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# The E295K DNA Polymerase Beta Gastric Cancer-Associated Variant Interferes with Base Excision Repair and Induces Cellular Transformation▽‡

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**Approximately 30% of human tumors examined for mutations in polymerase beta (pol β) appear to express pol β variant proteins (D. Starcevic, S. Dalal, and J. B. Sweasy, Cell Cycle 3:998–1001, 2004). Many of these variants result from a single amino acid substitution. We have previously shown that the K289M and I260M colon and prostate cancer variants, respectively, induce cellular transformation most likely due to sequence-specific mutator activity (S. Dalal et al., Biochemistry 44:15664–15673, 2005; T. Lang et al., Proc. Natl. Acad. Sci. USA 101:6074–6079, 2004; J. B. Sweasy et al., Proc. Natl. Acad. Sci. USA 102:14350–14355, 2005). In the work described here, we show that the E295K gastric carcinoma pol β variant acts in a dominant-negative manner by interfering with base excision repair. This leads to an increase in sister chromatid exchanges. Expression of the E295K variant also induces cellular transformation. Our data suggest that unfilled gaps are channeled into a homology-directed repair pathway that could lead to genomic instability. The results indicate that base excision repair is critical for maintaining genome stability and could therefore be a tumor suppressor mechanism.**

Tumor cells harbor significantly greater numbers of mutations than somatic cells, and the somatic mutation rate is not high enough to account for the numbers of mutations found in tumors (22). To account for these large numbers of mutations, it is hypothesized that cancer cells possess a mutator phenotype (22). This phenotype is thought to arise from mutations in genes that encode proteins that act to maintain genome stability (2). One example in support of the mutator phenotype hypothesis is the finding that the mutation of certain DNA mismatch repair genes results in hereditary nonpolyposis colon cancer (3, 10).

Base excision repair (BER) is a DNA repair pathway that removes as many 10,000 DNA lesions per cell per day (1, 21). The BER pathway recognizes and excises many types of DNA damage that arise endogenously, including oxidized and methylated bases (40). The simplest and perhaps most common form of BER is short-patch BER, which can be initiated by one of several different DNA glycosylases, each having preferences for specific types of lesions, but with overlapping specificities (11). Monofunctional DNA glycosylases recognize DNA lesions and catalyze the hydrolysis of the N-glycosidic bond to generate an abasic site (25). The abasic site is nicked at its 5′ side by AP endonuclease 1 (APE1), leaving a 3′OH and a 5′deoxyribose phosphate (dRP) (7). DNA polymerase beta (pol β) fills in the single nucleotide gap and catalyzes removal of the dRP group (23). Bifunctional glycosylases, which usually recognize oxidative lesions, generate an abasic site and then catalyze its removal via β elimination to generate a 3′dRP and 5′phosphate (25). APE1 then catalyzes removal of the 3′dRP, leaving a 3′OH, to which pol β can bind and fill in the resulting single nucleotide gap. In both cases, the XRCC1/ligase IIIα complex catalyzes ligation of the resulting ends (38). During long-patch BER, which appears to be a minor cellular repair pathway (33), DNA polymerases δ and/or ε bind to the 3′OH and fill in the gap with several nucleotides (12, 24). In this case, the Fen1 flap endonuclease removes the 5′dRP group after strand displacement synthesis has occurred (8). An alternative BER pathway that does not depend on APE1 is suggested to be utilized when the Neil glycosylases initiate repair (39). Neil 1, 2, and perhaps 3 catalyze excision of the damaged base via δ elimination, leaving a 3′phosphate and a 5′phosphate. The 3′phosphate is removed by polynucleotide kinase, leaving a gap that is most often filled by pol β.

Thirty percent of human tumors that have been studied express DNA pol β variant proteins that are not present in normal tissue (15, 36). Single amino acid substitutions are found in 48% of these tumors, 12% contain truncated variants, 14% harbor multiple alterations, and 25% express a protein in which exon 11 is deleted through alternative splicing. The exon 11 splice variant has also been found in normal tissue of other patients and in cell lines grown in culture (26), so its link to cancer is controversial.

We previously reported that expression of cancer-associated pol β variants in mouse cells could lead to a series of cancer-associated phenotypes, including an increased mutation frequency and the induction of cellular transformation (19, 37). Specifically, the K289M and I260M colon and prostate cancer-associated pol β variants, respectively, induce mutations within specific sequence contexts both in vivo and in vitro (6, 19). When expressed in established mouse cells, both of these vari-

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TABLE 1. DNA substrates employed in primer extension, gel shift, and BER assays

| Substrate | Sequences[a] |
|---|---|
| CII5bp | 5′ TTGCGACTTATCAACGCCCACAT GCTGTCTTCTCAGTTTC 3′ |
| | 3′ AACGCTGAATAGTTGCGGGTGTA**G**TCATCGACAGAAGAGTCAAAG 5′ |
| 45AG | 5′ GCCTCGCAGCCGTCCAACCAAC CAACCTCGATCCAATGCCGTCC 3′ |
| | 3′ CGGAGCGTCGGCAGGTTGGTTGA**A**GTTGGAGCTAGGTTACGGCAGG 5′ |
| LPSD | 5′ CTGCAGCTGATGCGC**U**GTACGGATCCCCGGGTAC 3′ |
| | 3′ GACGTCGACTACGCGGCATGCCTAGGGGCCCATG 5′ |
| HxB | 5′ GGATCATCGTTTTT(**Hx**) GCTACATCGC 3′ |
| | 3′ CCTAGTAGCAAAAA T CGATGTAGCG 5′ |

[a] The template base and the uracil and hypoxanthine residue are in boldface type in 1-bp gapped DNA, 5-bp gapped DNA, and BER substrate, respectively.

ants also induce focus formation and anchorage-independent growth. In this paper, we provide evidence that expression of the E295K gastric carcinoma-associated pol β variant (15) in mouse cells interferes with BER and induces sister chromatid exchanges (SECs) and cellular transformation. Our results are consistent with the interpretation that the E295K gastric cancer-associated variant plays a role in the induction of a mutator phenotype in cells that could lead to tumorigenesis or tumor progression. This is similar to the finding of the link between mutations in the MYH gene and colorectal carcinoma (for a review, see reference 5). Thus, BER is likely to be a tumor suppressor mechanism.

## MATERIALS AND METHODS

**Plasmids and cloning.** The E295K variant was generated by a site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer, using pET28a-WT pol β as a template, followed by DNA sequencing at the WM Keck facility at Yale University School of Medicine (16).

For transfection into mouse cells, hemagglutinin (HA)-tagged pol β was cloned into the Tet-regulated pRVYTet-Sis retroviral vector (18, 28, 37) using standard molecular cloning procedures. E295K was obtained by site-directed mutagenesis using the pRVYTET-WT pol β as a template with the following primers: GGGGGCGGA TGGTGTACTTATTGATTGTGAAGCCCTT and AAGGGCTTCACAATCAA TAAGTACACCATCCGCCCCC. In this vector, the left-hand retroviral long terminal repeat drives expression of tTA tetracycline (Tet) transactivator (37), the tetO/CMV promoter drives expression of pol β proteins in a tetracycline-repressible manner, and an internal SV40 early promoter drives expression of the hygromycin resistance gene. Thus, when Tet is present in the growth medium, expression of the wild-type (WT) or E295K proteins is turned off. However, expression of these proteins occurs when Tet is removed from the growth medium.

**Protein expression and purification.** For characterization in in vitro assays, WT pol β and variant E295K were overexpressed in *Escherichia coli* strain BL21 (DE3) and purified as described previously (6).

