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GRANT NUMBER DAMD17-94-J-4151

TITLE: Fidelity of DNA Replication in Normal and Malignant Human
Breast Cells

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REPORT DATE: July 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1998	3. REPORT TYPE AND DATES COVERED Final (1 Aug 94 - 31 Jul 98)	
4. TITLE AND SUBTITLE Fidelity of DNA Replication in Normal and Malignant Human Breast Cells			5. FUNDING NUMBERS DAMD17-94-J-4151	
6. AUTHOR(S) Jennifer Weeks Sekowski, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Maryland School of Medicine Baltimore, Maryland 21201			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
<div style="font-size: 2em; font-weight: bold; margin: 0;">19990511 082</div>				
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>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 synthesome 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 synthesome has been extensively demonstrated to carry out full length DNA replication <i>in vitro</i>, 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 synthesome 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 synthesome, we have initiated studies to map the possible interactions of the DNA synthesome 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 synthesome.</p>				
14. SUBJECT TERMS Breast Cancer, fidelity, DNA replication, multiprotein complex, cell proliferation DNA repair			15. NUMBER OF PAGES 44	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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RJ W. Sekowski, Ph.D. 7/31/98
RJ - Signature Date

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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 synthesome) from both malignant and non-malignant breast cells and have begun to determine the ability of the DNA synthesome 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 synthesome isolated from mammalian cells is fully competent to carry out large T-antigen-dependent DNA synthesis *in vitro* (27, 28). 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 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 synthesome 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 synthesome 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 synthesome 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 relationships of a number of key DNA repair proteins with the breast cell DNA synthesome. 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 characterize the association between the repair and replication 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 lac^P 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^aZ (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 (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 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. 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 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 the ATCC.

Fractionation scheme for the isolation of breast cell DNA synthesome.

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 synthesome 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 and Lamothe 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 μ l 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 lacZ α 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 lacZ α 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 1 μ g/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-pol α , 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 Mismatched and IDL containing DNA templates

Oligonucleotides of 40 base pairs containing a single G/T mismatch, 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]dCTP (50 μ Ci at 3,000Ci/mmol), 20 μ M dTTP, 20 μ M dATP, and 20 μ M 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 μ g/reaction), PCNA (mAb-10, 2.5 μ g/reaction), polymerase δ 20 μ g/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 I : 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 $2-5 \times 10^{-4}$. 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 1×10^{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 1 (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 [³²P]- 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 XLI-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 8.65×10^{-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 synthesize 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 synthesize derived from samples of benign breast disease demonstrated a slightly higher DNA replication fidelity (lower mutation frequency)(1.7×10^{-4} to 4.4×10^{-4} nucleotides) than that derived from the samples of normal breast reduction tissue and normal primary breast cell culture (mutation frequency of 6.4×10^{-4} to 8.7×10^{-4} nucleotides). Perhaps the higher DNA replication fidelity of the DNA synthesize 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 lacZ α 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 Z α) 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 synthesize examined. We have utilized the automated sequencing (UMAB sequencing facility) to sequence the β -galactosidase gene (Lac Z α). Using flanking primers (27 bp each), we were able to identify the types of errors which occur as a result of the DNA synthesize-mediated DNA replication. Interestingly, while the total number of errors created by the malignant and non-malignant breast cell DNA synthesizes 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 synthesize 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 both 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 Mismatched 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 1×10^{-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 from 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)(1.7×10^{-4} to 4.4×10^{-4} nucleotides) than that derived from the samples of normal breast reduction tissue and normal primary breast cell culture (mutation frequency of 6.4×10^{-4} to 8.7×10^{-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 Bechtel. 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.

