Expression of DNA polymerase β cancer-associated variants in mouse cells results in cellular transformation

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Thirty percent of the 189 tumors studied to date express DNA polymerase β variants. One of these variants was identified in a prostate carcinoma and is altered from isoleucine to methionine at position 260, within the hydrophobic hinge region of the protein. Another variant was identified in a colon carcinoma and is altered at position 289 from lysine to methionine, within helix N of the protein. We have shown that the types of mutations induced by these cancer-associated variants are different from those induced by the wild-type enzyme. In this study, we show that expression of the I260M and K289M cancer-associated variants in mouse C127 cells results in a transformed phenotype in the great majority of cell clones tested, as assessed by focus formation and anchorage-independent growth. Strikingly, cellular transformation occurs after a variable number of passages in culture but, once established, does not require continuous expression of the polymerase β variant proteins, implying that it has a mutational basis. Because DNA polymerase β functions in base excision repair, our results suggest that mutations that arise during this process can lead to the onset or progression of cancer.

base excision repair | DNA repair | mutagenesis

Spontaneous DNA damage occurs at a rate of ≈10,000 lesions per cell per day, and much of this damage is repaired by the base excision repair (BER) machinery (1, 2). The BER system plays a critical role in maintaining cellular genomic stability. During BER, damaged bases are removed by a DNA glycosylase, followed by incision of the DNA by AP endonuclease (APE) at a position that is usually 5′ to the lesion, leaving a nick with a 3′OH and a 5′ deoxyribose phosphate (2). DNA polymerase beta (pol β) binds to the nick, removes the deoxyribose phosphate with its DRP lyase activity (3), and fills in the single nucleotide gap, using its DNA polymerase activity (4).

Fifty-eight of the 189 tumors characterized to date express DNA pol β variant proteins (for review, see ref. 5) (6). Of these, 28 (48%) expressed variants with single amino acid alterations, seven expressed truncated forms of pol β, and eight expressed multiple variant forms of pol β. These mutations are absent from normal tissue from the same individuals and are not among the common polymorphisms found within the pol β gene (http://egp.gs.washington.edu/data/polb) (5, 7). In addition, an alternative splice variant of pol β in which exon 11 is deleted was expressed in 15 tumors. This splice variant appears to interfere with BER (8). This exon 11 splice variant has been detected in normal tissue, including normal tissue isolated from 2 of 15 patients with tumors, and its link to cancer etiology remains controversial (9–12). Each of the tumors characterized to date also contain the wild-type (WT) pol β allele.

The I260M variant of pol β was identified in a prostate carcinoma (13). Isoleucine 260 is located within a hydrophobic hinge region that appears to function in the movement of the fingers subdomain upon interaction of the polymerase with its nucleotide substrate (14). Alteration of other hinge residues of pol β results in polymerases with strong mutator activity, both *in vivo* and *in vitro* (15–19). Results from our laboratory show that the I260M variant has a mutational signature that is different from that of WT pol β. I260M expands dinucleotide repeats and induces frameshift mutations at dipyrimidine sequences at frequencies that are significantly higher than those of the WT enzyme. The I260M variant also induces base substitution mutations within a subset of DNA sequence contexts that differ from those in which the WT enzyme induces mutations (S. Dalal, K. Eckert, and J.B.S., unpublished data).

The K289M variant of pol β was identified in a colon carcinoma (9). Lysine 289 is located in α-helix N of the pol β protein (14), which is important for accurate DNA synthesis (20–23) because of its role in positioning the templating base during phosphodiester bond formation. We previously showed that the K289M protein (24) induces mutations within interrupted runs of like nucleotides in mouse cells at a 16-fold frequency over that of the WT enzyme. Repetitive sequences are known to be hotspots for mutagenesis both *in vivo* and *in vitro*, and the slippage within these sequences is known to occur within a significant number of colon carcinomas (25, 26). This previous study suggests that mutations induced by K289M may arise during misalignment of the DNA within the active site of this variant enzyme. Based on our biochemical studies, it is plausible that the I260M or K289M pol β variants could induce mutations in cells during the gap-filling step of BER, ultimately leading to tumorigenesis or tumor progression.

