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# Altered DNA Polymerase ι Expression in Breast Cancer Cells Leads to a Reduction in DNA Replication Fidelity and a Higher Rate of Mutagenesis

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## ABSTRACT

**The recently discovered human enzyme DNA polymerase ι (pol ι) has been shown to have an exceptionally high error rate on artificial DNA templates. Although there is a considerable body of *in vitro* evidence for a role for pol ι in DNA lesion bypass, there is no *in vivo* evidence to confirm this action. We report here that pol ι expression is elevated in breast cancer cells and correlates with a significant decrease in DNA replication fidelity. We also demonstrate that UV treatment of breast cancer cells additionally increases pol ι expression with a peak occurring between 30 min and 2 h after cellular insult. This implies that the change in pol ι expression is an early event after UV-mediated DNA damage. That pol ι may play a role in the higher mutation frequencies observed in breast cancer cells was suggested when a reduction in mutation frequency was found after pol ι was immunodepleted from nuclear extracts of the cells. Analysis of the UV-induced mutation spectra revealed that >90% were point mutations. The analysis also demonstrated a decreased C→T nucleotide transition and an increased C→A transversion rate. Overall, our data strongly suggest that pol ι may be involved in the generation of both increased spontaneous and translesion mutations during DNA replication in breast cancer cells, thereby contributing to the accumulation of genetic damage.**

## INTRODUCTION

One of the hallmarks of cancer cells is genetic instability. In addition to large chromosomal changes involving thousands of bp, another form of genetic instability results in an increased mutation rate at the nucleotide level due to a perturbation in nucleotide synthesis or in cellular processes such as DNA repair and replication (1). It has become increasingly recognized that cellular DNA normally sustains continuous damage requiring repair and resynthesis. The genome in all organisms is continually subjected to damaging agents, both endogenously, from hydrolysis and oxidation resulting from the process of metabolism, and exogenously, from UV light, ionizing radiation, and a wide variety of chemical carcinogens (2, 3). A homeostatic equilibrium exists in which cellular DNA damage is counterbalanced by multiple DNA repair pathways. In normal cells, most DNA damage is repaired without error. However, in tumor cells, this equilibrium may be skewed, resulting in the accumulation of multiple mutations. Among the multiple DNA damage responses available to cells, there are processes that permit a tolerance of unrepaired DNA damage as the genome is replicated. This particular pathway was termed translesion replication, damage tolerance, and lesion bypass (4, 5). Depending on the outcome of the process, the result can be classified as an error-free translesion or an error-prone translesion.

In the last 3 years, the number of prokaryotic and eukaryotic DNA polymerases has expanded to a total of 15. In addition, a major breakthrough has occurred with the discovery that several specialized DNA polymerases are clearly involved in translesion synthesis, these polymerases belong to the novel Y superfamily (UmuC/DinB/Rev1p/Rad30; Refs. 6–9). These DNA polymerases were found in a wide variety of organisms ranging from bacteria (*Escherichia coli*) to humans. A number of the Y family translesion polymerases have been identified in mammalian cells and include pol η(RAD30), pol ι(RAD30B), pol θ, pol κ, and Rev1 (10–14). One of their most prominent functional characteristics is a high error propensity during synthesis when using an undamaged DNA parental template. This error-prone property distinguishes these polymerases from known high-fidelity replicative DNA polymerases such as DNA polymerases α, δ, and ε (7, 15). Therefore, these translesion enzymes are alternatively known as error-prone DNA polymerases.

Human pol ι is a homologue of pol η, encoded by the *RAD30B* gene on chromosome 18, and is comprised of 715 amino acid residues (13). As with other translesion polymerases, it has low processivity and lacks an intrinsic 3′-5′-exonuclease activity with which to proofread mistakes (8, 16). On the basis of the *in vitro* studies, it appears that pol ι has the lowest fidelity of any eukaryotic polymerase studied to date. It exhibits misinsertion frequencies averaging 1 base/100 ($1 \times 10^{-2}$; Ref. 17). Recently, purified pol ι has been observed to be able to bypass several types of DNA lesions. For example, although pol ι is completely blocked by an intrastrand cisplatin deoxyguanine adduct and it can only traverse a cyclobutane pyrimidine dimer inefficiently (17, 18), it nonetheless can very efficiently bypass oxidized guanine and cytosine residues, as well as a variety of uracil lesions (19). It was also observed to efficiently insert 2 bases opposite a 6-4 pyrimidine pyrimidone photo adduct and to insert 1 base opposite a synthetic abasic site adduct (20). Not only can pol ι mediate translesion replication in damaged DNA very often in an error-prone manner, but it also can misincorporate bases in a template-dependent manner in undamaged DNA (15, 16). It is the only known DNA polymerase from any biological source that readily violates the Watson-Crick basepairing rule for nucleotide insertion opposite a parental template thymidine (T). The enzyme shows a preference for the misinsertion of G or T opposite parental template T (15, 21). Although published *in vitro* enzymatic properties shed light on the hypermutagenic potential of pol ι, to date, very little cellular data on pol ι have been described except to indicate that the protein plays a critical role in the somatic hypermutation of mammalian immunoglobulin genes (22).

In earlier work, we demonstrated that breast cancer cell lines and tissues mediate error-prone DNA replication, resulting potentially in higher spontaneous mutations (23). Recently, it was also reported that single nucleotide instability can be observed in breast cancer cells grown in culture (24). Taken together, these data lead us to propose that translesion DNA polymerases may be specialized and highly regulated in normal mammalian cells, but once escaped from their normal regulation, an error-prone translesion DNA polymerase can be a potential mutator. It could potentially interfere with the replicative DNA polymerases on undamaged DNA or even shift an error-free translesion pathway to an error-prone process when encountering DNA damage. These scenarios would potentially lead to an accumulation of mutation and genomic instability. To begin to address this hypothesis, we report data that show that there are elevated levels of

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pol ι in breast cancer cells and that this is correlated with the hypermutation status of the cells.

## MATERIALS AND METHODS

**Cell Culture and Plasmid.** MCF12A, Hs578bst, MCF7, Hs578T, and MDA-MB-468 cells were purchased from American Type Cell Culture (Manassas, VA). MCF10A cells were purchased from the Michigan Cancer Foundation. The malignant cell line Hs578T was grown in DMEM supplemented with 4.5 g/liter glucose, 10 units/ml bovine insulin, and 10% fetal bovine serum. The nonmalignant breast cell line Hs578Bst was grown in monolayer culture with modified DMEM, 30 ng/ml epidermal growth factor, and 10% fetal bovine serum. The malignant cell lines MCF7 and MDA-MB-468 were maintained in 90% DMEM and 10% fetal bovine serum. The nonmalignant MCF10A and MCF12A cell lines were maintained in a 1:1 mixture of DMEM and Ham's F-12 medium with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone (95%) and horse serum (5%).

Primary breast epithelial cells were purchased from the Clonetic Company (San Diego, CA). According to the information provided by the company, the normal human mammary epithelial cells were isolated from normal healthy human tissue. The breast cells supplied to us were determined to be basal epithelial cells. The basal epithelial cells stain positive for cytokeratins 14 and 18, whereas luminal epithelial cells stain positive for cytokeratin 19. The normal human mammary epithelial cells used in our studies did not stain for the cytokeratin 19 marker but tested positive for the basal epithelial cell markers cytokeratins 14 and 18. The primary cells were grown in mammary epithelial growth medium (Clonetic Company) supplemented with 2.5 μg/ml amphotericin B, 50 units/ml polymixin B sulfate, 10 ng/ml epidermal growth factor, 5 μg of insulin, 0.5 mg/ml hydrocortisone, and 52 μg/ml bovine pituitary extract.

The plasmid pSupFG1 was kindly provided by Dr. Gan Wang of Wayne State University. The pSupFG1 plasmid, derived from PSP189, contains a supF reporter gene, an ampicilin gene, a pBR327 replication origin for replication in *E. coli*, and a SV40 viral replication origin and the gene for the SV40 large T-antigen that permits replication of the plasmid in human cells (25, 26).