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FIGURE 1

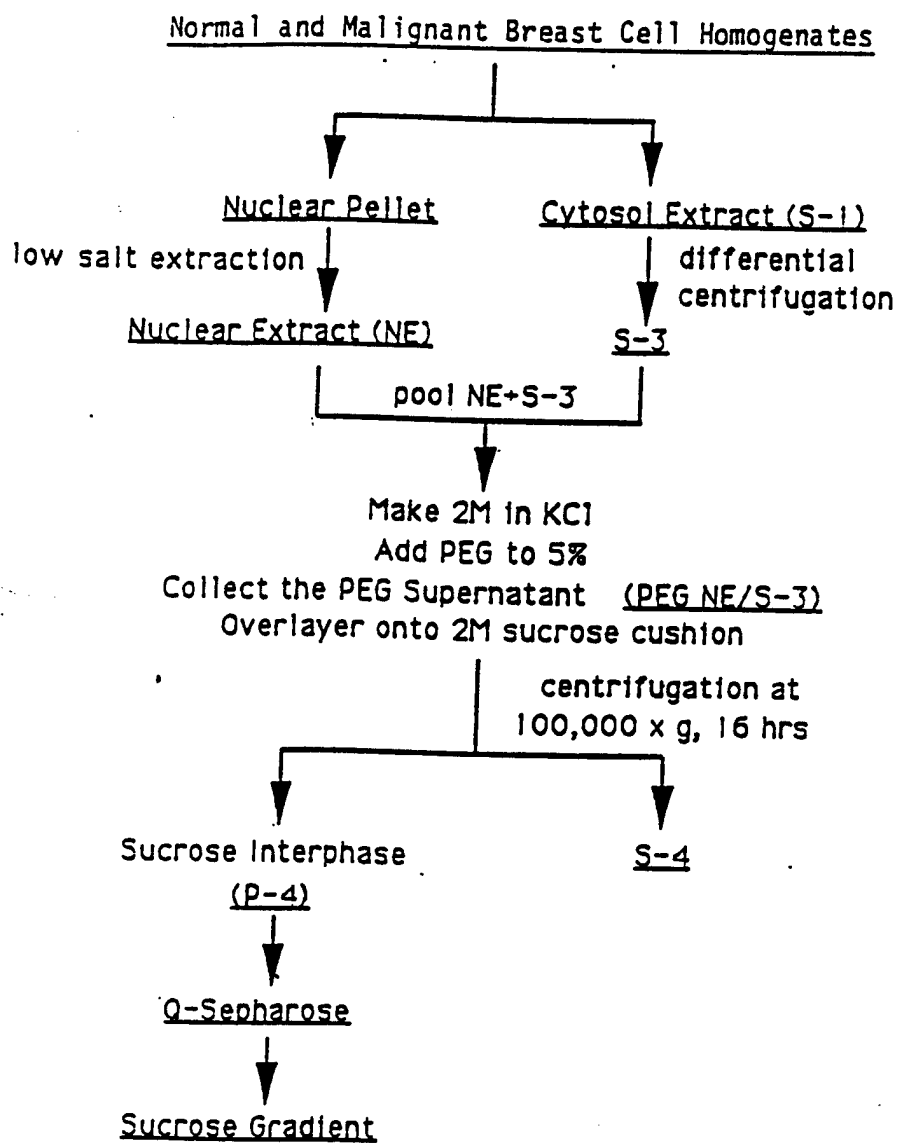


FIGURE 2

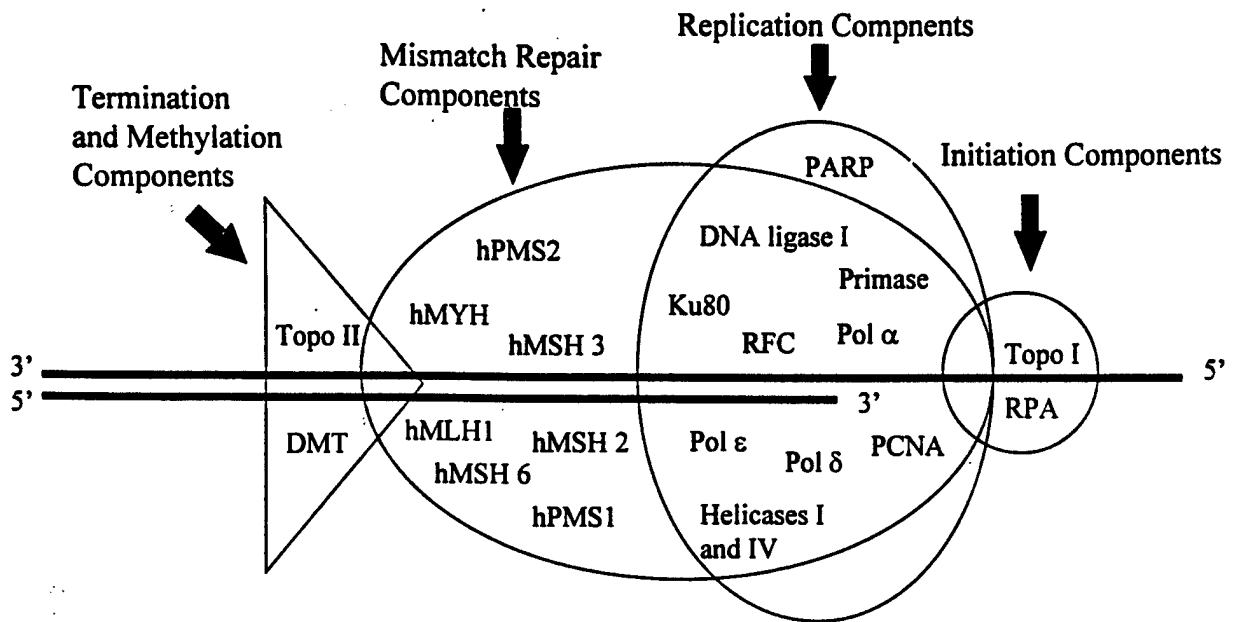


FIGURE 3

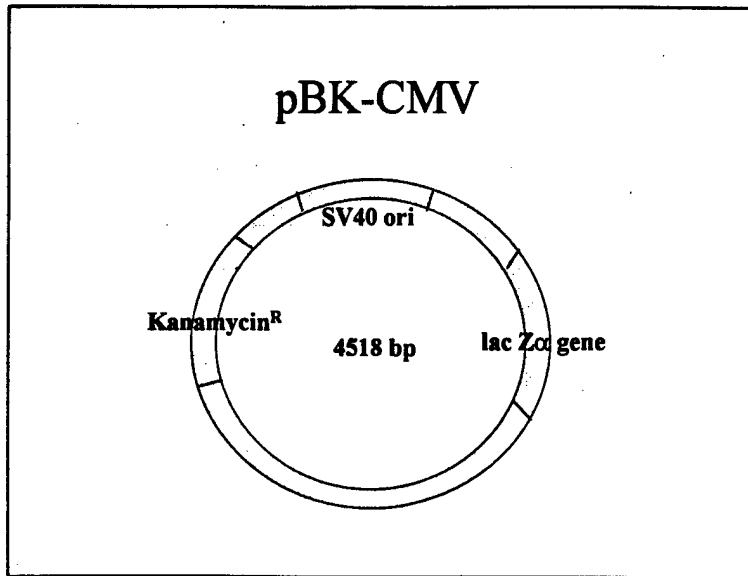


FIGURE 4

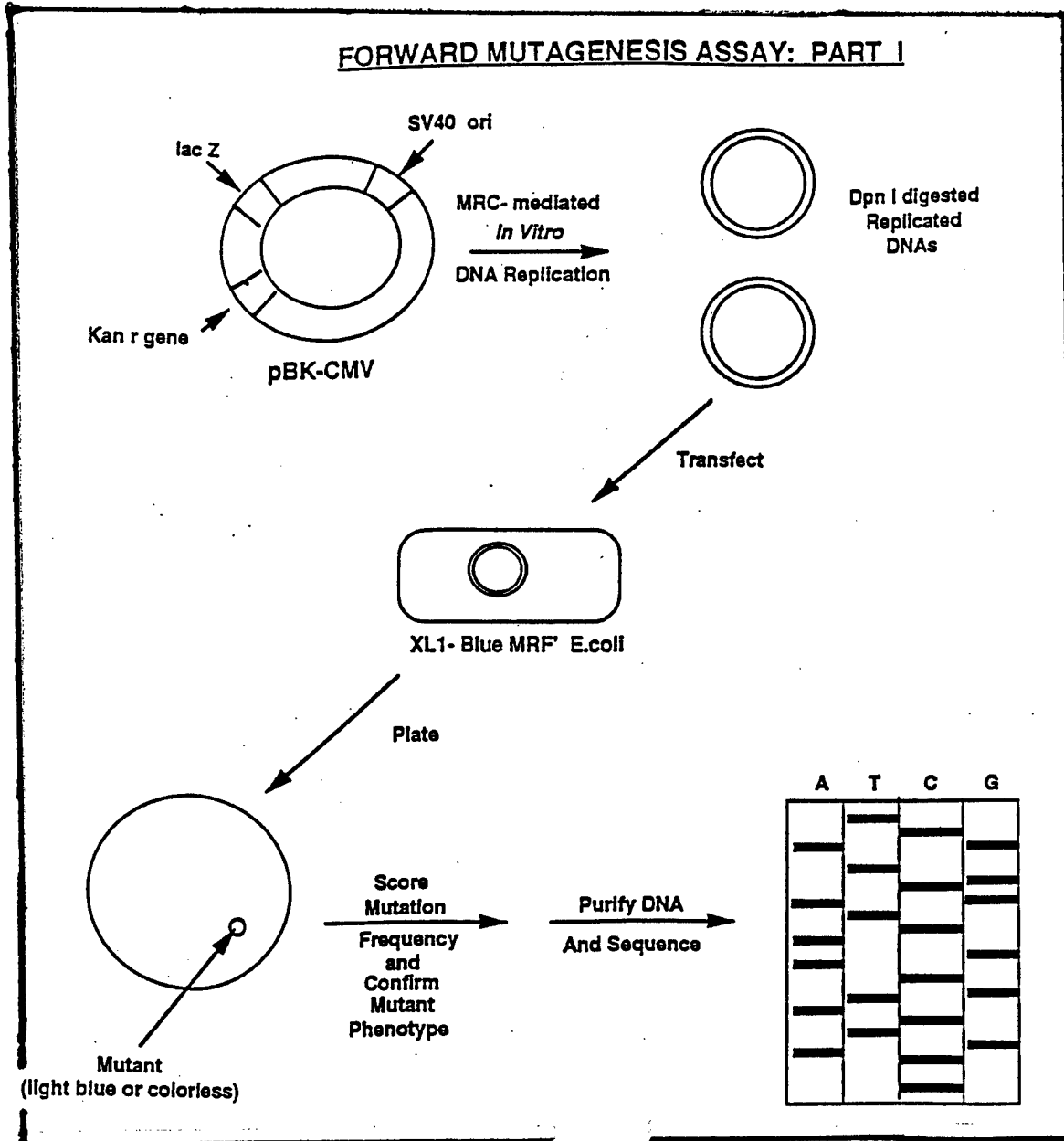


FIGURE 5

Table - DNA replication activity of the genetically matched malignant and non-malignant breast DNA synthesome.

Source of DNA synthesome	Units of T-antigen ^d dependent DNA replication activity ^e (x10 ⁻²)	Fold T-antigen ^d dependent replication activity ^f
MALIGNANT human breast cells		
^a tumor A (IDC) ^b	11.5	0.8
^a tumor B (IDC) ^b	11	1.3
^a tumor C (ILC) ^c	23.5	2.0
^a tumor D (ILC) ^c	12.5	1.0
average	14.6	1.3
NON-MALIGNANT human breast cells		
^a tissue A	15.0	1.0
^a tissue B	8.7	1.0
^a tissue C	11.5	1.0
^a tissue D	11.5	1.0
average	11.7	1.0

Legend for Table

^aSurgically 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 during data collection.

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

^c ILC= Infiltrating lobular carcinoma determined as above.

^d T- Ag = SV40 large T-antigen

^e *In vitro* DNA replication activity assays were performed as described previously.² 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.

^f 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.

FIGURE 6

Table - DNA replication fidelity of the malignant and non-malignant breast synthesesome.