In this study, we show that extended expression of the I260M or K289M variants of pol β in mouse C127 cells induces the cells to form foci and to exhibit anchorage-independent growth. Furthermore, focus-forming ability and anchorage independence was maintained when expression of the variant pol β proteins was extinguished. These results suggest that I260M and K289M induce mutations in the cells, presumably during BER, ultimately resulting in permanent cellular transformation. Our results suggest that faithful BER acts as a tumor suppressor mechanism, because it functions in the accurate repair of many lesions that arise endogenously in cells on a daily basis. Thus, faithful BER prevents genomic instability that could lead to cancer.

Materials and Methods

Construction of Tetracycline (Tet)-Regulated Retroviruses Expressing Pol β. The hemagglutinin (HA)-tagged WT human pol β cDNA or the I260M or K289M mutant pol β genes were inserted into pRVY-Tet to generate pRVYTet-WT, -I260M, and -K289M

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Abbreviations: BER, base excision repair; HA, hemagglutinin; pol β, polymerase β; Tet, tetracycline.

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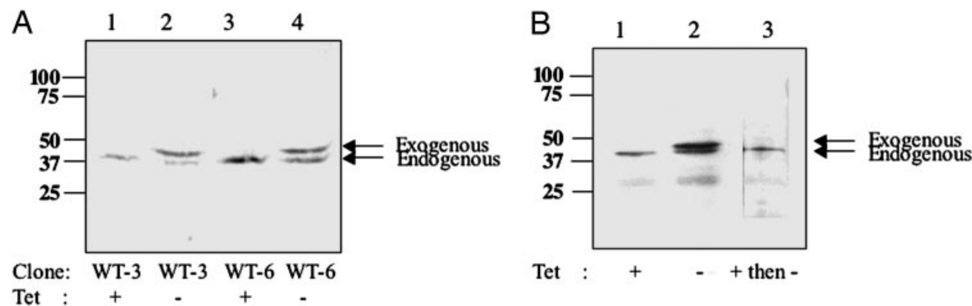

Fig. 1. Expression of WT pol β and the tumor-associated variants in C127 cells. Western blotting was performed on cellular extracts with monoclonal antibody raised against the pol β (Abcam). (*A*) WT clones. Lanes 1 and 3 are WT clones 3 and 6, respectively, grown in the presence of Tet. Lanes 2 and 4 are WT clones 3 and 6, respectively, grown in the absence of Tet. Quantification of the bands shows that the WT protein is present at levels 3 times higher than endogenous pol β in clone 3, and 1.5 times higher in clone 6. (*B*) I260M clones. Lanes 1–3, I260M, clone 2 grown in the presence (1), absence (2), and absence then presence (3) of Tet. Under inducing conditions, I260M is expressed at ≈2-fold higher levels than endogenous pol β (lane 2). The lower band represents endogenous pol β protein, and the upper band represents endogenous HA-tagged protein expressed from the retroviral vector.

constructs, respectively (27). In the retroviral vectors, the left-hand retroviral long terminal repeat drives expression of tTA Tet transactivator (28), the tetO/CMV promoter drives expression of pol β proteins in a Tet-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 WT, K289M, and I260M proteins is turned off. However, expression of these proteins occurs when Tet is removed from the growth medium. The constructs were packaged into retroviruses by using the Phoenix packaging cell line.

Cell Lines. C127 cells were obtained from American Type Culture Collection. C127 is a nontransformed clonal line derived from a mammary tumor of an RIII mouse (29). The cells were maintained in DME-10 (DMEM/10% FBS/penicillin-streptomycin).

To obtain C127 cells with either the I260M or K289M retroviral construct, an 80% confluent monolayer of cells was infected with 0.5 ml of the packaged retrovirus, containing the WT or either the I260M or K289M variant, in the presence of 4 μg of polybrene, and incubated at 37°C and 5% CO_2 for 2 h with occasional rocking. DME-10 and 16 μg of polybrene were then added to the cells, and they were incubated overnight at 37°C and 5% CO_2. On the next day, the cells were trypsinized, diluted, and plated into 100-mm plates in DME-10 with 2.5 μg/ml Tet. Twenty-fours hours later, 300 mg/ml HygB was added to select for individual clones having the construct. When individual colonies reached the size of a small cloning ring, they were picked, grown in six-well plates, and expanded into cell lines. After two passages in the presence of Tet, an aliquot of cells were passaged into DME10 containing Hyg but no Tet.

