Ag values minus (–)T-Ag values). One unit equals one picomole nascent DNA synthesized per hour per microgram synthesome protein. The values represent the average of two independent experiments. Replication values deviated by less than 3% from the average.

<sup>f</sup> Fold DNA replication activity was calculated by dividing the units of replication observed for the malignant breast cell DNA synthesome by the replication units observed for the DNA synthesome isolated from the genetically matched non-malignant breast cells. Each value represents the average of at least 2 experiments. Replication values deviated by less than 3% from the average.
### Table - DNA replication fidelity of the malignant and non-malignant breast synthesome.

<table>
<thead>
<tr>
<th>Source of DNA synthesome</th>
<th>total number of colonies scored</th>
<th>number of mutant colonies</th>
<th>mutant frequency (x 10^-5 nucleotides)</th>
<th>fold mutation frequency</th>
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</thead>
<tbody>
<tr>
<td><strong>MALIGNANT human breast cells</strong></td>
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</tr>
<tr>
<td>MCF 7</td>
<td>6.0 x 10^4</td>
<td>576</td>
<td>5.15</td>
<td>4.4</td>
</tr>
<tr>
<td>Hs578T</td>
<td>6.0 x 10^4</td>
<td>960</td>
<td>8.65</td>
<td>5.7</td>
</tr>
<tr>
<td>MDA-MB468</td>
<td>6.0 x 10^4</td>
<td>762</td>
<td>6.81</td>
<td>N/A</td>
</tr>
<tr>
<td>* tumor A (IDC h) (ER-, PR-, Ki-67 24% (high), HER-2/neu 52% (high), p53 positive)</td>
<td>3.0 x 10^4</td>
<td>141</td>
<td>2.52</td>
<td>3.6</td>
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<tr>
<td>* tumor B (IDC h) (ER+, PR+, diploid, 6% S-phase)</td>
<td>3.0 x 10^4</td>
<td>209</td>
<td>3.72</td>
<td>3.8</td>
</tr>
<tr>
<td>* tumor C (ILC h) (ER+ high, PR-, unknown ploidy, Ki-67 2% (low), HER-2/neu 42% (high), p53 negative)</td>
<td>3.4 x 10^4</td>
<td>122</td>
<td>1.92</td>
<td>2.4</td>
</tr>
<tr>
<td>* tumor D (ILC h) (ER+ low, PR+ high, diploid, S-phase 7.2% (high))</td>
<td>3.0 x 10^4</td>
<td>130</td>
<td>2.35</td>
<td>4.4</td>
</tr>
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<td><strong>NON-MALIGNANT human breast cells</strong></td>
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<tr>
<td>MCF 10A</td>
<td>4.0 x 10^4</td>
<td>66</td>
<td>1.18</td>
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<td>Hs578Bst</td>
<td>4.0 x 10^4</td>
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<td>* tissue A</td>
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<td>1.0 x 10^4</td>
<td>10</td>
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<td><strong>Benign breast pathology</strong></td>
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<td>juvenile fibroadenoma</td>
<td>4.0 x 10^4</td>
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<td>fibroadenoma</td>
<td>4.0 x 10^4</td>
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<td>benign phyllodes tumor</td>
<td>4.0 x 10^4</td>
<td>33</td>
<td>0.44</td>
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<td>ductal epithelial hyperplasia w/o atypia</td>
<td>4.6 x 10^4</td>
<td>17</td>
<td>0.35</td>
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<td><strong>NORMAL breast cells</strong></td>
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<tr>
<td>normal 1</td>
<td>1.0 x 10^4</td>
<td>12</td>
<td>0.64</td>
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<td>normal 2</td>
<td>2.0 x 10^4</td>
<td>22</td>
<td>0.59</td>
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<tr>
<td>primary breast cell culture 1</td>
<td>4.0 x 10^4</td>
<td>65</td>
<td>0.87</td>
<td>N/A</td>
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</tbody>
</table>
Legend for Table

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

b IDC= Infiltrating ductal carcinoma as determined by pathological diagnosis of tumor tissue.

c ILC= Infiltrating lobular carcinoma determined as above.

d Surgically resected breast reduction tissue from healthy females used to derived synthesome from frozen sample (tissue A) or from primary cultures (primary culture sample).

e 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 frequency (no mutations detected in $5 \times 10^{-8}$ colonies) divided by the chance of a nucleotide defect within the lacZ alpha gene if the colony expresses a white phenotype (0.5) divided by the number of sites in the target gene (373 bp). The lacZ alpha gene comprises 8.25% of the total pBK-CMV plasmid (4518bp). Each value reported in the table represents the average of at least 3 individual experiments, and the values did not deviate from the average by more than 5%.

f Values represent the fold increase in mutation frequency of the malignant cell synthesome as compared to its genetically matched non-malignant cell counterpart.

g Though not a genetically matched cell line, the fold mutation for the MCF7 cell derived synthesome was calculated using the mutation frequency measured for MCF10A cells. All other fold mutation calculations were made between genetically matched cell lines.