Source of DNA synthesesome	total number of colonies scored	number of mutant colonies	mutant frequency (x 10 ⁻⁵ nucleotides) ^e	fold mutation frequency ^f
MALIGNANT human breast cells				
MCF 7	6.0 x 10 ⁴	576	5.15	4.4
Hs578T	6.0 x 10 ⁴	960	8.65	5.7
MDA-MB468	6.0 x 10 ⁴	762	6.81	N/A ^g
^a tumor A (IDC ^b) (ER-, PR-, Ki-67 24% (high), HER-2/neu 52% (high), p53 positive)	3.0 x 10 ⁴	141	2.52	3.6
^a tumor B (IDC ^b) (ER+, PR+, diploid, 6% S-phase)	3.0 x 10 ⁴	209	3.72	3.8
^a tumor C (ILC ^c) (ER+ high, PR-, unknown ploidy, Ki-67 2% (low), HER-2/neu 42% (high), p53 negative)	3.4 x 10 ⁴	122	1.92	2.4
^a tumor D (ILC ^c) (ER+ low, PR+ high, diploid, S-phase 7.2% (high))	3.0 x 10 ⁴	130	2.35	4.4
NON-MALIGNANT human breast cells				
MCF 10A	4.0 x 10 ⁴	66	1.18	1
Hs578Bst	4.0 x 10 ⁴	113	1.50	1
^a tissue A	1.0 x 10 ⁴	13	0.70	1
^a tissue B	1.0 x 10 ⁴	18	0.96	1
^a tissue C	1.0 x 10 ⁴	15	0.80	1
^a tissue D	1.0 x 10 ⁴	10	0.54	1
Benign breast pathology				
juvenile fibroadcnoma	4.0 x 10 ⁴	13	0.17	N/A ^h
fibroadcnoma	4.0 x 10 ⁴	22	0.30	N/A ^h
benign phyllodes tumor	4.0 x 10 ⁴	33	0.44	N/A ^h
ductal epithelial hyperplasia w/o atypia	4.6 x 10 ⁴	17	0.35	N/A ^h
NORMAL breast cells^d				
normal 1	1.0 x 10 ⁴	12	0.64	N/A ^h
normal 2	2.0 x 10 ⁴	22	0.59	N/A ^h
primary breast cell culture 1	4.0 x 10 ⁴	65	0.87	N/A ^h