**Preparation of DNA substrates.** DNA substrates for in vitro assays were prepared from oligonucleotides. Oligonucleotides were synthesized by the WM Keck facility at Yale University. Oligonucleotide HxB was a generous gift from Leona Samson (Massachusetts Institute of Technology). The substrates used are shown in Table 1. CII5bp, 45AG, and LPSD were used for the primer extension, gel mobility shift, and base excision repair assays, respectively. The primer oligonucleotide was labeled at the 5′ end using T4 polynucleotide kinase (New England Biolabs) and [$\gamma$-$^{32}$P]ATP (Amersham). After purification by a Bio-Rad spin column to remove unincorporated deoxynucleoside triphosphates (dNTPs), annealing was performed by mixing phosphorylated template, radiolabeled primer, and phosphorylated downstream oligonucleotides in 50 mM Tris-HCl, pH 8.0, and 0.25 M NaCl. The mixture was incubated sequentially at 95°C for 5 min, slowly cooled to 50°C for 30 min, incubated at 50°C for 20 min, and immediately transferred to ice. Oligonucleotide HxB was labeled and annealed with complementary strand as described by Engelward et al. (9). The quality of annealing was assessed by resolving the product in an 18% native polyacrylamide gel, followed by autoradiography.

**In vitro primer extension assay.** Primer extension reactions were conducted in a solution containing 50 mM Tris-Cl buffer (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 20 mM NaCl, 10% glycerol, and 50 μM each of dATP, dCTP, dGTP, and dTTP (Sigma). Reactions were carried out at 37°C for various time lengths, after which they were stopped by addition of equal volumes of 90% formamide dye and 0.3 M EDTA. Samples were resolved by electrophoresis on 20% polyacrylamide gels containing 8 M urea (Sigma), visualized, and quantified using a phosphorimager. The primer extension assay was carried out under steady-state conditions (enzyme/DNA ratio = 1:60) and in single exponential conditions (enzyme/DNA ratio = 10:1).

**Gel mobility shift assay.** Various concentrations of pol β protein (0.1 to 1,000 nM) were incubated with 0.1 nM radiolabeled gapped 45AG DNA substrate in buffer containing 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and 0.1% Nonidet P-40 at room temperature (RT; 23°C) for 15 min. Samples were loaded onto a 6% native polyacrylamide gel with the current running at 300 V at 4°C. After the sample was loaded, the voltage was reduced to 150 V. Bound protein was quantified using ImageQuant software, after scanning the gel using a Molecular Dynamics PhosphorImager. Protein bound to DNA resulted in a shift of the DNA on the gel compared to DNA without bound protein. The fraction bound is the ratio of the intensity of all shifted species divided by the total. The dissociation constant for DNA ($K_D$) was estimated from fitting the bound protein ($Y$) versus protein concentration ($x$) with the equation $Y = [(mx)/(x + K_D)] + b$, where $m$ is a scaling factor and $b$ is the apparent minimum $Y$ value.

**Reconstituted base excision repair assay.** We conducted an in vitro assay to determine if E295K could function in BER. 5′-end-labeled LPSD substrate (Table 1) was used as a BER substrate. In a 20-μl reaction mixture, 20 nM uracil DNA glycosylase (UDG; New England Biolabs)-treated substrate was incubated for 5 min with 10 units of APE1 (New England Biolabs), 2 ng purified pol β (WT or E295K), and 50 units of T4 DNA ligase (New England Biolabs) in buffer B (45 mM HEPES [pH 7.8], 70 mM KCl, 7.5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 2 mM ATP, and 20 μM each of dNTP) at 37°C. Finally, formamide dye containing EDTA (30) was added to stop the reaction. The products were resolved on a 20% denaturing polyacrylamide gel followed by visualization on the PhosphorImager.

**Cell extract base excision repair assay.** Typically, AAG DNA glycosylase cleavage assays were performed at 37°C for 50 min in a 20-μl reaction mixture containing 100 fmol hypoxanthine containing oligonucleotide substrate and 20 μg of whole-cell extract diluted in glycosylase buffer (20 mM Tris-HCl [pH 7.6], 100 mM KCl, 5 mM EDTA, 1 mM EGTA, and 2 mM 2-mercaptoethanol) as described previously by Engelward et al. (9). Immediately after incubation, tubes were kept on ice for 5 min and then supplemented with BER buffer (20 mM Tris Hcl [pH 7.6], 10 mM $MgCl_2$, 2 mM ATP, 20uM dATP) and incubated again at 37°C for 1 min. Reactions were quenched by addition of an equal volume of formamide gel loading dye (90% formamide, 0.3 M EDTA). The percent primer extension was calculated by dividing the pixels of the n + 1 product by total pixels [n + (n + 1)] and multiplying by 100.

**5′dRP lyase assay.** The dRP lyase assay using purified pol β protein was performed as described previously (13) to determine if the E295K variant possessed dRP lyase activity. The 5′dRP-primer template was generated by treatment of 200 nM of the LPSD substrate with 12 units of uracil DNA glycosylase, followed by 20 units of APE1. Approximately 100 nM of this DNA substrate was used immediately in reaction mixtures (24 μl) containing either 200 nM WT or variant DNA polymerase in buffer R (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 20 mM KCl, and 2 mM DTT). Reaction mixtures were incubated at 37°C for 20 min. The reaction product was stabilized by the addition of 2 M sodium borohydride to a final concentration of 340 mM, followed by incubation on ice for 30 min. Stabilized (reduced) DNA products were ethanol-precipitated in the presence of 0.1 μg/ml of tRNA and resuspended in water, and an equal volume of formamide dye was added, followed by analysis on a 20% polyacrylamide gel, which

was visualized with an 860 PhosphorImager (Molecular Dynamics, Inc.). The dRP lyase assay with cell extracts was performed as described previously (35).

**Pull-down assays.** We performed a pull-down assay to determine if the E295K variant interacted with XRCC1 and DNA ligase IIII as does WT pol β. A cell extract was prepared from the 88Tag (pol $\beta^{-/-}$) cell line (see below), which is mouse embryo fibroblasts (MEFs) with pol β deleted, as we described previously (19). Approximately 20 μg of His-tagged WT pol β protein or His-tagged E295K was each separately added to 50 μg of the cell extract, and the mixture was then allowed to incubate for 15 min on ice. Approximately 15 μl of nickel beads were equilibrated by adding 150 μl nickel buffer (50 mM Tris-Cl, pH 7.6, 75 mM KCl, 0.1% IGPEAL, 1 mM DTT, 10 mM imidazole). Next, the proteins were added to the equilibrated nickel beads, and the mixture was incubated for one hour on ice with gentle mixing every 10 minutes. The beads were then centrifuged at 2,000 rpm for 1 min and washed seven times with 150 μl of nickel buffer. After the final wash, 2× sodium dodecyl sulfate (SDS) gel dye containing β-mercaptoethanol was added to the beads. The solution was boiled for 5 min and centrifuged at 10,000 rpm. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was then blocked with a 50-ml solution of 5% nonfat milk, 1× phosphate-buffered saline (PBS), and 0.1% Tween 20 for one hour at RT. After washing the blot twice with 1× PBS, primary antibody of anti-DNA ligase III (at a dilution of 1:1,000; BD Transduction Laboratories) or anti-XRCC1 (at a dilution of 1:1,000; Abcam) was added in a 5-ml solution of 5% nonfat milk and 1× PBS, and the blot was incubated overnight at 4°C. Subsequent to being washed with 1× PBS-0.1% Tween solution three times, the membranes were washed twice with 1× PBS. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (at a dilution of 1:10,000) for 1 h at RT. The membranes were then washed as described above. To detect the bands, an enhanced chemiluminescence kit was used according to the manufacturer's directions (PerkinElmer).