**Western Blotting.** The cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL), and the protein concentration was measured using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). For Western blot analysis of pol ι, cell lysates (150 μg of protein) were resolved by electrophoresis in a 10% SDS-PAGE gel. The resolved polypeptides were electrophoretically transferred to polyvinylidene difluoride membrane (Bio-Rad). The filter membranes were then blocked in Tris-buffered saline-Tween 20 (0.1% Tween 20) with 5% nonfat dry milk and then incubated with anti-pol ι antibody at a dilution of 1:300 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated antigoat IgG. The presence of pol ι was visualized by using a enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). Monoclonal antibodies to actin at a dilution of 1:200 (Santa Cruz Biotechnology) and tubulin at a dilution of 1:500 (Santa Cruz Biotechnology) were used to verify protein loading. The level of protein expression was quantified using a Scion image densitometry software (Scion Corporation, Frederick, MD).

**Relative Reverse Transcription-PCR (RT-PCR).** Total RNA was extracted from cells using the RNAqueous-4PCR kit (Ambion, Austin, TX). Using the RETROscript first strand synthesis kit for RT-PCR (Ambion), 2 μg the RNA sample were first denatured by heating at 85°C for 3 min with the 8 μM random decamers. First strand cDNA was synthesized in a reaction mixture (20 μl) containing 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 0.5 mM of each dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor, and 100 units of reverse transcriptase (MMLV-RT) and the denatured RNA mixture. After incubation at 42°C for 1 h, the reaction was stopped by heating at 92°C for 10 min. Finally, 250 nM each of the pol ι gene-specific PCR primers were added to a final volume of 50 μl containing 10 μl of first strand cDNA mixture for five cycles of PCR amplication, and 250 nM of each of the rig/S15 gene-specific primers were added for another 20 cycles of the PCR amplication. PCR was performed using TaqDNA polymerase (Sigma, St. Louis, MO) according to the following conditions: 94°C denaturation for 4 min, followed by 25 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, 40 s extension at 72°C, and 5-min final extension at 72°C. The PCR primers used were 5′-GAGAAGACCTGACCCGCTAC-3′ and 5′-TAGGGCACTGACGACTCTCAC-3′ for pol ι and 5′-TTCCGCAAGTTCACCTACC-3′ and 5′-CGGGCCGGCCATGCTTTACG-3′ for rig/S15. The amplified DNA fragments of pol ι and rig/S15, 805 and 361 bp, respectively, were analyzed by electrophoresis in a 1.8% agarose gel. The RT-PCR products were quantified by scanning the gel photographs followed by software analysis using Scion image densitometry software (Scion Corporation).

**Nuclear Extract Preparation.** The nuclear extracts were prepared as described previously with some modifications (27). Briefly, the cells were harvested from the culture medium by centrifugation (4°C) and washed twice with PBS. The cells were suspended in a volume of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT] that was five times the packed cell pellet volume. The resuspended cells were then incubated on ice for 10 min. The cells were then lysed by 20 strokes of a Kontes glass Dounce homogenizer (B-type pestle). The homogenate was centrifuged for 10 min at 25,000 × *g* (4°C) to pellet nuclei. The nuclei were resuspended in two volumes of Buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT] and lysed by 20 strokes of a Kontes glass Dounce homogenizer (B-type pestle). The resulting suspension was stirred gently for 30 min at 4°C and then centrifuged for 30 min at 25,000 × *g*. The clear supernatant was dialyzed against 50 volumes of Buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT] at 4°C for 6 h. The dialysate was centrifuged at 25,000 × *g* for 20 min, and the supernatant was frozen as aliquots in liquid nitrogen.

**Immunodepletion of Pol ι from Cell Nuclear Extracts.** Protein G-immobilized agarose beads (Sigma) were incubated overnight with antibody against pol ι (Santa Cruz Biotechnology) at 4°C. The antibody-bound agarose beads were collected by centrifugation at 4°C for 10 min and washed twice with PBS. MCF7 and MDA-MB-468 cell nuclear extracts were precleared with Protein-G immobilized agarose beads at 4°C for 2 h, and the extracts subsequently centrifuged at 4°C for 10 min. The supernatant was incubated with the anti-pol ι antibody-bound agarose beads at 4°C for 2 h. Protein G-agarose beads were used in the preparation of the negative control for these studies. The efficiency of pol ι immunodepletion was confirmed by Western blot analysis. The immunodepleted MCF7 and MDA-MD-468 cell nuclear extracts were used for DNA replication assays.

***In Vivo* DNA Replication Assay.** The plasmid pSupFG1 was irradiated with UVC (254 nm) in doses of 500, 1000, or 1500 J/$m^2$ by Stratalinker UV Crosslinker 2400 (Stratagene, La Jolla, CA). Non-UV-irradiated or -irradiated pSupFG1 plasmid was transfected into breast cells using Superfect transfection reagent (Qiagen, Inc., Valencia, CA). The cells were then incubated at 37°C for 48 h. The plasmid DNA was then isolated from the cells using the protocol described previously (25).

***In Vitro* DNA Replication Assay.** The reaction mixture (50 μl) contained 30 mM HEPES (pH 7.8), 7.5 mM $MgCl_2$, 0.5 mM DTT, 100 μM of each dATP, dGTP, dCTP, and dTTP, 200 mM of each rCTP, rUTP, and rGTP, 4 mM rATP, 40 mM phosphocreatinine, 5 μg of creatinine phosphokinase, 15 mM sodium phosphate (pH 7.5), 40 mM phosphocreatine, 1.0 μg of SV40 large T-antigen, 70 μg nuclear extract of different cells, and 100 ng of the non-UV-irradiated pSupFG1 plasmid or 125 or 250 J/$m^2$ UV-irradiated pSupFG1 plasmid. The reaction mixture was then incubated at 37°C for 3 h. The mixture was treated with 100 ng/μl proteinase K at 50°C for 1 h and then the plasmids were extracted with an equal volume of phenol-chloroform (28).

**Detection of Mutations Occurring in the supF Reporter Gene.** The pSupFG1 plasmid DNA isolated from transfected cells or from *in vitro* replication assay mixtures was digested with *Dpn*I restriction enzyme to eliminate any unreplicated parental plasmid. The plasmid DNA was then transformed into the *E. coli* SY204 (lacZ amber) strain by electroporation using a setting of 1800V/25μF. The transformed cultures were then plated on to Luria Bertani (LB) agar containing both ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg/ml), and isopropyl-1-thio-β-D-galactopyranoside (200 μg/ml) and plates incubated at 37°C overnight. Because the *E. coli* SY204 strain carries an amber mutation in the lacZ gene, a functional supF gene can suppress the mutation, resulting in blue bacterial colonies on the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plate, whereas a mutation in the supF reporter gene leads to colorless colonies. The mutation frequency was determined by comparing the number of mutant



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colonies to the total number of colonies on the plates. Mutant colonies were chosen from three independent experiments. Plasmid DNA was isolated from the mutant colonies using a QIAprep Spin Miniprep kit (Qiagen, Inc.) and analyzed by neutral agarose gel electrophoresis. Those plasmids without a detectable size change were sequenced using an Thermo Sequenase Cycle Sequencing kit (United States Biologicals, Cleveland, OH) as directed by the manufacturer. The oligonucleotide primer (5′-TTTGTGATGCTCGTCAG-GGG-3′) was synthesized by Qiagene Operon.

***In Vitro* DNA Repair Synthesis Assay.** The *in vitro* DNA repair synthesis assay was performed as previously described with some modifications (29). Briefly, the pSupFG1 plasmid was irradiated with UV light (254 nm) to generate DNA damage. The DNA repair assay was performed in a 25-μl reaction mixture containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 1 μg of plasmid DNA, 250 μM each of dATP, dCTP, dGTP, and dTTP, 10 μCi ($\alpha^{32}$P)dCTP (ICN Pharmaceuticals, Costa Mesa, CA), and 50 μg of the nuclear extract of each of the breast cell lines. The reactions were incubated at 30°C for 2 h, and the mixtures were then digested with proteinase K (100 ng/μl) at 50°C for 30 min. The reaction products were then extracted with phenol/chloroform, and the plasmid DNA was purified using a Centricon 30 apparatus (Millipore, Bedford, MA). The plasmid DNA was then digested with *Xho*I and analyzed by agarose gel electrophoresis using a 1% gel. Visualization of plasmid DNA and the incorporated ($\alpha^{32}$P)dCTP was achieved by ethidium bromide staining and autoradiography. Quantification of the incorporated ($\alpha^{32}$P)dCTP was determined using Scion image densitometry software (Scion Corporation).