Western Blotting. To determine whether the HygB-resistant cells expressed the WT or mutant pol β fused to the HA epitope, cell extracts were prepared from each cell line and subjected to Western blotting. Cells were grown to confluence in the presence or absence of Tet in 100 mM plates and washed with PBS. Approximately 200 μl of SDS gel loading buffer (30) heated to 80°C was added to each plate, and the cells were scraped and pipetted into an Eppendorf tube by using a wide-bore pipette tip. The extract was boiled for 10 min, and 25 μl was loaded onto a 10% SDS gel. The proteins in the extract were resolved by electrophoresis, blotted to an Immobilon-P membrane, and incubated with a 1:200 dilution of monoclonal antibody raised against the pol β (Abcam, Cambridge, U.K.) for at least 1 h at room temperature or overnight at 4°C. After extensive washing in PBS containing 1% Tween, the membrane was incubated with a 1:1,000 dilution of sheep anti-mouse secondary antibody that was conjugated to horseradish peroxidase for 1 h at room temperature, washed, and developed with ECL Plus (Amersham Pharmacia Biosciences), and the gel image was captured by scanning it on a Storm PhosphorImager (Molecular Dynamics). IMAGEQUANT software was used to quantify each band.

Focus Formation Assay. Cells were passaged every 3–4 days in the presence or absence of 2.5 μg/ml Tet. At various passages, ≈1 × 10^4 cells were seeded into each of two T25 flasks (Falcon). These cells were fed every 3–4 days with DME-10 containing or lacking Tet, as appropriate, and after 25 days they were stained with Giemsa to visualize foci. The presence of foci was also monitored by microscopic examination.

Anchorage-Independent Growth. Approximately 1 × 10^5 cells, grown in the presence (noninduced) or absence (induced) of Tet, were mixed with DME10 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. The number of colonies present in each of 10 microscope fields per plate was counted after 5 weeks of growth from four to six plates per experiment and is reported as an average from two independent determinations.

Results

Expression of the Cancer-Associated Pol β Proteins in Mouse C127 Cells. C127 cells were infected with the Tet-regulated retroviral constructs encoding pol β WT, I260M, or K289M, and individual drug-resistant clones were isolated and expanded into cell lines. To determine whether the HA-tagged mutant polymerases were expressed, extracts from early passage clonal cell lines were subjected to Western blotting with a monoclonal antibody raised against pol β. This permits visualization of both the endogenous protein and the HA-tagged protein that is expressed from the retroviral vector. As shown in Fig. 1*A*, WT clones 3 and 6 express HA-tagged pol β (upper band) only when they are grown in the absence of Tet (lanes 2 and 4). The WT protein is expressed at levels similar to or slightly higher than that of endogenous pol β. Similar results were obtained for I260M clone 2, as shown in Fig. 1*B*. WT clones 4 and 11, I260M clones 1, 3, 5, 6, 8, 10, and 12, and K289M clones 1, 4, and 5 also expressed exogenous polymerase in the absence of Tet at levels similar to endogenous pol β but did not express the exogenous polymerase in the presence of Tet (data not shown). Stripping and reprobing of the blot with antisera raised against HA shows that the

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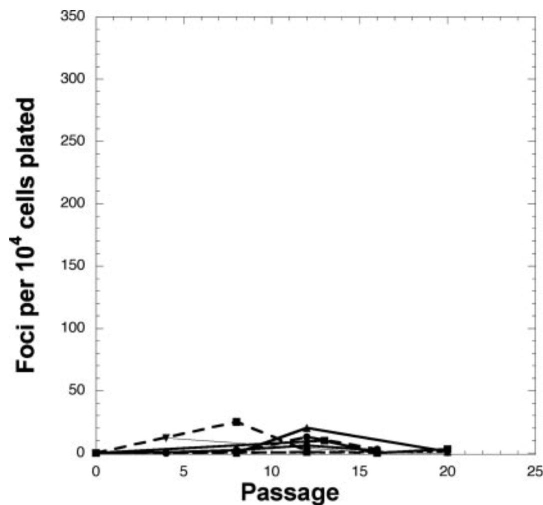

Fig. 2. Expression of WT pol β in mouse C127 cells does not result in focus formation. At the indicated passages, ≈10,000 cells were seeded in duplicate T25 flasks. These cells were fed every 3–4 days and stained with Giemsa after 25 days of growth. We observe little focus formation by four clones expressing WT pol β. ●, clone 2; ▼, clone 4; ■, clone 5; ▲, clone 11.

upper band of the doublet represents the HA-tagged pol β protein (data not shown).