N/A= no genetically matched counterpart available.
Table 3- Types of Replication Errors Produced by the Breast Cell DNA Synthetase

<table>
<thead>
<tr>
<th>Breast Cell Type (source of DNA synthetase)</th>
<th>Nucleotide Insertions</th>
<th>Nucleotide Deletions</th>
<th>Nucleotide Mismatches</th>
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<tr>
<td>MALIGNANT</td>
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<tr>
<td>MCF7 (n=3)</td>
<td>20</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>Hs578T (n=4)</td>
<td>16</td>
<td>5</td>
<td>79</td>
</tr>
<tr>
<td>Malignant tumor tissue (n=3)</td>
<td>20</td>
<td>14</td>
<td>66</td>
</tr>
<tr>
<td>Average</td>
<td>18% (+/- 2%)</td>
<td>9% (+/- 5%)</td>
<td>73% (+/- 7%)</td>
</tr>
<tr>
<td>NON-MALIGNANT</td>
<td></td>
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<tr>
<td>MCF10A (n=3)</td>
<td>13</td>
<td>6</td>
<td>81</td>
</tr>
<tr>
<td>Hs578Bst (n=4)</td>
<td>23</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>Non-malignant tissue (n=3)</td>
<td>8</td>
<td>4</td>
<td>88</td>
</tr>
<tr>
<td>Average</td>
<td>16% (+/- 7%)</td>
<td>9% (+/- 5%)</td>
<td>75% (+/- 12%)</td>
</tr>
</tbody>
</table>

Legend for Table:

* Percentages were derived from the numbers of nucleotide errors observed in the double-stranded nucleotide sequences of the replicated plasmid isolated from individual mutant bacterial transformant colonies produced using separate forward mutagenesis assays.

* All replication induced errors were measured by comparing double stranded nucleotide sequences of the lacZα gene of the replicated pBK-CMV template isolated from a mutant (white phenotype) bacterial transformant with that of a wildtype lacZα sequence. Mutant transformant colonies were selected from 3-4 individual forward mutagenesis assays. The sequences from normal transformant colonies (n=7) (blue wildtype phenotype) isolated from forward mutagenesis assays mediated by MCF7, MCF10A, Hs578Bst, Hs578T, resected breast tumor and non-malignant tissue derived DNA synthetase (data not shown) were checked against the published wildtype sequence for lacZα and were not found to contain any mutations.

* The averages reported were calculated to reflect the number of independent analyses of each cell line sampled, and each tumor sampled. The number of each type of error produced during the analysis of each tumor sample (n = 4) was summed and the average calculated or tissue tumor samples analyzed for each category of nucleotide sequence error.
FIGURE 8

DNA Synthesome Associated DNA Polymerase α and δ Activities and SV40 in vitro DNA Replication Co-purify through a Continuous Sucrose Gradient

A. Polymerase Alpha Activity

B. Polymerase Delta Activity

C. SV40 in vitro DNA Replication Activity
FIGURE 9

DNA Mismatch Repair and Replication Proteins Exclusively Co-Purify
with those Sucrose Gradient Fractions Containing Peak Replication and Polymerase
Activities of the DNA Synthesome

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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 10

DNA replication and mismatch repair proteins co-precipitate from the DNA synthesome with monoclonal antibody directed against DNA polymerase δ

A. Polymerase δ (125 kDa) *

large IgG fragment (50 kDa) -

B. large IgG fragment (50 kDa) -

PCNA (36 kDa) *

small IgG fragment (25 kDa) -

C. Polymerase α (180 kDa) *

large IgG fragment (50 kDa) -

D. hMLH1 (100 kDa) *

large IgG fragment (50 kDa) -
E. PMS1 (98 kDa) *
   large IgG fragment (50 kDa) -

F. PMS2 (98 kDa) *
   large IgG fragment (50 kDa) -
   small IgG fragment (25 kDa) -

G. GTBP (160 kDa) *
   large IgG fragment (50 kDa) -

H. Ku80 (80 kDa) *
   large IgG fragment (50 kDa) -
I.

MYH (63 kDa) *

large IgG fragment (50 kDa) -
FIGURE 11

DNA replication and DNA mismatch repair proteins co-precipitate from the purified DNA synthesome with monoclonal antibody directed against PCNA.