## RESULTS

**Overexpression of Pol ι in Malignant Breast Cell Lines.** Currently, there are no reports describing the expression of pol ι in human breast cancer cells. To develop a more accurate picture of the abundance of pol ι expressed in these cells, we analyzed a series of malignant and nonmalignant breast cell lines as well as a primary culture of normal breast epithelial cells for the expression of pol ι. The nonmalignant breast cell cultures used in this study included MCF10A, MCF12A, Hs578BST, and a primary culture. Two of the three breast cancer cell lines (MCF7 and MDA-MB-468) used in this study are epithelial in origin. MCF7 is both estrogen receptor and p53 positive, whereas MDA-MB-468 is estrogen receptor and p53 negative (30–32). The third malignant breast cell line (Hs578T) used in our studies is derived from the myoepithelia and is estrogen receptor and p53 negative. It is also genetically matched to the nonmalignant Hs578Bst cell line used in this study (33). The abundance of both pol ι and β actin expressed in these different cells was determined at the protein level using Western blotting (Fig. 1). It was observed that the expression of pol ι appears to be low in both the nonmalignant breast cell lines and the primary cell culture but significantly elevated in the breast cancer cell lines. The level of expression of pol ι was estimated to be 2–5-fold higher in the breast cancer cell lines (Fig. 1, *Lanes 5–7*) relative to the nonmalignant breast cell lines (Fig. 1, *Lanes 1–4*). The elevated level of expression in the malignant breast cell lines appears to be independent of the estrogen receptor and p53 status of these cells. The expression level of β-actin served as a control to assure that equal amounts of lysate were loaded into each well of the polyacrylamide gel.

To determine whether the increased pol ι protein level correlated with an increase in the expression of pol ι transcripts, the abundance of pol ι mRNA in the various cell lines was determined using RT-PCR (Fig. 2). The elevated mRNA expression level of pol ι in the three breast cancer cell lines implies that pol ι may be used far more extensively in breast cancer cells than in nonmalignant breast cells.

**Expression of pol ι Is Induced in Breast Cancer Cells after UV Irradiation.** The *in vitro* data obtained by others has shown that pol ι is a highly error-prone DNA polymerase (16). One of its predominant characteristics *in vitro* is the ability to misincorporate the nucleotide opposite some types of DNA lesions, which include cyclobutane pyrimidine dimer and 6-4 pyrimidine pyrimidone photo adduct distortion of UVC-damaged DNA (20, 34). To begin to evaluate whether these characteristics of pol ι may play a role in the process of DNA damage *in vivo*, we examined its expression in nonmalignant and malignant breast cells as they responded to genetic insult. UV irradiation was chosen as the DNA damage model because pol ι may take part in the translesion DNA replication process of UV-damaged DNA (20). MCF7 and MDA-MB-468 breast cancer cells and nonmalignant MCF10A breast cells were each irradiated with UV light (UVC, 254 nm) at a dose of 100J/m$^2$. Pol ι expression in these cells as they responded to damage was measured over the next 8 h (Fig. 3). It was observed that pol ι expression was rapidly induced both in the cancer and nonmalignant cells after their exposure to UVC light. The level of pol ι remained elevated in the cells for at least 2–8 h after UV irradiation. α-Tubulin levels were measured to assure that equivalent amounts of protein were loaded in each lane of the gels used to evaluate pol ι expression. These results suggest that pol ι may play a role in DNA damage response in both malignant and nonmalignant breast cells. The data also suggest that the induction of pol ι is an early event in the cell's response to DNA damage.

**The Error-Prone DNA Replication Status of Breast Cancer Cells Correlates with an Increased Frequency of Both Spontaneous and UV Damage-Induced Mutations.** To determine whether the high level of pol ι expression in breast cancer cells correlates with DNA replication hypermutagenesis, we compared the fidelity of DNA replication in both nonmalignant and malignant breast cancer cells. We chose the *supF* gene-based shuttle vector *in vivo* DNA replication system to detect both spontaneous and DNA damage induced mutations in breast cells. SV40-based shuttle vector plasmids carrying the *supF* gene, which encode a suppressor tyrosyl tRNA, have been widely used as a mutation marker for DNA replication fidelity in mammalian cells (35–37). Also, we again chose UV irradiation as the DNA damage model. Nonirradiated parental pSupFG1 plasmid DNA served as the template for the DNA replication process mediated on undamaged DNA, whereas pSupFG1 plasmid pretreated with UV light (UVC, 254 nm) was used as the template for the DNA replication process occurring after UV damage. The frequency of mutation produced in daughter DNA during *in vivo* DNA replication was deter-

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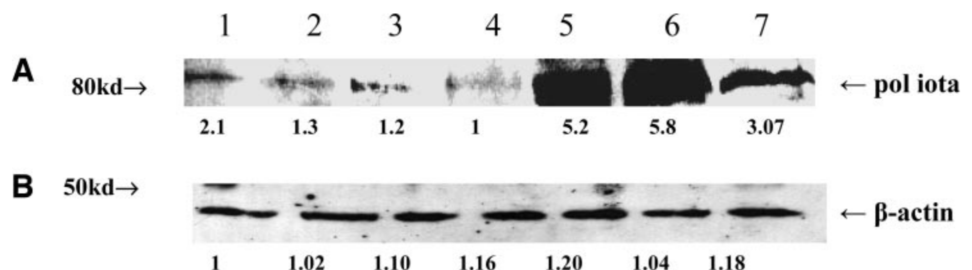


Fig. 1. Western blot analysis for the expression of pol ι in different breast cell lines. A total of 150 μg of whole cell lysate for each of the different cells was resolved by 10% SDS-PAGE gels, and the resolved polypeptides were then electrophoretically transferred to polyvinylidene difluoride membranes. *A*, membrane that was probed with antibody against the $NH_2$-terminal of human pol ι. *B*, membrane probed with antibody against β-actin. Densitometric values are indicated below each panel. *Lane 1*, primary cells; *Lane 2*, MCF-10A; *Lane 3*, MCF-12A; *Lane 4*, Hs578Bst; *Lane 5*, MCF-7; *Lane 6*, MDA-MD-468; and *Lane 7*, Hs578T.