**Cell lines and cell culture.** MEF cell lines 92TAg (WT), 88TAg (pol $\beta^{-/-}$), and 308TAg ($Aag^{-/-}$) were gifts from Leona Samson (Massachusetts Institute of Technology) (9, 32). Cells were maintained in Dulbecco modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), L-glutamine (Invitrogen), β-mercaptoethanol (Sigma), and penicillin-streptomycin (Invitrogen) at 37°C in a humidified 5% $CO_2$ incubator. C127 cells were obtained from ATCC. C127 is a nontransformed clonal line derived from a mammary tumor of an RIII mouse (20). The cells were maintained in DME-10 (Dulbecco modified Eagle's medium, 10% fetal bovine serum, penicillin-streptomycin).

**Transfection, infection, and expression analysis.** To infect the MEFs, the WT pol β and E295K constructs were packaged into retroviruses using the GP2-293 viral packaging cell line. Exponentially growing MEFs at approximately 50% confluence in 60-mm dishes were incubated with 1 ml of virus with 1 μg/ml Polybrene (Sigma) for 2 h. The medium was then removed and replaced with fresh medium containing 1 μg/ml Polybrene. After an overnight incubation, cells were split into 100-mm dishes at different cell densities. All dishes were incubated overnight and then supplemented with 400 μg/ml hygromycin B (HygB; Invitrogen) to select clones with stably integrated constructs. Tetracycline (Sigma) at a concentration of 4 μg/ml was also present in the medium to repress expression of the WT or E295K proteins. Individual cell colonies were cloned using cloning cylinders and expanded in the same growth medium.

**Western blotting.** Western blotting experiments were performed to assess expression of the WT and E295K constructs in vivo. Each cell clone was seeded into duplicate wells of six-well plates at the same cell density. Cells were grown in either the presence or absence of Tet. After 2 days, the wells were washed with PBS, and 100 μl 80°C 1× SDS loading buffer was added into each well. The cell lysate was placed into an Eppendorf tube and boiled for 5 min. Approximately 12 μl of cell lysate was resolved on a 10% SDS-PAGE gel. Protein was transferred onto a polyvinylidine difluoride membrane using a semidry transfer apparatus. Western blotting was carried out as described previously (37) using monoclonal anti-pol β antibody (Abcam; Ab1831).

**MMS sensitivity assay.** Sensitivity to methyl methanesulfonate (MMS) was determined by a growth inhibition assay. Cells were seeded at 1,500 cells per well and left at 37°C overnight to attach. Cells were treated by multiple dilutions of MMS for one hour at 37°C, washed in fresh medium, and incubated for 72 h under normal growth conditions. The numbers of viable cells were determined by the CellTiter 96 AQueous one-solution cell proliferation assay (Promega) as recommended by the manufacturer. At least four replicas for each clone were averaged, and at least two clones for each variant were tested. Data are expressed as the percentage of growth control (no MMS treatment). Statistical analysis and graphs were made by Prizm software (GraphPad). Differences were considered statistically significant if $P$ was $<0.05$ by the Mann-Whitney test.

**Immunofluorescence.** Immunofluorescence experiments were conducted to determine if cells expressing WT or E295K harbored DNA breaks. Cells were split into six-well plates containing acid-treated cover slides and incubated overnight. The cover slides were then washed with 1× PBS, fixed with 3.7% formaldehyde in 1× PBS for 30 min, and then washed with PBS three times, 15 min each time. Triton X-100 (0.05%) was then added for 10 min to permeabilize the cells. After three washes with 1× PBS, slides were blocked with 3% bovine serum albumin (Sigma)-1:200 normal donkey serum (Jackson Immunoresearch) in 1× PBS for one hour and then incubated with rabbit anti-γ-H2AX (Upstate) in the blocking reagents for 2 h at 37°C in a humidified box. The slides were washed with 1× PBS four times, 10 min each time. Secondary antibody conjugated with FITC (fluorescein isothiocyanate; Jackson Immunoresearch) was then incubated with slides for one hour, followed by washing with 1× PBS and staining with DAPI (4′,6′-diamidino-2-phenylindole). The mounted slides were viewed with a Zeiss Axioscope, and images were captured with a charge-coupled-device camera. Monocolor pictures were merged and enhanced with Adobe Photoshop. The numbers of γ-H2AX foci in 100 nuclei were counted for each type of cells.

**SCE assay.** The sister chromatid exchange (SCE) assay was carried out as described by Sobol et al. (34) with minor changes to determine if cells expressing WT or E295K harbored SCEs. Briefly, one million cells were seeded into a 100-mm tissue culture dish and incubated for 8 h. Cells were then treated or not treated with MMS. For MMS treatment, cells were exposed to 0.2 mM MMS for 1 h. The medium was then changed to McCoy's 5A (Invitrogen) media supplemented with 10 uM bromodeoxyuridine (Sigma). All dishes were incubated for 18 h, and then 0.1 μg/ml Colcemid (Invitrogen) was added into each dish. The cells were incubated for another 2 h before they were harvested by mitotic shake-off. They were washed once with 1× PBS, resuspended with hypotonic buffer (0.075 M KCl), and incubated at 37°C for 30 min. Cells were then centrifuged and fixed with methanol/acidic acid (3:1). Finally, cells were dropped onto wet slides and dried. Slides were stained with 5 μg/ml Hoechst 33258 for 20 min and washed with 0.067 M Sorensen's buffer (pH 6.8; equal volumes of $Na_2HPO4$ and $KH_2PO4$). A coverslip was applied onto each slide, and then the edges of the coverslip were sealed with rubber cement. Slides were heated under a 60 W light bulb overnight and then incubated at 65°C in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min, washed with water, and stained in a 5% Giemsa solution in 0.067 M Sorensen's buffer for 5 min. Again they were washed with water and dried at RT. All pictures were taken under a lens with a 100× objective. Fifty metaphase spreads were counted per data point.

**Focus formation assay.** Cells were passaged every 3 to 4 days in the presence or absence of 4 μg/ml Tet. At various passages, approximately $1 \times 10^4$ cells were seeded into each of two T25 flasks (Falcon). These cells were fed every 3 to 4 days with DME-10, and after 21 days, they were stained with Giemsa to visualize foci. The presence of foci was also monitored by microscopic examination as described previously (37).

**Anchorage-independent growth.** Approximately $2 \times 10^5$ cells, grown in the presence (noninduced) or absence (induced) of Tet for 20 passages, were mixed with DME-10 containing 0.3% Difco Noble agar containing or lacking Tet, as appropriate. This mixture was poured onto a layer of DME-10 containing 0.6% Difco Noble agar in a 60-mm dish. Cells were fed twice weekly with 1 ml of DME-10 containing 0.3% Difco Noble agar in the presence or absence of Tet, as described previously (37). The number of colonies present in each of five microscope fields per plate from a total of three plates per experiment was counted after 7 weeks of growth.