Expression of WT Pol β in C127 Cells Does Not Induce Focus Formation. Four different clones of C127 cells expressing the exogenous WT pol β gene were serially passaged in DME-10 with or without Tet, to repress or induce expression, respectively, of WT pol β protein. At various passages, 1×10^4 cells were seeded into T25 flasks and incubated without further passaging for an additional 21 days in the same medium. The cells were then stained to visualize foci. Expression of WT pol β in each of the four clones we tested did not result in significant numbers of foci, as shown in Fig. 2. In contrast to our studies, Hoffman and colleagues (31) reported that overexpression of WT pol β induces focus formation in Chinese hamster ovary cells and that these cells form tumors in nude mice. These conflicting results can most likely be explained by the use of different cell lines and different expression systems. Thus, C127 cells are suitable for assessing the focus formation activity of pol β tumor-associated variants.

The I260M and K289M Pol β Variant Proteins Induce Focus Formation in C127 Cells. Several individual clones harboring either the human I260M or K289M pol β constructs were serially passaged in DME-10 in the presence or absence of Tet and examined for focus formation as described for WT pol β. An example of a confluent monolayer of I260M-expressing cells at early passage illustrating the macroscopic appearance of foci is shown in Fig. 3 along with a monolayer of cells that did not express I260M. The microscopic appearance of a typical focus from the I260M-expressing cells is also shown in Fig. 3. Note that the cells are highly refractile and grow densely on top of each other in a disorganized manner, which is typical of C127 cells transformed by other oncogenes such as the bovine papillomavirus E5 gene.

The kinetics of focus formation is shown in Fig. 4 and reveals that focus formation induced by expression of the variant pol β polymerases depends on growth in the absence of Tet and passage number. Fig. 4*A* shows the results for six clones of cells expressing I260M. These six I260M-expressing clones generated progressively increasing numbers of foci that eventually became too numerous to count accurately (Fig. 4*A*, solid

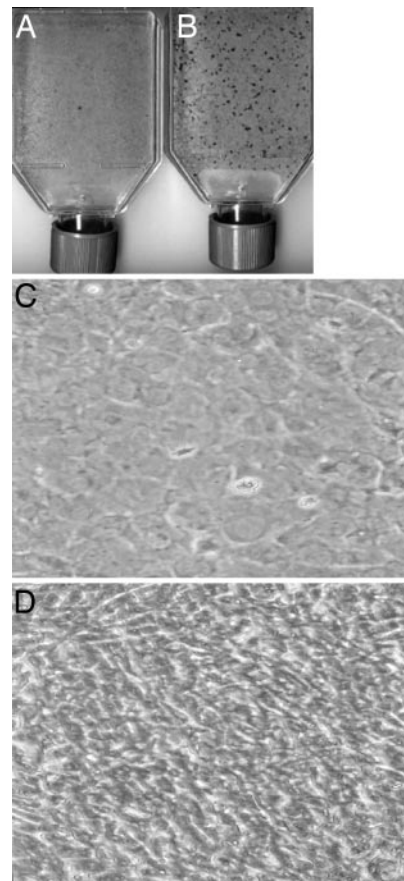

Fig. 3. Morphology of cells in the presence and absence of Tet. *A* and *B* are examples of monolayers of I260M, clone 6 cells, grown for 20 passages in the presence and absence of Tet, respectively. Cells were then seeded into flasks and fed every 3–4 days with DME-10. After 25 days, the cells were stained with a 1:10 dilution of Giemsa in PBS. *C* and *D* are close-up views of confluent cells of I260M, clone 2 grown for six passages in the presence and absence of Tet, respectively. Cells were then seeded into flasks and fed every 3–4 days with DME-10. After 25 days, the cells were stained with a 1:20 dilution of Giemsa in PBS.

lines). At late passage numbers, we estimate that 1–10% of the 1×10^4 cells plated formed foci. Some clones generated many foci at early passage number, whereas others displayed a more gradual increase in focus formation with continued passage. In contrast, very few foci were observed for most of the clones when they were passaged in the presence of Tet (Fig. 4*A*, dashed lines). One uninduced clone, clone 12 (⊙), generated appreciable numbers of foci ≈10 passages later than its induced counterpart, whereas a second clone, clone 8, generated a small number of foci at very late passages that did not increase in number with continued passage. Two additional I260M clones, numbers 1 and 3, did not generate appreciable numbers of foci during 20 passages in the absence or presence of Tet (data not shown), even though these clones expressed the I260M polymerase (data not shown).