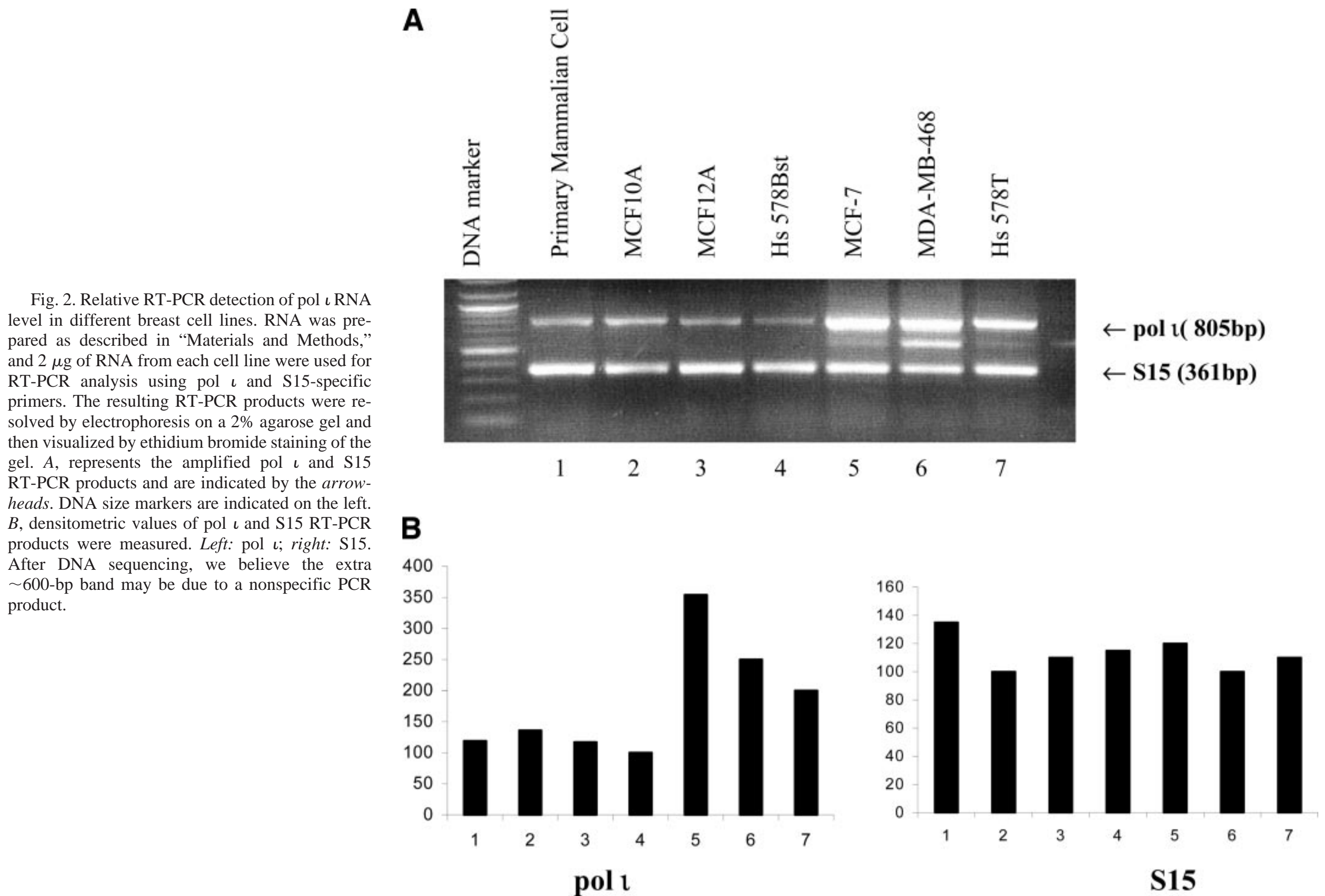


Fig. 2. Relative RT-PCR detection of pol ι RNA level in different breast cell lines. RNA was prepared as described in "Materials and Methods," and 2 μg of RNA from each cell line were used for RT-PCR analysis using pol ι and S15-specific primers. The resulting RT-PCR products were resolved by electrophoresis on a 2% agarose gel and then visualized by ethidium bromide staining of the gel. *A*, represents the amplified pol ι and S15 RT-PCR products and are indicated by the *arrowheads*. DNA size markers are indicated on the left. *B*, densitometric values of pol ι and S15 RT-PCR products were measured. *Left:* pol ι; *right:* S15. After DNA sequencing, we believe the extra ~600-bp band may be due to a nonspecific PCR product.

mined. Nonmalignant MCF10A, MCF12A, Hs578Bst, and primary mammary epithelial cells and the breast cancer cells MCF7, MDA-MB-468, and Hs578T were used in this study. The cells were transfected with either UV-irradiated or nontreated pSupFG1 plasmid and then incubated for 48 h. Replicated daughter DNA was collected, and the detection of mutations in the pSupFG1 reporter gene was performed as described in "Materials and Methods." The mutation frequencies measured from the replication of nondamaged plasmid template represents the spontaneous mutagenesis level, whereas those produced from replication of the UV-pretreated plasmid template represent the level of mutations that result from UV damage-induced mutagenesis.

The frequency of mutations within the reporter gene made during *in vivo* DNA replication of the UV treated and nontreated plasmids is shown in Table 1. In nonmalignant breast cells, nonirradiated parental plasmid DNA generated comparably low levels of background mutations [MCF10A, MCF12A, Hs578Bst, and primary mammary epithelial cells ($0.4 \times 10^{-3}$, $0.34 \times 10^{-3}$, $0.37 \times 10^{-3}$, and $0.33 \times 10^{-3}$, respectively)]. In contrast, nonirradiated plasmid DNA replicated in malignant cells (MCF7, MDA-MB-468, and Hs578T) had a 2–3-fold higher mutation frequency ($1.02 \times 10^{-3}$, $1.10 \times 10^{-3}$, and $0.87 \times 10^{-3}$, respectively). These data indicate that breast cancer cells exhibit a higher spontaneous mutation frequency than nonmalignant breast cells. This conclusion was consistent with the *in vitro* mutagenesis frequency data we have previously published (23), suggesting that the DNA replication processes in breast cancer cells may be inherently more mutagenic relative to nonmalignant breast cells.

When the UV-irradiated shuttle vector was used as the parental DNA in *in vivo* DNA replication experiments in the malignant and nonmalignant breast cells, the resulting daughter DNA from the malignant cells contained more mutations than daughter plasmids rescued from the nonmalignant breast cell lines (Table 1). The frequency of mutation associated with the UV-irradiated plasmids rescued from the malignant and nonmalignant cells was also consistently higher than the background levels of mutations seen with the untreated plasmid DNA. The increase in the mutation frequency of the rescued plasmids directly correlated with the dose of UV light used to pretreat the plasmid DNA. For example, at the maximum dose of $1500 J/m^2$, the mutation frequency of the plasmid rescued from the malignant cells was $\sim 15 \times 10^{-3}$, whereas the mutation frequency of the plasmid rescued from the nonmalignant cells was 2.5-fold lower at just $\sim 6 \times 10^{-3}$. This observation provides the first *in vivo* evidence, indicating that there is an error-prone DNA replication process in breast cancer cells and that it reflects the hypermutability of malignant breast cells relative to that of the nonmalignant breast cells.

**The Response of Breast Cancer Cells to UV-Induced DNA Damage Correlates with the Altered Enzymatic Properties of Pol ι in These Cells.** It has been demonstrated that the mutant daughter plasmids rescued after heavy UV irradiation ($300 J/m^2$) of pSP189 plasmid are all due to independent events and are not siblings (38). Because of this, the shuttle vector pSupFG1, derived from pSP189 (25, 26), was an appropriate model for us to use to analyze mutation spectra after UV damage because we used a UV power of $1500 J/m^2$ to induce DNA damage in our studies.

Agarose gel electrophoresis revealed that most (>90%) of the rescued plasmids containing supF mutant remained unchanged in their size (data not shown), suggesting that most of the UV generated mutations are either point mutations or very small deletions. The



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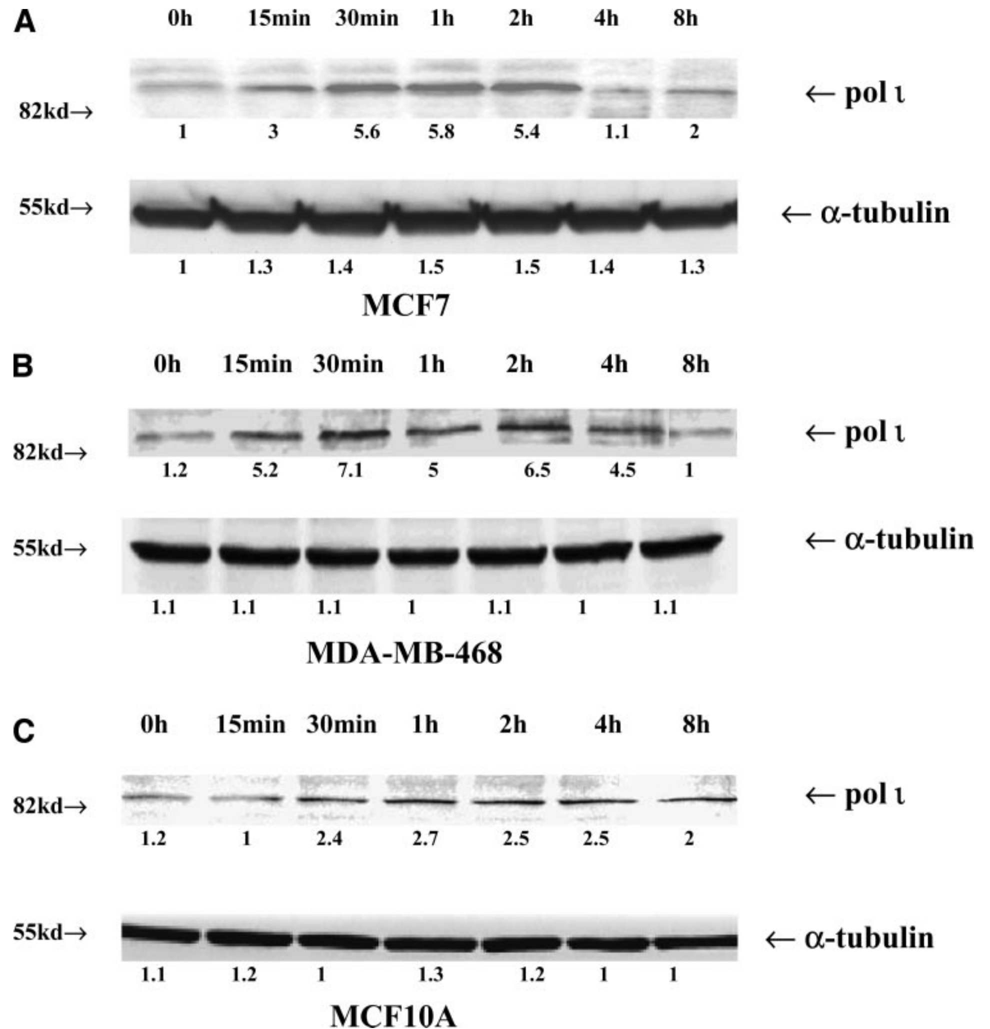