## RESULTS

**The E295K gastric cancer-associated variant is inactive.** In the original study describing the E295K gastric cancer pol β variant, the authors showed with an in vitro assay that HeLa cell extracts harboring this variant appeared to be deficient in BER (15). Examination of the crystal structure of pol β shows that in a catalytically inactive open form of pol β, amino acid residue D192 interacts with R258. However, once pol β assumes its closed and catalytically active form, residue E295 interacts with R258, sequestering R258 away from D192 and freeing D192 to participate in catalysis (17). These combined results suggested to us that alteration of E295 to K would eliminate the interaction between residues 295 and 258 and lead to inefficient DNA synthesis. In fact, we show in Fig. 1 that

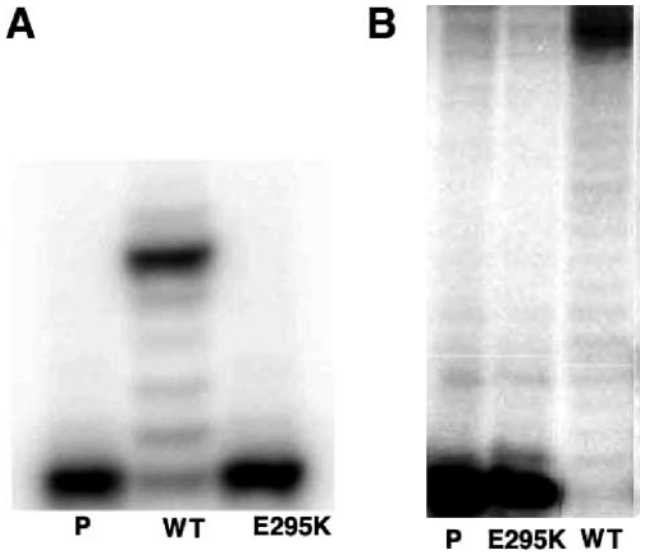


FIG. 1. E295K has no polymerase activity. (A) An in vitro primer extension assay under steady-state conditions. Typically, 5 nM pol β and 300 nM DNA substrate were incubated with 10 mM $MgCl_2$ and 50 μM each of dATP, dCTP, dGTP, and dTTP at 37°C for 2 min. (B) An in vitro primer extension assay with enzyme in excess of DNA. Five hundred nanomolar pol β and 50 nM substrate were incubated with 10 mM $MgCl_2$ and 50 μM each of dATP, dCTP, dGTP, and dTTP at 37°C for 30 min. Note that under these conditions, WT pol β exhibits strand displacement synthesis. P represents the primer alone; WT and E295K indicate the results obtained from the reactions with WT and E295K pol β proteins, respectively.

the E295K variant is inactive in in vitro primer extension reactions conducted either under steady-state reaction conditions or when the enzyme was in vast excess of the DNA substrate. To be certain that this was not due to an inability of the E295K pol β variant to bind to DNA, we determined its apparent equilibrium dissociation constant ($K_D$) for DNA as described previously (6). E295K and WT pol β had apparent $K_D$s of 28 nM and 12 nM, respectively, for the 45AG one-base pair gapped DNA substrate (Table 1). Thus, the E295K variant is catalytically inactive but is able to bind to DNA with an affinity similar to that of WT pol β.

**E295K interferes with WT pol β in an in vitro primer extension assay.** Next, we wanted to determine whether E295K interferes with DNA synthesis by WT pol β. We mixed various amounts of each protein along with dNTPs, buffer, and DNA substrate in a primer extension reaction described in Materials and Methods, resolved the products on a sequencing gel, and quantified them. In Fig. 2, we demonstrate that when E295K and WT are present in equal amounts, there is less DNA synthesis than when WT is present alone. In fact, even half the concentration of E295K compared to that of WT reduces the amount of DNA synthesis products. This suggested that E295K could interfere with WT in gap filling during BER, as proposed by Iwanaga et al. (15).

**E295K cannot support BER of uracil.** To determine if E295K could function in BER of uracil, we used a reconstituted system of purified proteins that support short-patch BER. In this system, both the gap-filling step and the removal of the dRP group are dependent upon pol β. As shown in Fig. 3A, in the presence of WT pol β (lane 5), we observe a fully ligated product, but in the presence of E295K (lane 6), no repaired product is observed. This appears to be due to the inability of E295K to synthesize DNA, as evidenced by the lack of an n + 1 gap-filling product. We demonstrate in Fig. 3B that E295K has dRP lyase activity. Thus, the inability of E295K to support BER is likely due to its lack of DNA synthesis, at least in our in vitro BER assay. Finally, in Fig. 3C, we show that the E295K protein can interact physically with both XRCC1 (lane 1, XRCC1) and DNA ligase III (lane 4, ligase III), as assessed by a pull-down assay. This suggests that E295K is able to interact with BER proteins in a manner similar to that of WT pol β. Taken together, these results indicate that the reason E295K is unable to support BER is due to its inability to catalyze DNA synthesis.

**Expression of E295K and WT in MEFs.** Because our in vitro studies showed that E295K interfered with WT pol β, we wished to determine if this also occurred in cells. WT, pol $\beta^{-/-}$, or $Aag^{-/-}$ MEFs were infected with retrovirus-contain-

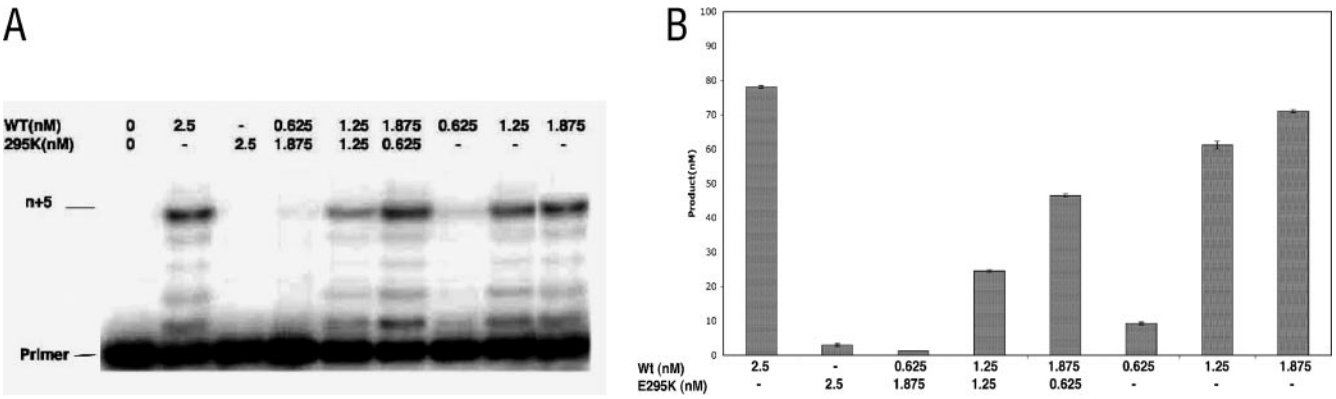


FIG. 2. E295K interferes with polymerase activity of WT. (A) Autoradiograph showing the results of the primer extension assay using the CII5bp 5-nucleotide gapped DNA substrate and various concentrations of WT pol β and E295K. Three hundred nanomolar radiolabeled 5-bp gapped DNA (CII5bp) was incubated with WT (2.5 nM) or E295K (2.5 nM) alone. For the interference studies, DNA was incubated with WT and E295K together (final concentration was 2.5 nM) with three different E295K/WT ratios (3:1, 1:1, and 1:3). A control experiment was performed with various concentrations (as used in interference study) of WT alone. (B) Quantification of the results presented in panel A.