Legend for Table

^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 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 synthesize 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 5×10^8 colonies) divided by the chance of a nucleotide defect within 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 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 synthesize 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 synthesize was calculated using the mutation frequency measured for MCF10A cells. All other fold mutation calculations were made between genetically matched cell lines.

^h N/A= no genetically matched counterpart available.

FIGURE 7

Table 3- Types of Replication Errors Produced by the Breast Cell DNA Synthesome

Breast Cell Type (source of DNA synthesome)	Percentage ^b Replication Induced Errors ^a		
	Nucleotide Insertions	Nucleotide Deletions	Nucleotide Mismatches
MALIGNANT			
MCF7 (n=3)	20	10	70
Hs578T (n=4)	16	5	79
Malignant tumor tissue (n=3)	20	14	66
Average	18% (+/- 2%)	9% (+/- 5%)	73% (+/- 7%)
NON-MALIGNANT			
MCF10A (n=3)	13	6	81
Hs578Bst (n=4)	23	14	63
Non-malignant tissue (n=3)	8	4	88
Average	16% (+/- 7%)	9% (+/- 5%)	75% (+/- 12%)

Legend for Table :

^a 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.

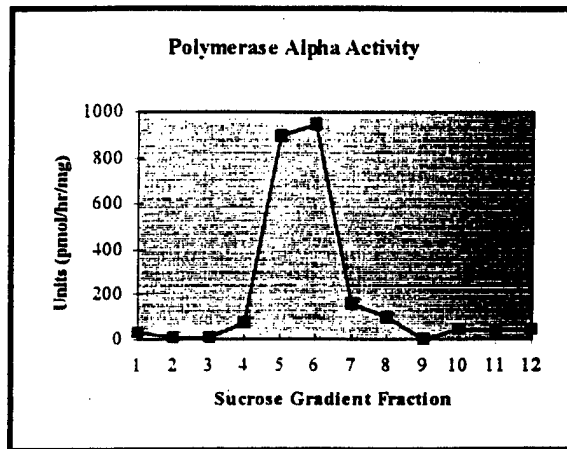
^b All replication induced errors were measured by comparing double stranded nucleotide sequences of the lacZ α gene of the replicated pBK-CMV template isolated from a mutant (white phenotype) bacterial transformant with that of a wildtype lacZ α sequence. Mutant transformant colonies were selected from 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 synthesome (data not shown) were checked against the published wildtype sequence for lacZ α and were not found to contain any mutations.

^c 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.

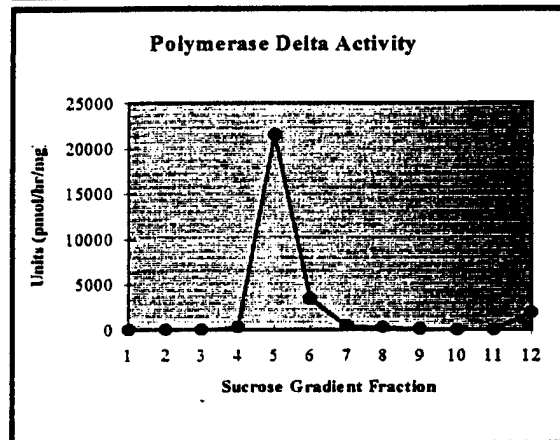
FIGURE 8

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

A.



B.



C.

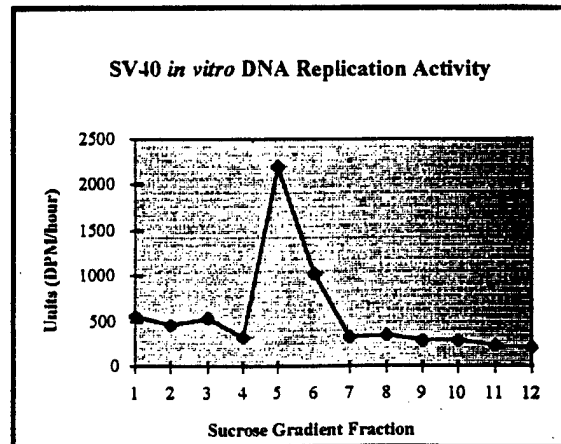
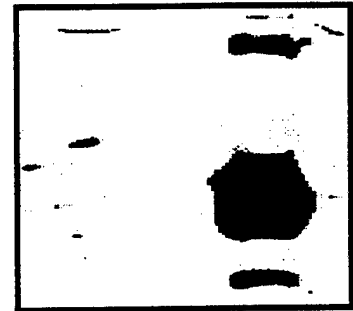