<table>
<thead>
<tr>
<th>Control</th>
<th>Supernatant</th>
<th>Co-Im pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

A. large IgG fragment (50 kDa) -

PCNA (36 kDa) *

small IgG fragment (25 kDa) -

B. Polymerase δ (125 kDa) *

large IgG fragment (50 kDa) -

C. Polymerase α (180 kDa) *

large IgG fragment (50 kDa) -

D. hMLH1(100 kDa) *

large IgG fragment (50 kDa) -
E. hMSH2 (107 kDa) *
   large IgG fragment (50 kDa) -
   small IgG fragment (25 kDa) -

F. hMSH6 (160 kDa) *
   large IgG fragment (50 kDa) -

G. hPMS1 (98 kDa) *
   large IgG fragment (50 kDa) -

H. hPMS2 (98 kDa) *
   large IgG fragment (50 kDa) -
I. hMYH (63 kDa) *
   large IgG fragment (50 kDa) -
   small IgG fragment (25 kDa) -

J. Ku80 (80 kDa) *
   large IgG fragment (50 kDa) -
   small IgG fragment (25 kDa) -
FIGURE 12

DNA replication and DNA mismatch repair proteins co-precipitate from the purified DNA synthesome with polyclonal antibody directed against hMSH2

<table>
<thead>
<tr>
<th>Control</th>
<th>Supernatant</th>
<th>Co-IP pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

A. hMSH2 (107 kDa) *
   large IgG fragment (50 kDa) -

B. large IgG fragment (50 kDa) -
   PCNA (36 kDa) *
   small IgG fragment (25 kDa) -

C. Polymerase α (180 kDa) *
   large IgG fragment (50 kDa) -

D. hMSH6 (GTBP) * (160 kDa)
   large IgG fragment (50 kDa) -
E. hPMS 2 (98 kDa) *
   large IgG fragment (50 kDa) -
   small IgG fragment (25 kDa) -

F. MYH (63 kDa) *
   large IgG fragment (50 kDa) -
FIGURE 13

TABLE 4 Summary of Co-immunoprecipitation Data

Proteins Co-immunoprecipitated

<table>
<thead>
<tr>
<th>Precipitating Antibody</th>
<th>DNA Pol α</th>
<th>DNA Pol δ</th>
<th>PCNA</th>
<th>hMSH2</th>
<th>hMSH6</th>
<th>hMLH1</th>
<th>hPMS1</th>
<th>hPMS2</th>
<th>Ku80</th>
<th>MYH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Pol δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hMSH2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+L</td>
<td>+L</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+L = As suggested by Acharya et al., 1996; and Unar et al., 1996
DNA synthesome specifically recognizes and binds to DNA templates containing an IDL2, IDL4, or a G/T or A/8-oxoguanine (A/GO) mismatch

**FIGURE A - IDL2**

Fold Competitor IDL2 DNA

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
<th>600</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Bound IDL2

Free IDL2

**FIGURE B - IDL4**

Fold Competitor IDL4 DNA

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
<th>600</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Bound IDL4

Free IDL4
FIGURE C-G/T

Fold Competitor G/T DNA

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
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<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Bound G/T
Free G/T

FIGURE D-A/GO

Fold Competitor A/GO DNA

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
<th>600</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

Bound A/GO
Free A/GO
FIGURE DNA synthesome has a higher affinity for a DNA template containing a mismatch (IDL2, IDL4, G/T, or A/GO) than for a perfectly matched DNA template.

<table>
<thead>
<tr>
<th>Fold Competitor</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
<th>600</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation-Free DNA</td>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Bound single mismatch or IDL DNA template

Free single mismatch or IDL DNA template
Addendum I

Principal Investigator: Jennifer Weeks Sekowski, Ph.D.                      Grant# DAMD17-94-J-4151

PUBLICATIONS:


PRESENTATIONS:


I, Jennifer Weeks Sekowski, was awarded a Ph.D. on May 22, 1998. My graduate education was funded by this U.S. Army Breast Cancer Research Program Fellowship.
List of Personnel Funded by this Fellowship

Jennifer Weeks Sekowski          Ph.D. candidate

Addendum III

100% effort