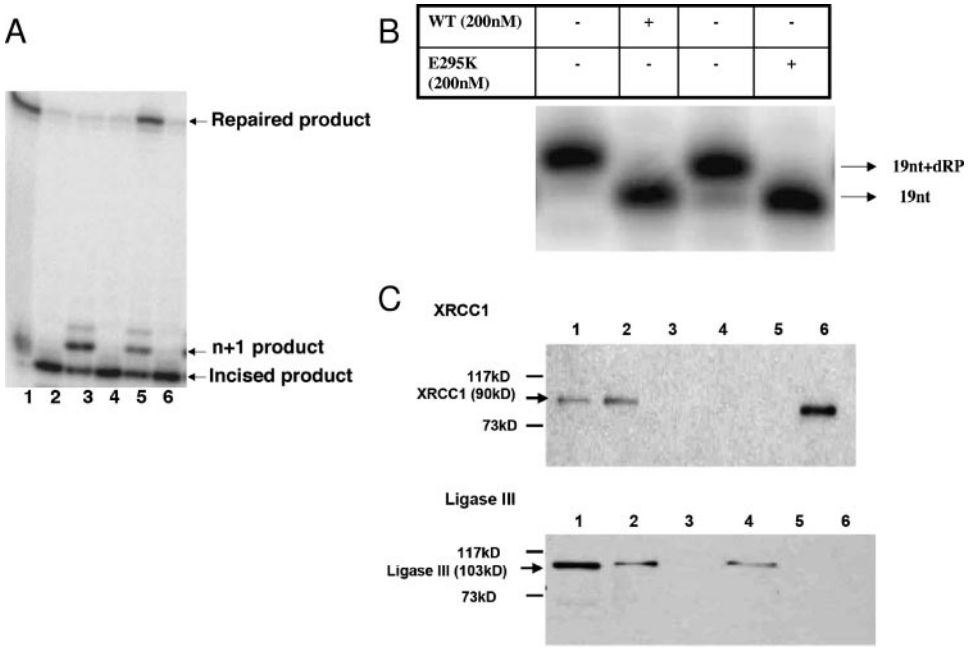


FIG. 3. E295K cannot support BER. (A) E295K is unable to support BER. Lane 1, UDG-treated substrate; lane 2, 20 nM UDG-treated substrate incubated with 10 units APE1; lane 3, UDG-treated substrate incubated with 10 units APE1 and 2 ng WT pol β; lane 4, UDG-treated substrate incubated with 10 units APE1 and 2 ng E295K pol β; lane 5, UDG-treated substrate incubated with 10 units APE1, 2 ng WT pol β, and 50 units T4 DNA ligase; lane 6, UDG-treated substrate incubated with 10 units APE1, 2 ng E295K pol β, and 50 units T4 DNA ligase. (B) E295K has dRP lyase activity. The 5′dRP-containing DNA substrate (100 nM) was incubated in buffer R at 37°C for 20 min with 200 nM of pol β, followed by the addition of 340 mM $NaBH_4$ and stabilization of the reaction product as indicated in Materials and Methods. The products were analyzed by a denaturing 20% polyacryamide gel and visualized by autoradiography. (C) E295K interacts with XRCC1 and ligase III. An Ni-NTA pull-down experiment was carried out as described in Materials and Methods. Twenty micrograms of purified His-tagged β or E295K variant polymerase and 50 μg of the pol β null MEF cell line extract (Δβ MEF) were used for pull-down assays. The interaction was confirmed by Western blot analysis using antisera raised against either XRCC1 (top panel) or ligase III (bottom panel), denoted at the top of the figure. Western blot analysis of the interaction of WT and E295K with XRCC1 (top panel): lane 1, E295K and Δβ MEF pulldown; lane 2, WT and Δβ MEF pulldown; lane 3, Δβ MEF alone with beads; lane 4, E295K alone with beads; lane 5, WT alone with beads; lane 6, 50% Δβ MEF input. Western blot analysis of the interaction of WT and E295K with ligase III (bottom panel): lane 1, 50% Δβ MEF input; lane 2, WT and ΔβMEF pulldown; lane 3, WT alone with beads; lane 4, E295K and Δβ MEF pulldown; lane 5, E295K alone with beads; lane 6, WT alone with beads.

ing pRVYTET with either the WT or the E295K construct and selected with Hyg B for successful viral integration, and stable clones were isolated and expanded. To determine whether the proteins were expressed in the cells, we prepared cell lysates and performed a Western blotting experiment with a monoclonal antibody raised against pol β. As shown in Fig. 4A, both the WT and E295K proteins are expressed in the MEFs. Because exogenous E295K and WT are tagged with the HA epitope, they migrate more slowly on the SDS-PAGE gel than the endogenous pol β protein. WT HA-pol β expression is inducible in the MEFs by removal of Tet from the growth medium, and the levels of endogenous and exogenously expressed WT pol β are similar. However, we found that we were not able to shut off expression of HA-E295K in the MEFs by

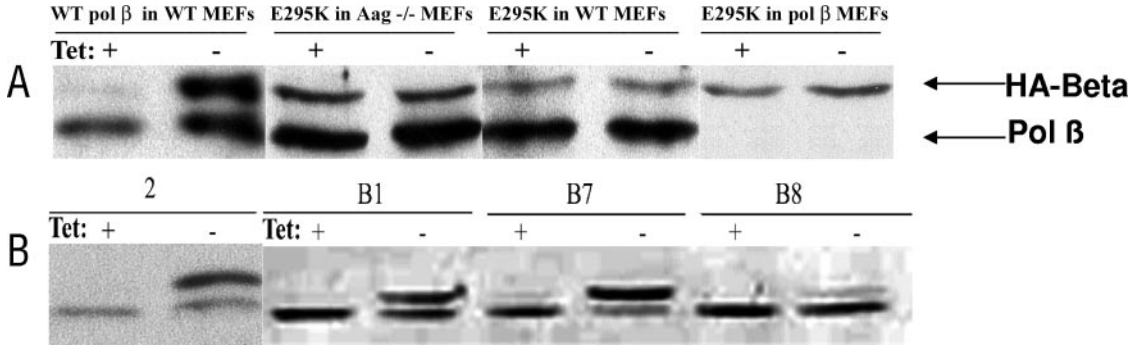


FIG. 4. Western blot showing the expression of WT pol β and E295K in mouse cells. (A) WT HA-pol β or HA-E295K is expressed in MEFs; (B) HA-E295K is expressed in C127 cells. Arrows denote endogenous and exogenous pol β. pol β is the endogenous form, and HA-pol β is exogenous pol β.

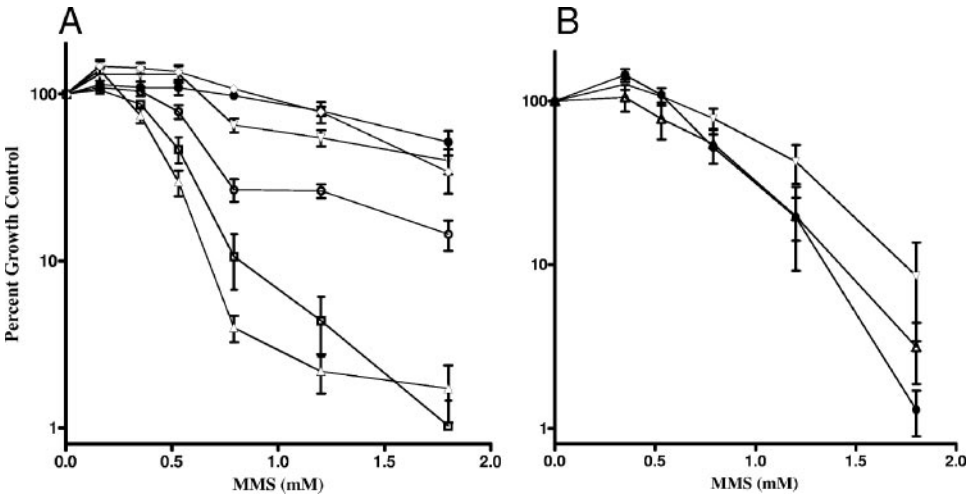


FIG. 5. The E295K pol β variant interferes with BER. (A) Expression of E295K sensitizes cells to MMS. WT or pol βΔ MEFs expressing either WT or E295K pol β were exposed to MMS, and percent growth control was assessed as described in Materials and Methods. ◆, WT MEFs; ●, WT MEFs expressing WT pol β; ○, WT MEFs expressing E295K pol β; △, pol βΔ MEFs; □, pol βΔ MEFs expressing E295K pol β; ▼, pol βΔ MEFs expressing WT pol β. (B) The E295K pol β variant does not sensitize AAG-deficient cells to MMS. ▼, AAG-deficient MEFs; △, AAG-deficient MEFs expressing E295K pol β; ●, AAG-deficient MEFs expressing WT pol β. Note that all AAG-deficient MEFs express endogenous WT pol β.

removing Tet from the growth medium. HA-E295K is expressed at less than half the level of endogenous WT pol β in the MEFs.