FIGURE 10

FIGURE 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) *

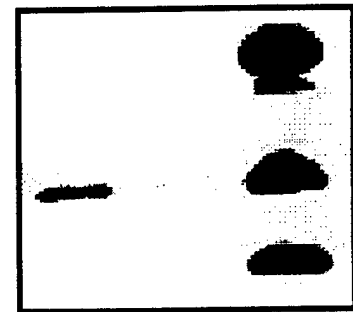
large IgG fragment (50 kDa) -



B. large IgG fragment (50 kDa) -

PCNA (36 kDa) *

small IgG fragment (25 kDa) -



C. Polymerase α (180 kDa) *

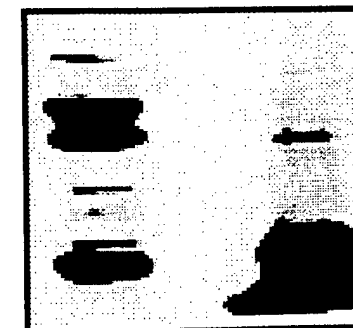
large IgG fragment (50 kDa) -



D.

hMLH1 (100 kDa) *

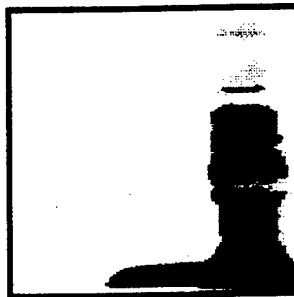
large IgG fragment (50 kDa) -



Control
Supernatant
Co-Ip pellet
1 2 3

E. PMS1 (98 kDa) *

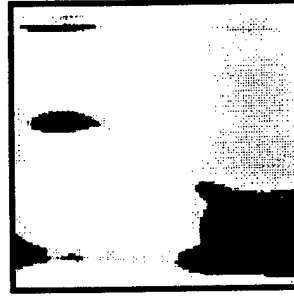
large IgG fragment (50 kDa) -



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) -

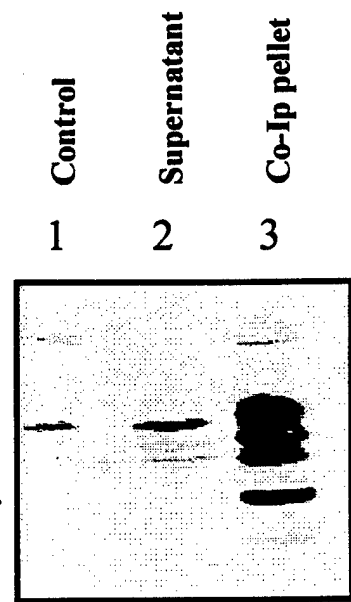
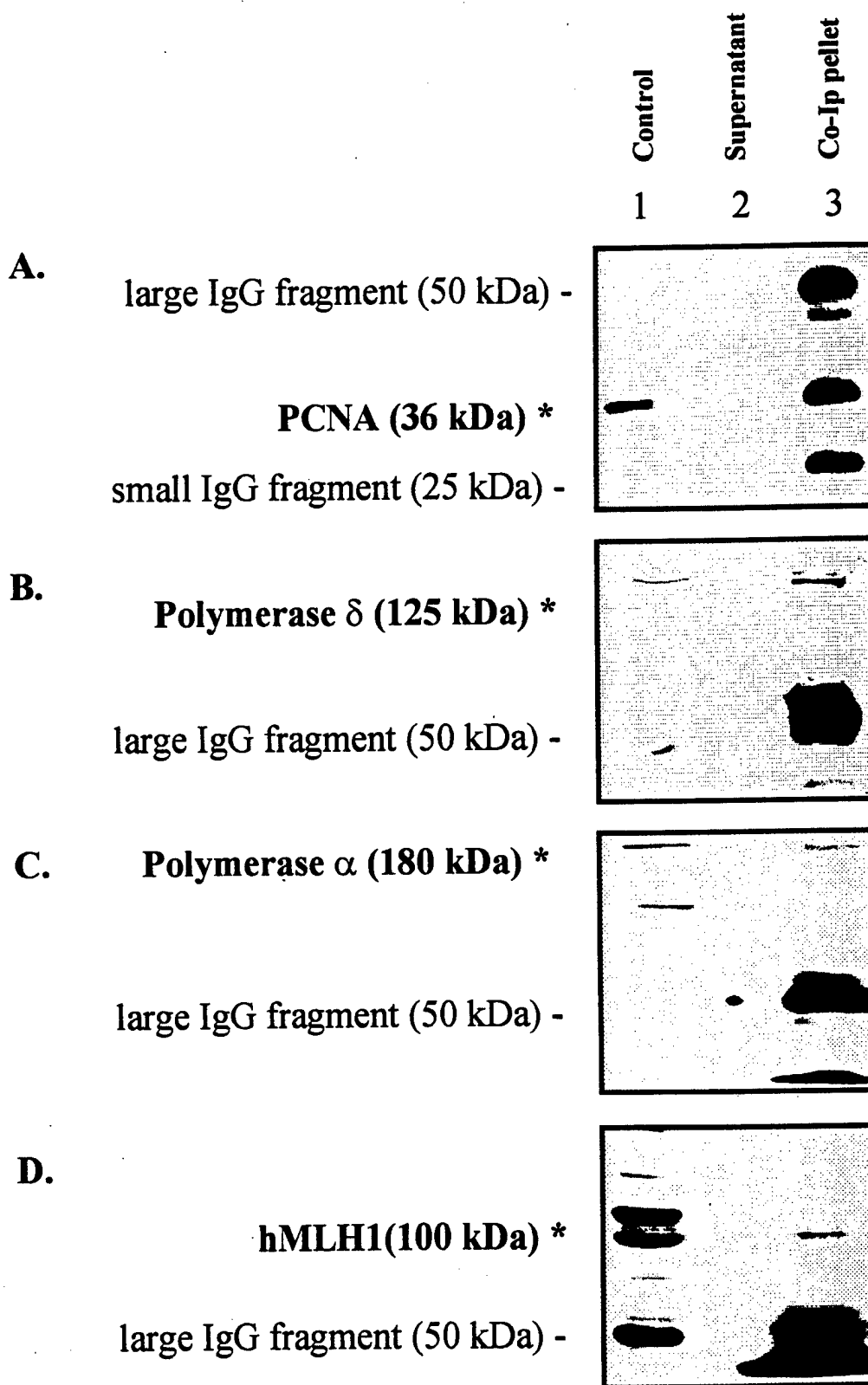