Fig. 3. Western blot analysis of induced expression of pol ι at different times after UVC irradiation. Following cell line exposure to 100 J/m$^2$ UVC (254 nm), the cells continued to be cultured in complete medium for varying lengths of time and then were lysed. A total of 150 μg of each cysate was subjected to electrophoresis and Western blotting. The times indicated are post-UV irradiation time. *A*, MCF7; *B*, MDA-MD-468; and *C*, MCF10A. The results are representative of three independent experiments. Densitometric values are indicated below each panel.

outcome of our DNA sequence analysis, of 46–49 independently selected supF mutants rescued from the MCF7 and MCF10A cells (MCF10A, 49; MCF7, 46), showed that only three of each cell line contained deletions ranging from 5 to 100 nucleotides in length, whereas 43–46 contained point mutations (MCF10A, 46; MCF7, 43; Fig. 4, *A* and *B*). The point mutations were primarily single base changes but did include multiple and tandem substitutions as well. Our sequence analysis clearly demonstrated that all of the point mutations occurred at the 3′-site of the pyrimidine dimers that resulted from treating the plasmid with UV light. Three types of pyrimidine dimers are found in both nonmalignant and malignant cells, and each of these dimers is related to the type of UV photoadduct formed in these cells (39). If these mutations resulted from the nucleotide excision repair process, the mutations would have been expected to be randomly located within a 30-bp stretch of DNA containing the UV-induced damage site. This is because the incision and excision step of the mammalian cell nucleotide excision repair process creates a 30-bp long repair patch containing the damage site (40). Our data are consistent with the observations of other investigators who excluded the nucleotide excision repair process as the primary cause of the error-prone repair of pyrimidine dimers and indicated that the mutations retained in the rescued plasmid DNA are caused by an error-prone translesion process (41).

The most important finding was that there was a significantly different qualitative distribution of the type of transitions and transversions created around the UV-induced pyrimidine dimers between the malignant MCF7 cell line and the nonmalignant MCF10A cell line (Fig. 4, *A* and *B*). It has been demonstrated that the mutation spectrum of UV damage in nonmalignant cells is composed of up to 90% C→T transitions with the supF-based shuttle vector system (42, 43). Our own findings of the mutation spectrum in the nonmalignant breast cell line MCF10A were consistent with this observation. We found that the majority of the point mutations are of the C→T transition type (83%) in MCF10A cells with a smaller percentage of C→A (6.5%), T→A (4.3%), C→G (4.3%), and T→C (2.1%; Fig. 4*A*). Our results were also consistent with the work of others examining the effect of UV mutagenesis on the HPRT gene in nonmalignant cells and of cell extracts on the lacZ gene. These studies indicate the C→T transition as the predominant type of mutation (44, 45). As for the malignant breast cancer cell MCF7, although the C→T transition was still the main type of UV-directed mutation, its occurrence decreased significantly to ~51%. In contrast, a large increase in the number of C→A (21%), T→A (7%) transversions and T→C (14%) and C→G (6.9%) transitions was observed (Fig. 4*B*).

*In vitro* kinetic analyses of pol ι has provided a considerable body of data that has been useful for understanding the contribution of pol ι to the mutagenesis process *in vivo*. In these studies, it was observed that pol ι prefers to misincorporate a G or a T opposite the 3′-T of cyclobutane pyrimidine dimer and the 5′-T of a 6-4 pyrimidine pyrimidone photoadduct, including T-T dimers, T-C dimers, or C-C dimers (18, 34). These observations make our discovery of a large increase in the frequency of C→A (misincorporation of T opposite



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Table 1 *Mutation frequency of SupF gene*

| | Total colonies scored | Mutation colonies | Mutation frequency ($\times 10^{-3}$) (mean ± SE)* |
|---|---|---|---|
| Spontaneous† | | | |
| Nonmalignant cell | | | |
| Primary cells | $5.2 \times 10^4$ | 21 | 0.40 ± 0.020 |
| MCF10A cell line | $4.0 \times 10^4$ | 13 | 0.34 ± 0.020 |
| MCF12A | $3.0 \times 10^4$ | 11 | 0.37 ± 0.026 |
| Hs578Bst | $3.5 \times 10^4$ | 12 | 0.33 ± 0.017 |
| Malignant cell | | | |
| MCF7 | $3.5 \times 10^4$ | 36 | 1.02 ± 0.036 |
| MDA-MD-468 | $5.6 \times 10^4$ | 62 | 1.10 ± 0.062 |
| Hs578T | $4.5 \times 10^4$ | 40 | 0.87 ± 0.045 |
| UVC (500 J/m$^2$) | | | |
| Nonmalignant cell | | | |
| Primary cells | $2.2 \times 10^4$ | 35 | 1.60 ± 0.265 |
| MCF10A | $2.0 \times 10^4$ | 42 | 2.10 ± 0.247 |
| MCF12A | $2.5 \times 10^4$ | 58 | 2.32 ± 0.080 |
| Hs578bst | $2.7 \times 10^4$ | 32 | 1.20 ± 0.183 |
| Malignant cell | | | |
| MCF-7 | $3.0 \times 10^4$ | 144 | 4.80 ± 0.278 |
| MDA-MD-468 | $2.0 \times 10^4$ | 107 | 5.35 ± 0.346 |
| Hs578T | $2.5 \times 10^4$ | 80 | 3.20 ± 0.236 |
| UVC (1000 J/m$^2$) | | | |
| Nonmalignant cell | | | |
| Primary cells | $1.5 \times 10^4$ | 44 | 2.93 ± 0.187 |
| MCF10A | $2.0 \times 10^4$ | 60 | 3.00 ± 0.155 |
| MCF12A | $1.5 \times 10^4$ | 51 | 3.40 ± 0.156 |
| Hs578bst | $1.2 \times 10^4$ | 36 | 3.00 ± 0.101 |
| Malignant cell | | | |
| MCF7 | $1.8 \times 10^4$ | 100 | 5.60 ± 0.278 |
| MDA-MD-468 | $1.5 \times 10^4$ | 102 | 6.80 ± 0.361 |
| Hs578T | $2.0 \times 10^4$ | 89 | 4.43 ± 0.153 |
| UVC (1500 J/m$^2$) | | | |
| Nonmalignant cell | | | |
| Primary cells | $1.2 \times 10^4$ | 75 | 6.25 ± 0.312 |
| MCF10A | $1.5 \times 10^4$ | 106 | 7.06 ± 0.379 |
| MCF12A | $1.0 \times 10^4$ | 65 | 6.50 ± 0.300 |
| Hs578bst | $1.2 \times 10^4$ | 71 | 5.92 ± 0.426 |
| Malignant cell | | | |
| MCF7 | $2.0 \times 10^4$ | 306 | 15.33 ± 1.470 |
| MDA-MD-468 | $1.8 \times 10^4$ | 289 | 16.00 ± 0.896 |
| Hs578T | $1.0 \times 10^4$ | 147 | 14.70 ± 0.985 |

* Each data from at least three independent cell transfection experiment. SE: standard error.

† Plasmid pSupFG1 were exposed to different dose of UVC ( 0, 500, 1000, and 1500 J/m$^2$).