**Expression of E295K sensitizes WT cells to MMS.** MMS is an SN2 alkylating agent that forms predominantly 7-methylguanine and 3-methyladenine adducts in DNA (14, 31), which are repaired by the BER pathway. It has been previously shown that pol β null MEFs are much more sensitive to MMS than WT MEFs, demonstrating that pol β plays a key role in BER (33). This sensitivity can be rescued by expression of either the full-length pol β or its 8-kDa dRP lyase domain (33). We found that expression of E295K in WT MEFs sensitized the cells to MMS, as shown in Fig. 5A. In comparison, WT cells expressing WT pol β were not sensitive to MMS. This suggested that the E295K variant interfered with BER in the cells. Interestingly, expression of E295K in pol β-deleted MEFs did not restore the MMS resistance of these cells as does WT pol β, even though this variant possesses dRP lyase activity.

The DNA glycosylase AAG-deficient cells are not sensitive to MMS, whether or not they are pol β proficient, because the methylated bases cannot be converted to abasic sites by AAG and therefore are not toxic to cells (9). To determine if the E295K variant disrupts BER, we expressed it in $Aag^{-/-}$ MEFs and characterized cell survival in the presence of MMS. As shown in Fig. 5B, there is no significant difference in MMS sensitivity when either pol β WT or the E295K variant is expressed in the $Aag^{-/-}$ MEFs. This suggests that E295K interferes with BER in vivo, perhaps by binding to gaps in the DNA and not allowing WT pol β to access these gaps.

Extracts prepared from cells expressing E295K cannot support BER of hypoxanthine. Hypoxanthine is a substrate for the Aag DNA glycosylase (9). To determine if E295K can support BER of this adduct, we prepared extracts from MEFs that express E295K and monitored BER in vitro, as shown in Fig. 5. In this assay, the DNA substrate containing hypothanine is first incubated in buffer that supports the Aag reaction. As can be seen from the figure, only a small percentage of the DNA substrate is acted upon by Aag and Ape1 in the extracts, as observed by others (L. Samson, personal communication), resulting in the excised product (Fig. 6). This product becomes the substrate for extension by pol β. Extracts prepared from pol βΔ MEFs expressing WT pol β support BER (Fig. 6, lanes 4 and 5), as can extracts prepared from pol β-WT MEFs that express WT pol β (Fig. 6, lanes 10 and 11). However, extracts from pol βΔ cells that express the E295K variant do not support BER, apparently because the DNA substrate cannot be extended to generate the n + 1 product (Fig. 6, lanes 6 and 7). Interestingly, expression of E295K in pol β-WT cells appears to interfere with BER in vitro (Fig. 6, lanes 12 and 13), which is consistent with the primer extension assay shown in Fig. 3. Cell extracts from Aag-deficient cells do not support the BER of hypoxanthine, because they cannot generate the excised product (n). These cell extracts do support BER of hypoxanthine if Aag enzyme is added to the extract (data not shown). Thus, E295K does not support BER of hypoxanthine and interferes with the repair of this adduct in the presence of WT pol β. Note that E295K exhibits dRP lyase activity in cell extracts as shown in Fig. S1 in the supplemental material.

**Expression of E295K increases the amount of SCEs.** In S phase, DNA double-strand breaks are often repaired through error-free homology-directed repair (29). Cells that are defective in double-strand break repair, such as Blooms Syndrome cells or cells which have many double-strand breaks, have an increased level of SCEs (4, 27). Next, we quantified the numbers of SCEs in WT MEFs expressing either WT or E295K pol β in the presence and absence of MMS; examples of nuclei with SCEs are shown in Fig. 7A and B. As shown in Fig. 7C, MEFs expressing E295K have larger numbers of SCEs per nucleus than MEFs expressing pol β WT protein. In fact, less than 5% of the nuclei of cells expressing WT pol β have more than 20 SCEs, whereas 20% of the nuclei expressing E295K have more than 20 SCEs. Treatment of the cells with MMS

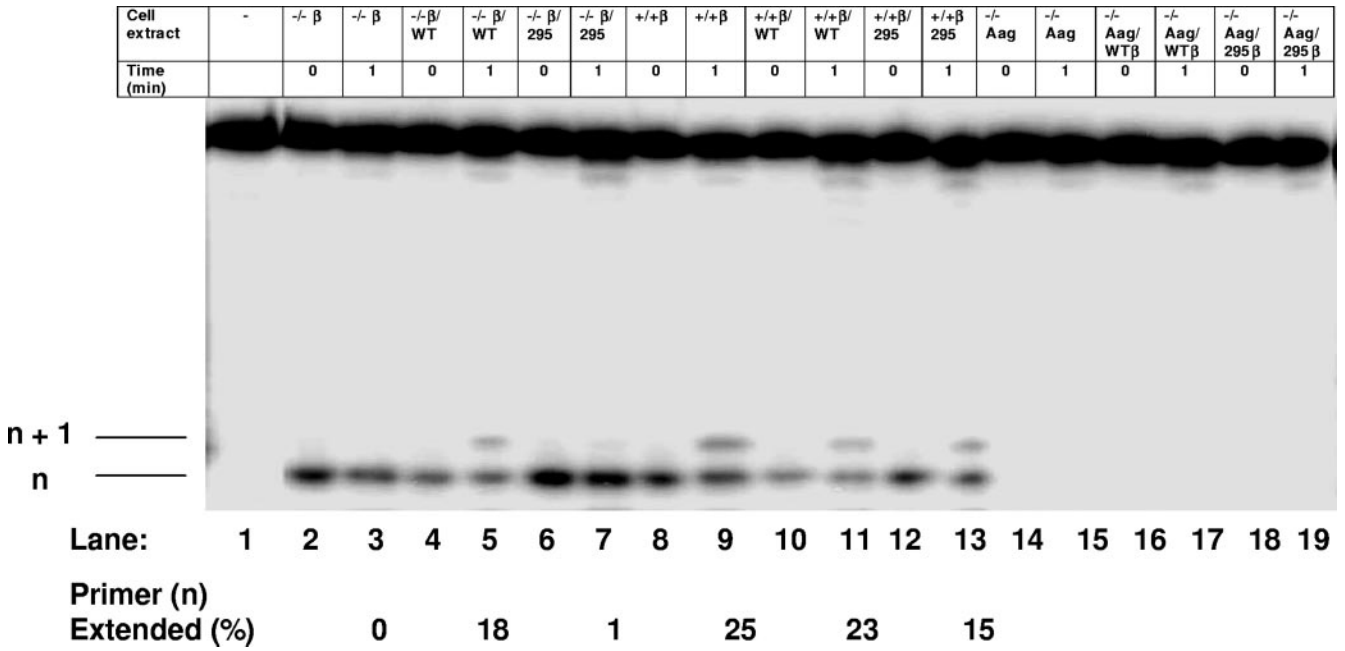


FIG. 6. BER assay of MEF cell lines using Aag lesion as substrate. The BER assay was carried out as described in Materials and Methods. Lane 1, radiolabeled substrate; lanes 2 and 3, extract from pol βΔ cells after incubation with BER buffer for 0 and 1 min, respectively; lanes 4 and 5, extract from pol βΔ cells expressing WT pol β; lanes 6 and 7, extract from pol βΔ cells expressing the E295K variant; lanes 8 and 9, extract from WT MEFs; lanes 10 and 11, extract from WT MEFs expressing WT pol β; lanes 12 and 13, extract from WT MEFs expressing E295K; lanes 14 and 15, extract from AagΔ cells; lanes 16 and 17, extract from AagΔ cells expressing WT pol β MEF; lanes 18 and 19, extract from AagΔ cells expressing the E295K variant.