FIGURE 11

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



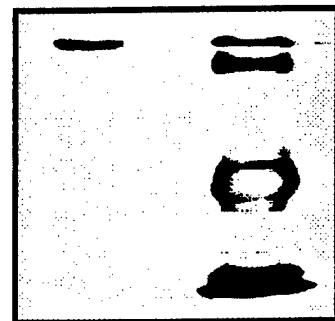
E.

hMSH2 (107 kDa) *

large IgG fragment (50 kDa) -

small IgG fragment (25 kDa) -

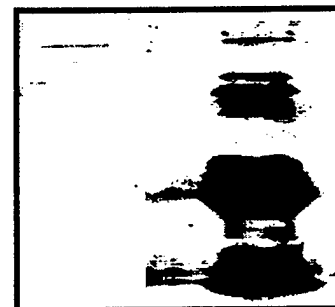
Control
1
Supernatant
2
Co-Ip pellet
3



F.

hMSH6 (160 kDa) *

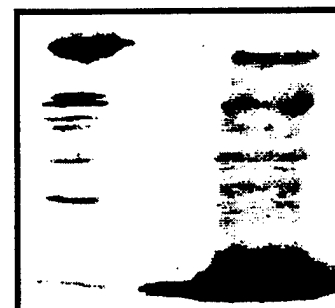
large IgG fragment (50 kDa) -



G.

hPMS1(98 kDa) *

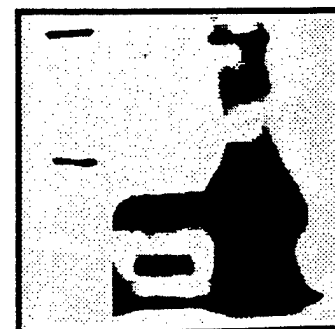
large IgG fragment (50 kDa) -



H.

hPMS2(98 kDa) *

large IgG fragment (50 kDa) -



I.

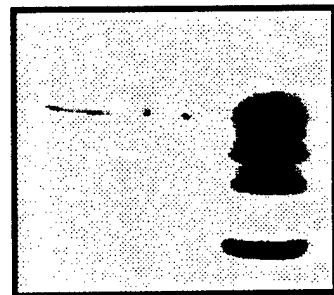
hMYH (63 kDa) *

large IgG fragment (50 kDa) -

small IgG fragment (25 kDa) -

Control
Supernatant
Co-IP pellet

1 2 3



J.

Ku80 (80 kDa) *

large IgG fragment (50 kDa) -

small IgG fragment (25 kDa) -

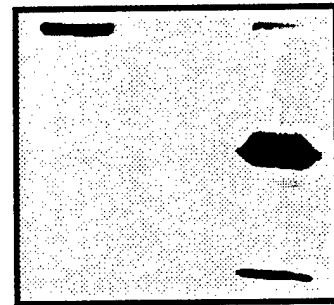
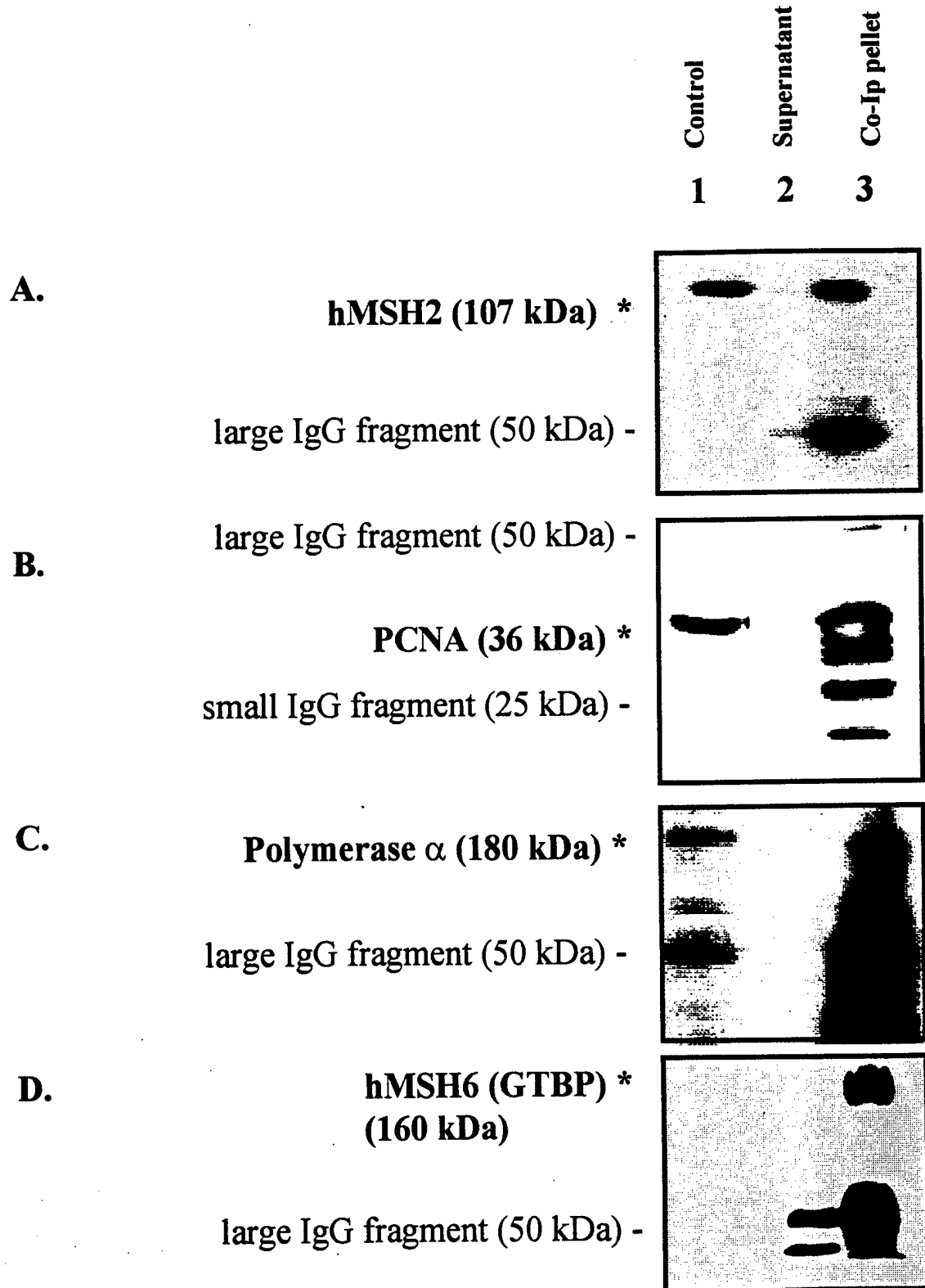