Similar results were obtained with cells harboring the K289M retrovirus, as shown in Fig. 4*B*. Each of the three tested clones of K289M generated large numbers of foci when the cells were passaged in the absence of Tet, and few or no foci when they were passaged in its presence. As was the case with the I260M cell lines, foci were generated with different kinetics in the different clones. These results demonstrate that expression of two cancer-associated variants of pol β can induce focus formation in mouse fibroblasts. The differing patterns of focus formation in the various lines of cells expressing I260M or

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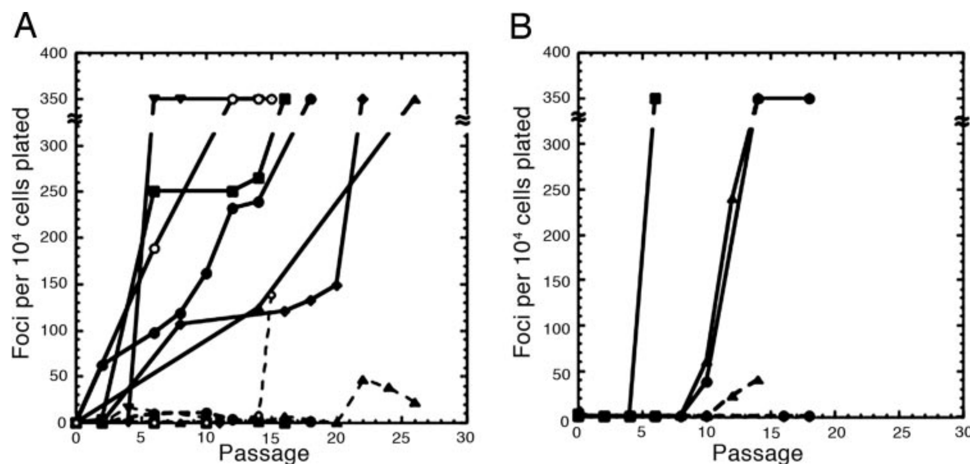

Fig. 4. Expression of I260M and K289M in mouse cells results in focus formation. (*A* and *B*) Expression of I260M (*A*) or K289M (*B*) in C127 cells for various passages results in focus formation. (*A*) Focus formation by various clones expressing I260M. ●, clone 2; ■, clone 5; ♦, clone 6; ▲, clone 8; ▼, clone 10; ⊙, clone 12. (*B*) Focus formation by various clones expressing K289M. ●, clone 4; ■, clone 5; ▲, clone 1. Numbers of foci are averages obtained from two T25 flasks. The solid line represents focus formation of cells grown under inducing conditions (no Tet in medium), and the dashed line is focus formation of cells grown under noninducing conditions (Tet in medium). When the counts reach 350–500 foci per 1×10^4 cells plated, there are too many foci to count accurately, as represented by the break in the *y* axis and through the plots.

K289M are consistent with the interpretation that focus formation in these lines results from a random process, which could involve the mutation of one or more genes at different passage numbers. In preliminary studies, the D160N gastric cancer-associated pol β variant also induces focus formation in C127 cells (J.B.S., unpublished data).

Cells That Form Foci also Exhibit Anchorage-Independent Growth. Another characteristic of transformed cells, besides focus formation, is anchorage-independent growth. To confirm that the ability of the cells to form foci reflected a transformed cellular phenotype, we assessed the capacity of C127 cells expressing I260M or K289M to grow in soft agar. Examples of typical microscope fields of cells containing K289M that were plated in soft agar after growing with and without Tet, respectively, are shown in Fig. 5. C127 cells not expressing K289M exhibit little growth in soft agar, as shown in Fig. 5*A*. In contrast, C127 cells that express K289M exhibit appreciable colony formation in soft agar, as shown in Fig. 5*B*. As shown in Table 1, the average number of colonies per field for C127 cells that do not express K289M is 3.0 ± 1.0, whereas it is 27.0 ± 7.0 for cells that express K289M, a 9-fold difference. C127 cells expressing the I260M pol β variant also exhibited appreciable growth in soft agar (25.0 ± 4 colonies per field) compared with noninduced cells (7.0 ± 1). These results confirm that expression of either the K289M or I260M pol β variant induces cellular transformation in C127 cells.