increases the numbers of SCEs per nucleus in both cell lines. Importantly, the histogram of WT cells treated with MMS overlaps that of cells expressing E295K in the absence of exogenous DNA damage. This suggests that cells expressing E295K pol β harbor some type of damage, perhaps an unfilled gap, that leads to double-strand break formation at the replication fork. We have observed that cells expressing the E295K variant have large numbers of γH2AX foci, whereas cells expressing WT pol β display few foci (T. Lang and J. B. Sweasy, unpublished results), which would be consistent with the formation of double-strand breaks in the cells.

**Expression of E295K in C127 cells induces cellular transformation.** E295K is associated with human gastric cancer; therefore, we tested whether this mutant protein could cause cellular transformation, a necessary early step toward cancer. We infected C127 cells with retrovirus carrying the pRVYTET vector with the E295K construct, isolated colonies, and expanded them into cell lines as described above for the MEFs. As shown in the Western blot in Fig. 4B, the E295K variant was expressed in the C127 cells at levels similar to (clone B1) or at levels greater (clones 2 and B7) or lesser (clone B8) than the endogenous WT pol β protein. Unlike what we observed with the MEFs, expression was inducible in the C127 cells; in the presence of Tet there was no detectable expression of the HA-tagged E295K protein, but once Tet was removed from the growth medium, E295K expression was induced, as shown in Fig. 4B.

Next, we determined whether expression of E295K induced focus formation. We have previously shown (37) that expression of WT pol β in C127 cells does not induce focus formation. As shown in Fig. 8, four independent E295K-expressing clones induced focus formation in the absence of Tet. Focus formation occurred in each of these cell lines by passage 10. When Tet was present in the growth medium, suppressing E295K expression, significantly fewer foci were observed, especially at low passage numbers.

Another criterion that is used to assess the ability of proteins to induce cellular transformation is anchorage-independent growth. Therefore, we characterized the E295K-2 clone for anchorage-independent growth and found that an average (± the standard deviation) of 10.4 ± 1.4 large and multicellular colonies was observed per microscope field when cells were grown in the absence of Tet. In contrast, an average of 0.83 ± 0.042 colonies per field was observed when cells were grown in the presence of Tet. In combination with our focus formation data, our results demonstrate that expression of E295K in mouse cells induces cellular transformation.

## DISCUSSION

The goal of this study was to determine whether the E295K gastric cancer-associated pol β variant has a phenotype that could be related to the etiology of human cancer. We discovered that E295K is unable to catalyze DNA synthesis but that it binds to DNA and possesses dRP lyase activity at a level similar to that of WT pol β. We found that when expressed in MEFs, E295K conferred a dominant-negative phenotype. Cells expressing E295K were sensitized to MMS and induced more SCEs per nucleus than did cells expressing WT pol β. An intact BER pathway appears to be important for the manifes-

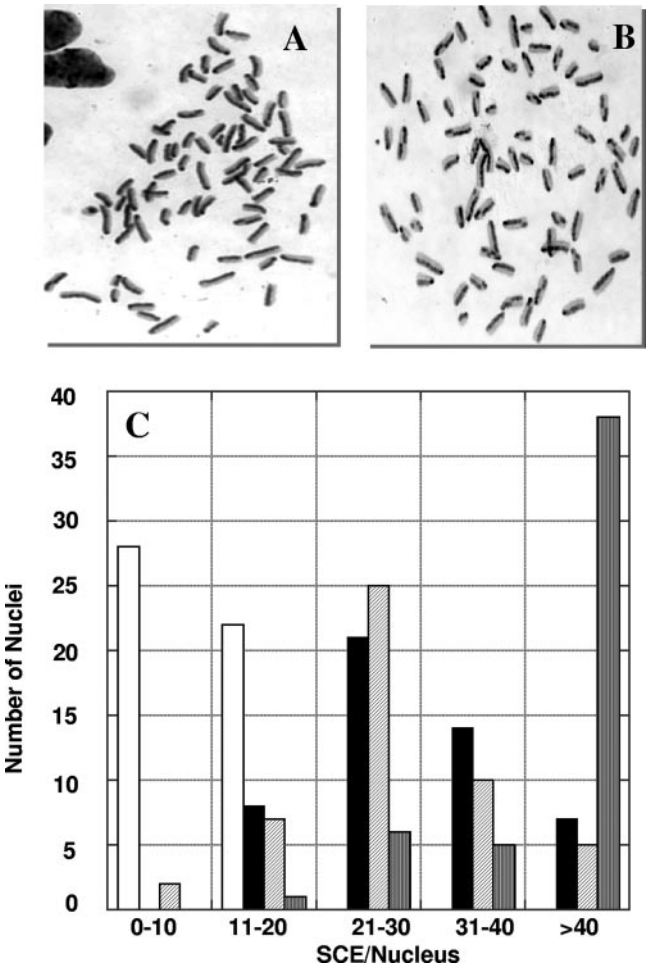


FIG. 7. Expression of E295K increases the amount of SCEs. Representative SCE images of nuclei from WT MEFs (A) and WT MEFs expressing E295K pol β (B). The distribution of the numbers of SCEs per nucleus is shown in panel C. SCEs from 50 images of metaphase spreads of each cell line were counted. Open bars, WT MEFs expressing WT pol β; closed bars, WT MEFs expressing WT pol β and treated with MMS; slashed bars, WT MEFs expressing E295K pol β; bars with vertical lines, WT MEFs expressing E295K pol β.

tation of these phenotypes, because elimination of the AAG DNA glycosylase fully or partially abrogated them. We conclude that the E295K variant interferes with BER, most likely during the gap-filling step. Because large numbers of γH2AX foci are induced upon expression of E295K in cells, it is suggested that interfering with BER leads to the generation of double-strand breaks. Perhaps these double-strand breaks arise during replication when forks encounter unrepaired lesions or lesions bound by an inactive pol β protein. In this study, we also show that expression of the E295K variant induces cellular transformation. Our results are consistent with the possibility that the genomic instability induced by E295K leads to cellular transformation. Thus, the presence of the E295K variant in gastric cells could lead to tumorigenesis or play a role in cancer progression.

**E295K interferes with BER.** In the initial study of the status of pol β in gastric carcinomas, Iwanaga and colleagues found that 6 of 20 tumors exhibited different missense mutations within the pol β cDNA (15). Interestingly, they also found that each of the tumors expressed WT pol β in addition to the variant. Thus, in order for E295K to be related to the etiology of cancer, it likely exerts its effect in the presence of WT pol β.