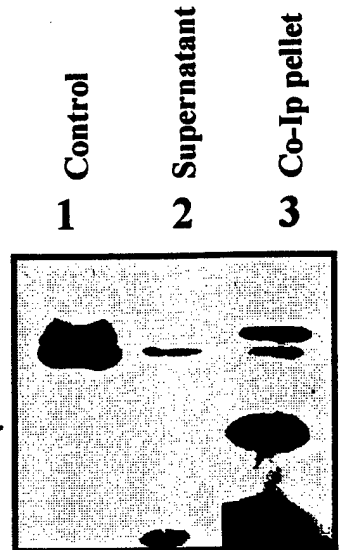
FIGURE 12

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



E.

hPMS 2 (98kDa) *
large IgG fragment (50 kDa) -
small IgG fragment (25 kDa) -



F.

MYH (63 kDa) *
large IgG fragment (50 kDa) -

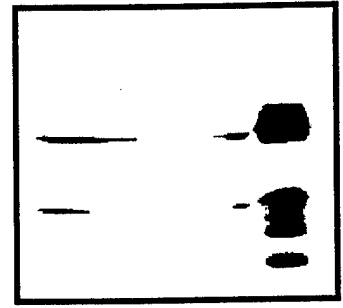


FIGURE 13

TABLE 4 Summary of Co-immunoprecipitation Data

Precipitating Antibody	Proteins Co-immunoprecipitated									
	DNA Pol α	DNA Pol δ	PCNA	hMSH2	hMSH6	hMLH1	hPMS1	hPMS2	Ku80	MYH
DNA Pol δ	+	+	+	-	+	+	+	+	+	+
PCNA	+	+	+	+	+	+	+	+	+	+
hMSH2	+	-	+	+	+	+L	+L	+	-	+