C), T→A (misincorporation of T opposite T), and T→C (misincorporation of G opposite T) transversions consistent with the utilization of pol ι by breast cancer cells to correct pyrimidine dimers and suggests that translesion DNA synthesis by pol ι may play an important role in mediating the lower fidelity and higher mutation frequency exhibited by breast cancer cells.

On the other hand, when comparing the mutation distribution shown in Fig. 4, *A* and *B*, it was observed that mutations were predominantly at a few sites, in particular, at positions 155 and 156bp in the supF sequence of the plasmid rescued from MCF10A cells. This indicates that the 3′-cytosine in a 5′-TC sequence context was the most frequently mutagenized site. Unlike in MCF10A cells, there were few obvious hot spots detected in the plasmids rescued from MCF7 cells. It may be the translesion synthesis that different polymerases bypass different types of DNA lesions or may require varying signals in the form of specific DNA structure distortions that are related to the nucleotides found adjacent to the DNA lesion. If either of these explanations is correct, it would suggest that different DNA polymerases may function to mediate the translesion replication process in malignant and nonmalignant breast cells.

**Both Spontaneous and UV-Directed Mutagenesis in Breast Cancer Cells Are Dependent on DNA Pol ι.** To confirm that pol ι is the error-prone DNA polymerase involved in bypass synthesis of UV-induced DNA damage, we used an *in vitro* DNA replication fidelity assay to test whether UV-induced damage could be reversed either completely or in part when pol ι was depleted from cancer cell nuclear extracts prepared from MCF7 and MDA-MB-468 cells. The *in vitro* SV40 DNA replication assay has been shown by a variety of laboratories to adequately reflect many aspects of the human cell DNA replication process carried out *in vivo* and, when coupled with a plasmid containing a reporter gene, is capable of determining the fidelity status of the DNA replication process (23, 46). We, like other investigators (47, 48), have observed that the SV40 origin containing plasmid pSupG1, subjected to a high level of UV irradiation (500 J/m$^2$), barely supports *in vitro* DNA replication (data not shown). To evaluate the role of mutagenesis by pol ι in UV-damaged DNA synthesis, we UV irradiated the parental DNA at lower doses that still supported SV40 DNA replication *in vitro*. Therefore, we optimized the *in vitro* DNA replication assay using UV-damaged DNA that had been irradiated with doses of UV light equivalent to 125 and 250J/m$^2$. To eliminate any unreplicated parental plasmid DNA produced during the *in vitro* DNA replication assay, the replication products were subsequently digested with *Dpn*I. The replication products surviving *Dpn*I digestion consisted of those plasmids that were completely replicated during the *in vitro* replication reaction. Therefore, the daughter DNA plasmids containing mutations generated by UVC irradiation and survived digestion by *Dpn*I had to result from error-prone lesion bypass DNA synthesis.

For our study we prepared nuclear extracts from MCF7 and MDA-MB-468 cells ("Materials and Methods"). Immunodepletion of DNA pol ι from the nuclear extracts was accomplished using an anti-pol ι antibody ("Materials and Methods") and verified as shown in (Fig. 5*A*). The extracts were then assayed for their *in vitro* replication fidelity. The level of mutation in the daughter DNA molecules produced by the pol ι-depleted nuclear extracts was compared with that of non-pol ι-depleted extracts (Fig. 5*B*). The frequency of mutations made in the pol ι-depleted extracts, prepared from the MCF7 and MDA-MB-468 cells, were significantly lower when compared with that in extracts that had not been depleted of pol ι. These results provide evidence suggesting that pol ι contributed to the error-prone DNA synthesis observed in breast cancer cells and that this polymerase functions during both lesion bypass repair synthesis and potentially plays a role in the creation of mutations in newly replicated undamaged DNA.

**Comparison of the DNA Repair Ability in Nonmalignant and Malignant Breast Cells.** Mutations can be created in DNA when there are deficiencies in the DNA repair processes (49). Genomic insults can also be created during the translesion DNA replication process when the translesion DNA polymerases replicate parental DNA containing damaged nucleotides (50). To exclude the possibility that malignant breast cells accumulate mutations because they are unable to repair damaged DNA as efficiently as nonmalignant breast cells, we compared the DNA repair ability of the nonmalignant MCF10A cell line to that of the breast cancer cell lines MCF7 and MDA-MD-468. To monitor the level of DNA repair activity in the cell lines, we used an *in vitro* DNA repair assay ("Materials and Methods") and monitored the abundance of ($\alpha$-$^{32}$P)dCTP incorporated into UV pretreated or undamaged plasmid pSupFG1 DNA. The results of the assay are shown in Fig. 6. Fig. 6, *Lanes 1*, *3*, and *5*, contains the reaction products formed during the assay when an undamaged plasmid was used. Fig. 6, *Lanes 2*, *4*, and *6*, shows the reaction products formed during the assay when the plasmid had been irradiated. These data indicate that there is no significant difference in the ability of the three cell lines to repair damaged DNA. This result implies that the overexpression of pol ι, as well as the increase in the replication mutation frequency in the breast cancer cells, was not associated with a deficiency in the ability of these cells to repair damaged DNA.



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**A**

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GAATTCGAGAGCCCTGCTCGAGCTGTGGGGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAA
CTTAAGCTCTCGGGACGAGCTCGACACCCCCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTT
        81        91        101       111       121       131       141       151       161

TCCTTCCCCCCCCACCACCCCCTCCCCCTCGGCCGAAATTCGGTACCCGGATCCTTAGCGAAAGCTAAGGATTTTTTTTACGCGTGAGCTC
AGGAAGGGGGGGGTGGTGGGGGAGGGGGAGCCGGCTTTAAGCCATGGGCCTAGGAATCCCTTTCGATTCCTAAAAAAAATGCGCACTCGAG
    171       181       191       201       211       221       231       241       251
```

**Point mutation: Total: 46 ( C-T: 38 C-A: 3 T-A:2 C-G:2 T-C:1 )**

**Deletion: Total: 3 ( nt 84-174, nt 94-97, nt 153-255)**

**B**

```
GAATTCGAGAGCCCTGCTCGAGCTGTGGGGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAA
CTTAAGCTCTCGGGACGAGCTCGACACCCCCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTT
        81        91        101       111       121       131       141       151       161