Based upon structural studies of pol β, we reasoned that the E295K variant would be unable to synthesize DNA due to its inability to interact with Arg258 and sequester it away from D192. We demonstrated that E295K was unable to catalyze DNA synthesis and showed that E295K could interfere with DNA synthesis by WT pol β. This most likely occurs because E295K and WT pol β have similar affinities for DNA. We suggest that during BER, once APE1 nicks the DNA, E295K and WT pol β have equal chances of binding to the 3′OH. If WT pol β binds, it will catalyze DNA synthesis and removal of the dRP moiety, preparing the DNA for ligation by DNA ligase IIIα. If E295K binds to the DNA, it will be unable to fill in the single nucleotide gap. Some of the unfilled gaps could lead to cell death, and this is consistent with our demonstration that expression of E295K in the presence of WT pol β in cells sensitizes them to MMS. Because the presence of AAG DNA glycosylase is important for E295K to induce cell death in response to treatment with MMS, we suggest that E295K interferes predominantly with BER.

In this study, we demonstrate that E295K possesses dRP lyase activity, yet it is unable to support BER in a reconstituted system. We also show that E295K is unable to complement the MMS sensitivity of the pol β-deficient MEFs. In combination, these results indicate that both the DNA polymerase and the dRP lyase activities of pol β are important for BER. It has previously been suggested that the pol β dRP lyase activity alone was able to complement the MMS sensitivity of pol β-deficient MEFs (35). We suggest that the E295K mutant is unable to complement the pol β-deficient cells because it binds with high affinity to the gap and precludes WT pol β or other DNA polymerases from accessing this gap. It is likely that unfilled gaps lead to cytotoxicity. Interestingly, the polymerase inactive D256A pol β variant is able to complement the pol β-deleted MEFs. Although we are uncertain why this variant, but not E295K, complements the MMS sensitivity of the pol β-deficient cells, one possibility is that the D256A protein does not have high affinity for the gap and does not preclude access to other polymerases. The R283A pol β variant also restores MMS resistance to the pol β-deficient cells (35), even though its activity is significantly less than that of the WT protein. In our hands, pol β variants with significantly less activity than WT pol β are able to support BER in cell extracts (data not shown). Thus, R283A may have enough DNA polymerase activity to function in BER. In summary, we suggest that E295K precludes other polymerases, including WT pol β, from accessing the gap. This likely results in a lack of complementation of the MMS sensitivity of pol β-deficient MEFs.

**Interference with BER leads to genome instability.** We propose that unfilled gaps created during aberrant BER in the presence of E295K are channeled into a double-strand break repair pathway, based upon the presence of γH2AX foci in cells expressing this variant. Double-strand breaks could be created when the replication fork reaches an unfilled gap or by the induction of a single-strand break created by an unsuccessful attempt to repair a lesion that is induced opposite to the

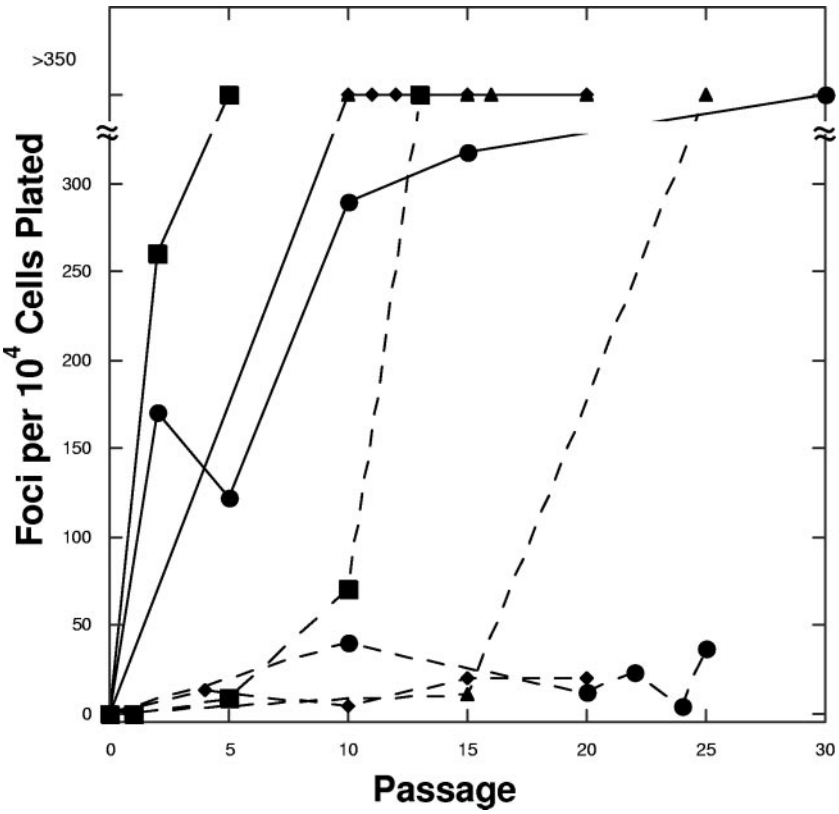


FIG. 8. Expression of E295K in C127 cells induces cellular transformation. Expression of E295K (●), clone 2 (■), clone B8 (◆), and clone B7 (▲). Numbers of foci are averages obtained from two T25 flasks. The solid lines represent focus formation of cells grown under inducing conditions (no Tet in medium), and the dashed lines are focus formation of cells grown under noninducing conditions (Tet in medium). When the counts reach 350 to 500 foci per $10^4$ cells plated, there are too many foci to count accurately, as represented by the break in the *y* axis and through the plots.

unfilled gap. If left unrepaired, these breaks could lead to cell death. If repaired aberrantly, they could lead to genome instability.

Our demonstration of an increased frequency of SCEs in cells expressing E295K strongly suggests that expression of E295K induces genomic instability. The instability appears to be a direct result of the interference of E295K with BER. We suggest that once it is created, an abasic site becomes a substrate for APE1 endonuclease, which nicks the DNA, resulting in a 3′OH and a 5′dRP moiety. If E295K binds to the 3′OH, no gap filling will occur. Based upon the detection of an increased frequency of SCEs in cells deleted of pol β versus pol β-proficient cells, and the demonstration that the dRP lyase 8-kDa domain of pol β was sufficient to prevent cell death from MMS, Sobol and colleagues (33) suggested that DNA substrates containing the dRP group could induce genomic instability (9). The results from our study suggest that gaps that remained unfilled during BER may also be a source of genomic instability.

**Cellular transformation could result from genomic instability.** We have shown that E295K induces an increased frequency of SCEs, which is a hallmark of genomic instability. Whether the cellular transformation results from an increased level of SCEs remains to be determined. If mutations were induced in key growth control genes during strand exchange and DNA synthesis, it is possible that uncontrolled growth may result, leading to cellular transformation. Alternatively, unfilled gaps could be resected by an exonuclease, leading to an increased frequency of deletions. Mutations resulting from these processes could result in cellular transformation. Unfilled gaps could also be filled in by polymerases that substitute for pol β if they are able to gain access to the gap in the presence of E295K. These polymerases could insert incorrect nucleotides, especially if they are members of the low-fidelity Y family of DNA polymerases. E295K could remove the dRP moiety and create a ligatable end in the presence of DNA synthesis, leading to the fixation of mutations. If these mutations occurred in key growth control genes, cellular transformation could result.

BER is responsible for the repair of at least 10,000 lesions per cell per day, most of which arise endogenously due the inherently unstable nature of DNA. Thus, BER is responsible for the repair of the majority of cellular DNA damage. The results from this study suggest that when the BER process is unable to be completed, genomic instability results. This and previous studies from our laboratory have demonstrated that pol β cancer-associated variants appear to induce mutations during the gap-filling step of BER, either by misincorporation (6, 19) or by interfering with BER. These studies suggest that the pol β cancer-associated variants that are found in tumors are related to the etiology of human cancer. Importantly, our studies demonstrate that BER is critical for genome maintenance and suggest that aberrant BER leads to tumorigenesis or contributes to cancer progression.

## ACKNOWLEDGMENTS

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