+L= As suggested by Acharya et al., 1996; and Unar et al, 1996

FIGURE 14

FIGURE 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

0	10	30	100	300	600	900
1	2	3	4	5	6	7

Bound IDL2

Free IDL2

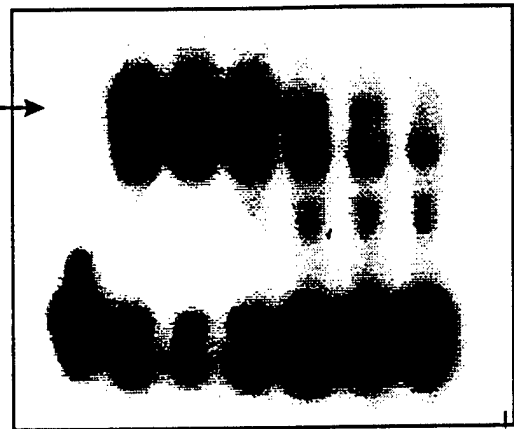


FIGURE B- IDL4

Fold Competitor IDL4 DNA

0	10	30	100	300	600	900
1	2	3	4	5	6	7

Bound IDL4

Free IDL4

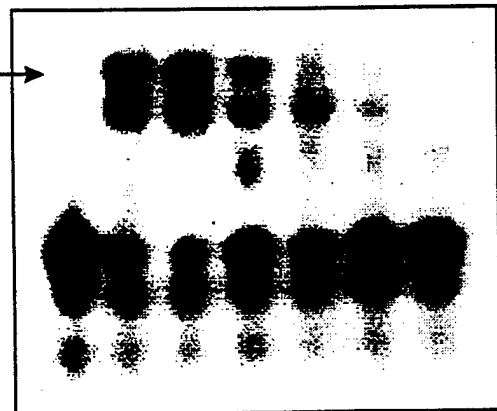


FIGURE C-G/T

Fold Competitor G/T DNA

0	1	10	30	100	300	600	900
1	2	3	4	5	6	7	8

Bound G/T

Free G/T

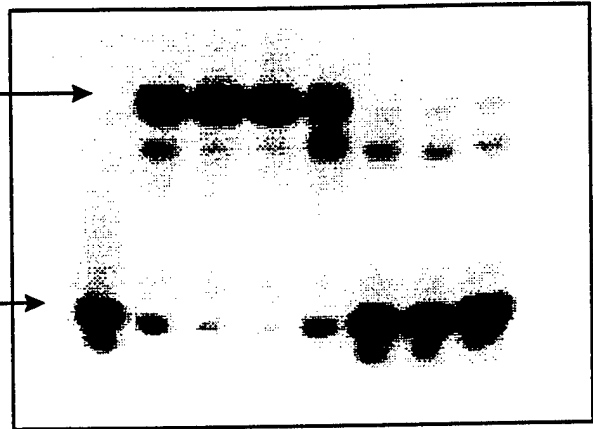


FIGURE D-A/GO

Fold Competitor A/GO DNA

0	1	10	30	100	300	600	900
1	2	3	4	5	6	7	8

Bound A/GO

Free A/GO

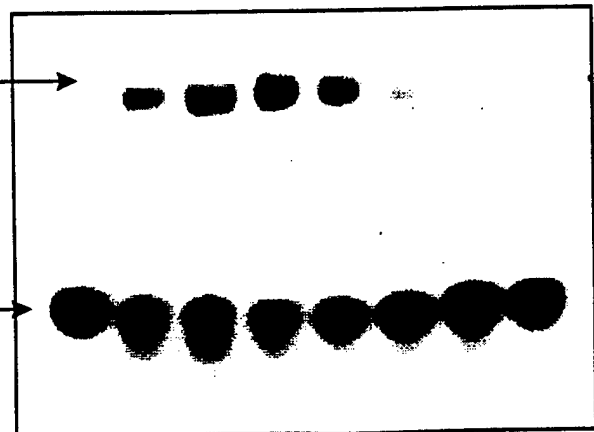
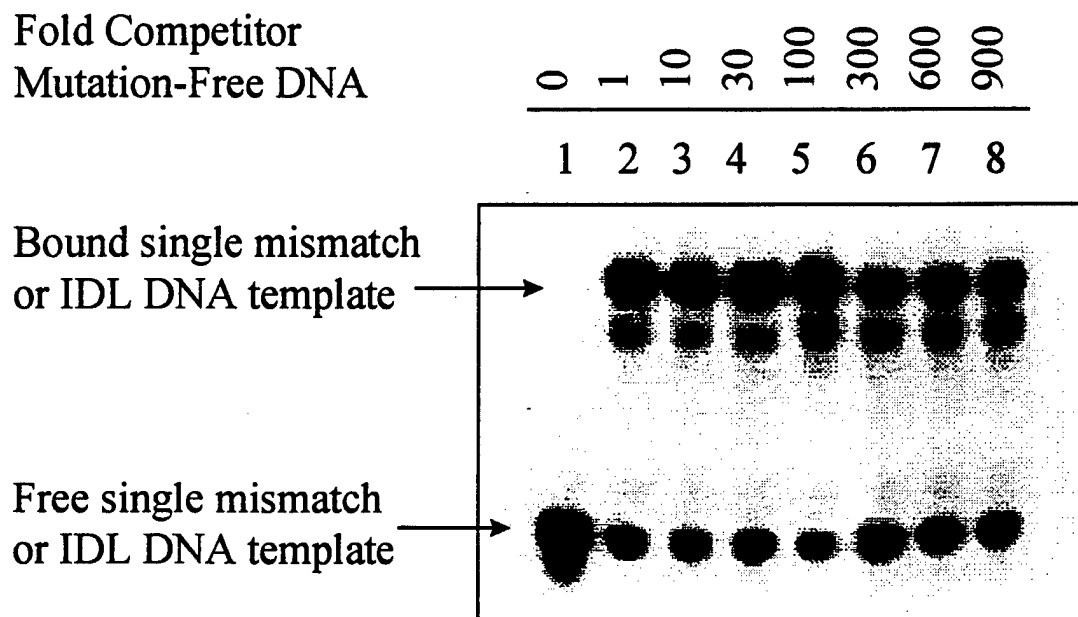


FIGURE 15

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



Principal Investigator: Jennifer Weeks Sekowski, Ph.D.

Grant# DAMD17-94-J-4151

PUBLICATIONS:

- Coll, J.M, **Sekowski, J.W.**, Hickey, R.J., Schnaper, L., Yue, W., Brodie, A.M.H., Syvaiojqa, J., and Malkas, L.H. The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture. (1996) *Oncology Research*, Vol. 8, Nos 10/11, pp.435-447.
- 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.
- Sekowski, J.W.**, Applegren, N., Hickey, R.H., Tuteja, N., Wilson, S., Uitto, L., Syvaioja, J., and Malkas, L.H. (1998). The human cell DNA synthesome: Evaluation of its DNA replication mechanism and the identification of additional components. *J. Cell Biochem.*, in press.
- Sekowski, J.W.**, Malkas, L.H., Schnaper, L., Bechtel, P., and Hickey, R.J. (1998) Human breast cancer cells contain an error-prone DNA replication apparatus. *Cancer Res*, in press.
- Bechtel, P.E, Hickey, R.J., Schnaper, L., **Sekowski, J.W.**, Long, B.J., Freund, R., Lui, N., Rodriguez-Valenzuela, and Malkas, L.H. (1988) A unique form of proliferating cell nuclear antigen (PCNA) is present in malignant breast cells. *Cancer Res.*, in press.
- Sekowski, J.W.**, Hoelz, D.J., Malkas, L.H., Lu, A.-L., Han, S., Lee, M.Y.T., and Hickey, R.J. (1998) Human Mismatch DNA Repair Proteins are Coupled to the Cellular DNA Replication Apparatus. In preparation.

PRESENTATIONS:

- J.W. Sekowski, D.J. Hoelz, L.H. Malkas, A.-L. Lu, S. Han, M.Y.T., Lee, and R.J. Hickey** (1998) Human Mismatch DNA Repair Proteins are Coupled to the Cellular DNA Replication Apparatus. AACR, New Orleans, LA . 3/28-4/2.
- J.W. Sekowski, L.H. Malkas, L. Schnaper, R.J. Hickey** (1997) Human Breast Cells Contain An Error-Prone DNA Replication Apparatus. 20th Annual San Antonio Breast Cancer Symposium, San Antonio, TX. 12/4-12/8.

Degrees Awarded as a Result of this Fellowship

Addendum II

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

Addendum III

Jennifer Weeks Sekowski

Ph.D. candidate

100% effort