TCCTTCCCCCCCCACCACCCCCTCCCCCTCGGCCGAAATTCGGTACCCGGATCCTTAGCGAAAGCTAAGGATTTTTTTTACGCGTGAGCTC
AGGAAGGGGGGGGTGGTGGGGGAGGGGGAGCCGGCTTTAAGCCATGGGCCTAGGAATCCCTTTCGATTCCTAAAAAAAATGCGCACTCGAG
    171       181       191       201       211       221       231       241       251
```

**Point mutation: Total :43 ( C-T: 22 C-A: 9 T-C : 6 T-A: 3 C-G: 3 )**

**Deletion: Total: 3 ( nt 76-111, nt 108-165 , nt 152-210)**

Fig. 4. Sequence analysis of mutations generated by UVC in supF report gene. UV (1500 J/m$^2$) induced mutations in the replicated plasmids that were rescued from MCF7, and MCF10A cells were analyzed from three independent experiments. *A*, mutations obtained from MCF10A cells (total 49 mutants) *B*, mutations obtained using MCF7 cells (total 46 mutants). Point mutations produced by UV are indicated above each bp, with the listed base representing a change from the top strand. Deletion mutations are presented below the supF gene sequence and indicated by *dashed lines*.

## DISCUSSION

We have demonstrated that high levels of pol ι were expressed in a panel of breast cancer cell lines when compared with that of nonmalignant cells and that the levels of pol ι can be additionally elevated with UV damage. We also have shown that DNA replication in breast cancer cells is of low fidelity, resulting in higher spontaneous mutations. In addition, it was observed that UV damage of breast cancer cells also results in an increase in mutations. Mutation spectra analysis of the UV-induced mutations showed that there was an increased mutation frequency of transversion, which correlated with the pol ι misinsertion property. Furthermore, when we immunodepleted pol ι from cell extracts, it was found that the low fidelity of DNA replication in breast cancer cells can be reversed. Taken together, we conclude that pol ι plays a critical role in the mutagenesis of breast cancer cells and may play a significant role in breast cancer genomic instability.

It is an increasingly accepted concept that cancer cells exhibit a mutator phenotype (51). The mutator phenotype hypothesis proposes that the phenotype is the result of mutations in genes that maintain genomic stability in normal cells. Mutations in target genes that control the fidelity of DNA replication or the efficacy of DNA repair can generate a cascade of mutations in other genes that may maintain the tumor phenotype (52). Among the possible mutagenic pathways involved, the recent discovery of so many sluggish DNA polymerases (the error-prone polymerases) provides us with a new hint that could possibly explain the origin of many cancer cell DNA mutations. Considering that different DNA polymerases exhibit varying degrees of fidelity of DNA synthesis, mutation rates might be altered by



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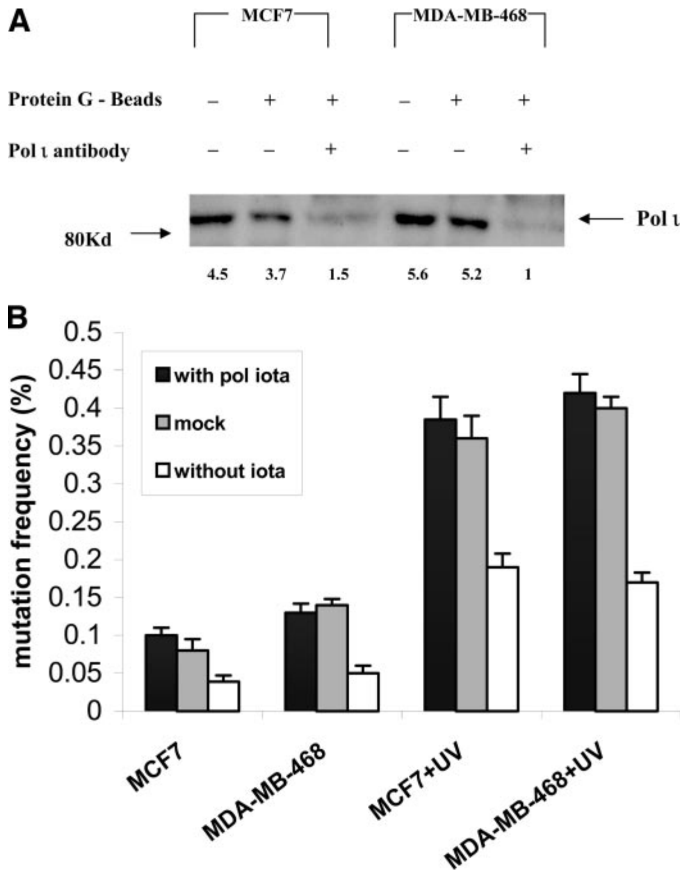


Fig. 5. Detection of mutation frequency generated in SupF reporter gene upon immunodepletion of pol ι from cancer cell nuclear extracts. *A*, Western blot analysis of pol ι after immunodepleting the protein from cell nuclear extracts. Pol ι in the nuclear extracts of MCF7 and MDA-MB468 was depleted using pol ι antibody prebound to IgG beads. Protein G-agarose beads were also used in the preparation of the negative control for these studies (indicated as mock samples). Fifty μg each of MCF7 and MDA-MB-468 nuclear extracts, as well as MCF7 and MDA-MB-468 nuclear extracts immunodepleted of pol ι, and the negative control were subjected to electrophoresis and Western blotting. Densitometric values are indicated below each panel. *B*, detection of mutation frequency generated in SupF reporter gene upon immunodepletion of pol ι from cancer cell nuclear extracts. DNA replication fidelity assays were then performed using pSupFG1 plasmid as described in the text and "Materials and Methods." The data are from three independent experiments. *Error bars*, ±SE.

varying the relative utilization of the various DNA polymerases as well as by mutations that render these enzymes error-prone. We proposed the hypothesis that translesion DNA polymerases may be specialized and highly regulated in normal mammalian cells, but if able to escape from their normal regulation, an error-prone translesion DNA polymerase can be a potential mutator. It could potentially interfere with the replicative DNA polymerases on undamaged DNA or even shift an error-free translesion pathway to an error-prone process when encountering DNA damage. As a result of the sluggish character of the translesion DNA polymerases in DNA replication, their biochemical characteristics of lacking a 3′-5′-exonuclease proofreading function, and low processivity, they are strictly distributive and tightly regulated in normal cells. Some of the DNA polymerases, however, have been found to be elevated in tumor cells and tissues. For example, the level of pol β is significantly elevated in some human adenocarcinomas as well as ovarian cell lines, and deregulation of error-prone DNA pol β is a potential source of genomic alterations (53, 54). Similarly, DNA pol κ, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer (55). Our results show that pol ι is also overexpressed, both in its RNA and protein levels, in malignant breast cell lines as compared with nonmalignant cell lines and that it likely participates in the high mutagenesis observed in breast cancer cells.

So, how is pol ι regulated by the DNA damage response? To answer this question, we measured the protein expression level of pol ι in breast cells, and we found that pol ι became elevated in just 30 min after UV exposure and reached peak levels in ~1–2 h. Until now, among the translesion polymerases, *DinB* transcription has also been found to be up-regulated in response to damage and replication pertubation, and although yeast *RAD30*, which encodes yeast pol η, is also induced by DNA damage, mammalian pol η transcription is not affected by UV irradiation (56). It is thought that under different types of DNA damage, different organisms can choose different translesion DNA polymerases to bypass the lesion. Because up-regulation of pol ι starts in just 30 min after UV irradiation, it suggests that the translesion process is a relatively quick response to DNA damage. When the replication machinery stalls upon encountering DNA damage, the translesion polymerases move very quickly to the damage site and bypass the lesion to rescue the DNA replication process. It is quite clear pol η is primarily responsible for error-free DNA replication past the UV-induced cyclobutane pyrimidine dimer (57–59), and our data show that pol ι was expressed at a very limited elevated level after UV exposure in normal breast cells (Fig. 3). However, in breast cancer cells, pol ι reaches a much higher level after UV irradiation, which suggests that there is a delicate regulation mechanism limiting the error-free pathway and that pol ι plays an important role in translesion synthesis in breast cancer cells. This regulation mechanism limits the error-prone polymerases in nonmalignant breast cells, but in breast cancer cells, this regulation mechanism is defective and allows the error-prone DNA pol ι to escape regulation. The translesion process may actually be a part of the checkpoint response, which regulates cell-cycle transition and facilitates DNA repair processes, or it may induce apoptosis when the organism is faced with genomic stress. There is evidence showing that when there are mutations in the

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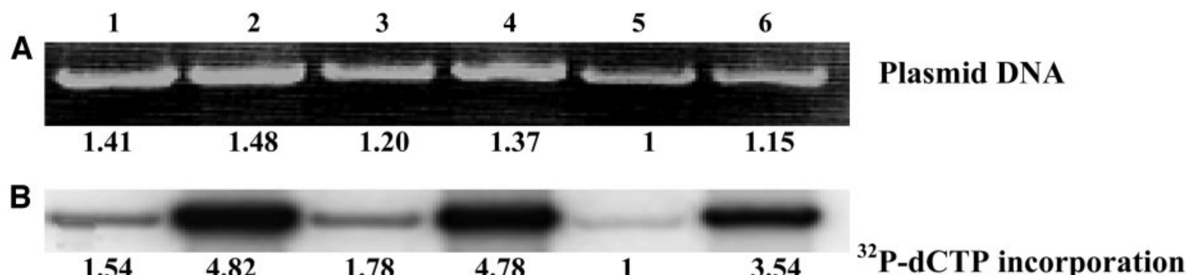


Fig. 6. UV damaged DNA repair of pSupFG1 in nuclear extracts prepared from MCF10A, MCF7, and MDA-MD-468 cells. pSupFG1 plasmid was irradiated with UVC (500 J/m$^2$). *A*, visualization of the plasmid DNA by ethidium bromide staining and autoradiography. *B*, autoradiogram of the same gel showing labeled nucleotide incorporation indicative of DNA repair synthesis. Densitometric values are indicated below the gel panel. *Lane 1*, untreated pSupFG1DNA + MCF10A nuclear extract; *Lane 2*, UV-treated pSupFG1 plasmid DNA + MCF10A nuclear extract; *Lane 3*, untreated pSupFG1 DNA + MCF7 nuclear extract; *Lane 4*, UV-treated pSupFG1 DNA + MCF7 nuclear extract; *Lane 5*, untreated pSupFG1 DNA + MDA-MD-468 nuclear extract; and *Lane 6*, UV-treated pSupFG1 DNA + MDA-MD-468 nuclear extract.

$G_1$-S checkpoint gene, DNA replication is allowed in the presence of unrepaired lesions and results in enhanced mutagenesis (60). We propose the possibility that any failure in the sensors/transducers that detect genomic stress or the effector kinases that relay the signals in the whole chain of the checkpoint process will cause an imbalance of the translesion DNA polymerases, resulting in genomic instability of cancer cells.

When cells encounter UV damage, the removal of the UV lesion is dependent on nucleotide excision repair. However, the relationship between nucleotide excision repair and translesion replication has not been fully investigated, although it has been proposed by others that defective nucleotide excision repair brings about increased DNA mutation formation after UV irradiation because defective nucleotide excision repair leaves more unrepaired lesions than translesion synthesis can handle (61, 62). To determine whether the elevated pol ι-associated error-prone translesion synthesis observed in breast cancer cells is stimulated by defective nucleotide excision repair, we tested nucleotide excision repair activity and showed that nucleotide excision repair appears to be normal in breast cancer cells. We therefore exclude the possibility that the accumulation of mutations in breast cancer cells is the result of a compensation activation of translesion synthesis because of defective nucleotide excision repair. On the other hand, the early response of up-regulated pol ι in breast cancer cells after UV irradiation supports the premise that the translesion process is not a postnucleotide excision repair event and is therefore not regulated by nucleotide excision repair.

Moreover, how does elevated pol ι contribute to mutagenesis in breast cancer cells? First, mutations can result from nucleotide misincorporation because of elevated pol ι copying nondamaged DNA templates during DNA replication or even during DNA repair synthesis. Very recently, Kannouche *et al.* (63) demonstrated that pol ι is located in the replication foci of undamaged cells, suggesting that it may play a role in the maintenance of the genome's integrity during DNA replication. High levels of error-prone DNA pol ι may interfere with the replication machinery, containing the replicative DNA polymerases such as pol δ, pol α, or pol ϵ, when copying the undamaged template via an interaction with accessory molecules such as proliferating cell nuclear antigen (64). It potentially could substitute for a more accurate DNA polymerase and therefore may cause increased spontaneous mutation during the DNA replication process. Secondly, there is increasing evidence that cellular metabolic processes also generate reactive chemical intermediates with the potential to damage DNA and, as a result, might also be a source of spontaneous mutations in cancer especially by reactive oxygen species. So, the spontaneous mutation can be alternatively explained by error-prone damage tolerance. Lastly, when cells are exposed to exogenous DNA damage, it is easy to understand that elevated pol ι may be more active to facilitate the error-prone translesion process and to render more misincorporations at a lesion bypass. Thirdly, concerning UV damage, recent genetic and biochemical studies suggest that translesion replication passed a cyclobutane pyrimidine dimer-TT (*cis*-syn TT dimer) or a 6,4-TT lesion can be facilitated by four DNA polymerases, pol η, ι, ζ, and κ. According to the translesion model raised by Woodgate *et al.* (65), when normal cells encounter UV damage, pol η can efficiently and correctly insert two As opposite two Ts in a *cis*-syn T-T dimer, which is the major photoadduct formed during UV damage. Although pol η has the ability to bypass the 6,4-TT dimer, which distorts the DNA helix to a greater extent than the *cis*-syn TT dimer, it is error-prone with a preference for inserting a G opposite a 3′-T, but they are not extended efficiently by pol η and may be substrates for another enzyme such as pol ζ. Pol ι also has a limited capacity to replicate damaged DNA and/or extend from misinserted bases. Considering the major type of DNA damage is the cyclobutane pyrimidine dimer pyrimidine-pyrimidone dimer, related to the error-free bypass of pol η, it is evident that the predominant error-free/accurate mode of translesion synthesis is dependent upon pol η (58, 66–68), and the relative low mutation formation in the normal cell. So, what if the pol η is defective? What if elevated pol ι occurs in tumor cells? Actually, defects in pol η cause the variant form of xeroderma pigmentosum V (XPV) characterized by UV-induced hypermutability and a strong sunlight-induced skin cancer incidence (69, 70). Interestingly, the spectrum of UV-induced mutations in the hypermutable xeroderma pigmentosum V cells is very different from that of wild-type (71, 72). The UV-induced substitutions are mainly transversions (C→A) up to 48%, whereas in wild-type cells, transition (C→T) predominates up to 90%. Assuming pol η is an "A" rule polymerase (73), pol η can then insert an A opposite a C in the TC and/or CC dimer and bring the C→T transition in wild-type cells. Although pol ζ could conceivably bypass the UV damage, the C→A predominant mutation spectrum observed in xeroderma pigmentosum V cells is strikingly similar to pol ι-dependent misinsertion of a 3′-T opposite a cyclobutane pyrimidine dimer adduct, suggesting that most of the mutagenic events scored in xeroderma pigmentosum V are probably pol ι dependent. These findings also raise another question about the relationship between pol η and pol ι. Is pol ι just a backup to pol η during translesion synthesis, or is there competition between pol η and pol ι? Our research presents the idea that pol ι is elevated in its expression in breast cancer cells and that the UV mutation spectrum of breast cancer cells is quite different from that of nonmalignant breast cells but quite similar to that of xeroderma pigmentosum V. Transversion is largely increased (34%) in breast cancer cells, especially the frequency of C→A transversion which jumps from 5% in normal cells to 20% in cancer cells. This indicates that there is an increased pol-dependent error-prone translesion synthesis in breast cancer cells. Pol η and pol ι share 20–30% homogeneity and contain similar catalytic domains. From recent data published by Kannouche *et al.* (63), it was demonstrated that the localization of pol η and pol ι to the replication machinery is tightly correlated in human cells and that pol ι directly interacts with pol η. Therefore, we raise the hypothesis that there is competition between pol ι and pol η. Because of the very low expression level of pol ι in normal cells, this potential competition is limited to low levels, but once pol η is defective, pol ι can substitute for it because the protein appears to localize into nuclear foci after UV irradiation. However, pol ι-dependent translesion synthesis may also need the help of pol ζ to be a misinsertion extender. On the other hand, if the regulation of the translesion polymerases is defective and therefore results in the elevated level of pol ι as in breast cancer cells, it may efficiently compete with pol η and cause hypermutation and eventually contribute to mutagenesis.

Because of the multistage nature of the carcinogenesis process, two overlapping mechanisms were initially and independently proposed for genomic instability: mutation and selection (3, 74). The 2–3-fold higher mutagenesis observed by overexpression of pol ι, as a potential mutator phenotype gene, could initiate a cascade of mutations, which may randomly create mutations of other mutator genes, such as those involved in DNA repair, cell cycle checkpoints, tumor suppressor genes, and many others. A low level of continuous mutation could eventually contribute to the large number of mutations found in breast cancer cells, including those found in oncogenes and genes specifying tumor phenotype. However, an increased mutation frequency of only 2–3-fold still may not fully explain the number of mutations observed in cancer cells. Therefore, selection of mutated clones is a second crucial mechanism contributing to the mutator phenotype of these cells. According to evolutionary theory, the selection of major mutated genes would be done by clonal proliferation of mutated cells with growth advantage in the local environment. From both the



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experiments in bacteria and model studies, it has been demonstrated that even with a small growth advantage; mutated cells will rapidly overtake and give rise to clonal progeny (75, 76). Mao *et al.* (76) showed in bacteria that after only four rounds of selection, the spontaneous mutation rate progressively increased from 1/100,000 to 1/200, and if exposed to mutagen, the population could reach 100% mutants. As a result of continuous mutation and selection processes, it is reasonable that overexpressed pol ι, as a potential mutator gene, could inevitably, and perhaps rapidly, lead to the genomic instability observed in breast cancer cells. In the future, we will determine whether the hypermutagenetic property of pol ι is specific to breast cancer or a general feature of cancer itself.

## ACKNOWLEDGMENTS

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