Cellular Transformation Does Not Require Continuous Expression of Pol β Mutants. If a heritable change such as a mutation responsible for focus formation occurred in the I260M or K289M cells during passaging, then continuous expression of the mutant polymerase would not be required for maintenance of the transformed phenotype. Alternatively, if transformation does not have a mutational basis, it is possible that continuous expression of the mutant polymerase may be required to maintain transformation. To distinguish between these mechanisms, we shut off expression of the mutant polymerase by the addition of Tet to cells at a passage number where they formed numerous foci. Western blotting confirmed that the addition of Tet shut off expression of the mutant polymerase in I260M, clone 2, as shown in Fig. 1 and K289M, clone 4 (data not shown). Next, we characterized focus formation in these cell lines in which expression of the pol β variant had been extinguished, as shown in Fig. 6. In each graph, we plotted the number of foci as a function of passage number for cells

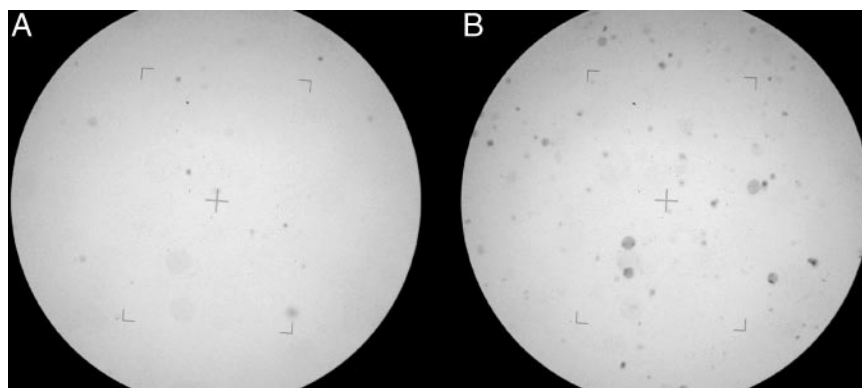

Fig. 5. Cells expressing K289M exhibit anchorage-independent growth. Approximately 1×10^5 K289M clone 4 cells, grown in the presence (noninduced) or absence (induced) of Tet for 34 passages, were mixed with DME-10 containing 0.3% Difco Noble agar. This mixture was poured onto a layer of DME-10 containing 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 with (*A*) or without (*B*) Tet. Colonies were counted after 5 weeks of growth. (*A*) Noninduced. (*B*) Induced.

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Table 1. Transformed cells exhibit anchorage independence

Growth condition	Colonies per field: K289M, clone 4	Colonies per field: I260M, clone 2
Noninduced	3.0 ± 1	7.0 ± 1
Induced	27.0 ± 7	25.0 ± 4
Extinguished	31.0 ± 7	60.0 ± 4

Cells were passaged in the presence of Tet (noninduced), absence of Tet (induced), and in the absence then presence of Tet (extinguished) before plating in soft agar containing (noninduced and extinguished) or lacking (induced) Tet. Colonies per microscope field were counted and averaged as described in *Materials and Methods.*

continuously growing in the absence (● and solid line) and presence (● and dashed line) of Tet, as we did in Fig. 4. Focus formation for each clone in which expression was extinguished (■) after growth under inducing conditions is also plotted. The passage number at which Tet was added to the medium is denoted by an arrow. In each case, the cells formed numerous foci after expression of the mutant polymerase was extinguished. Similar results were also obtained with each of the other I260M and K289M clones that induced focus formation (data not shown). In addition, I260M and K289M cells in which expression had been extinguished exhibited anchorage-independent growth, as shown in Table 1. These results show that continuous expression of neither I260M nor K289M is required for focus formation and anchorage independence, and suggest that focus formation results from a heritable change. In contrast, repression of the BPV E5 oncogene expressed from the pRVY-Tet vector resulted in rapid reversion of the transformed phenotype of C127 cells (27), consistent with the mechanism of action of the E5 protein as an activator of cellular mitogenic signaling pathways.

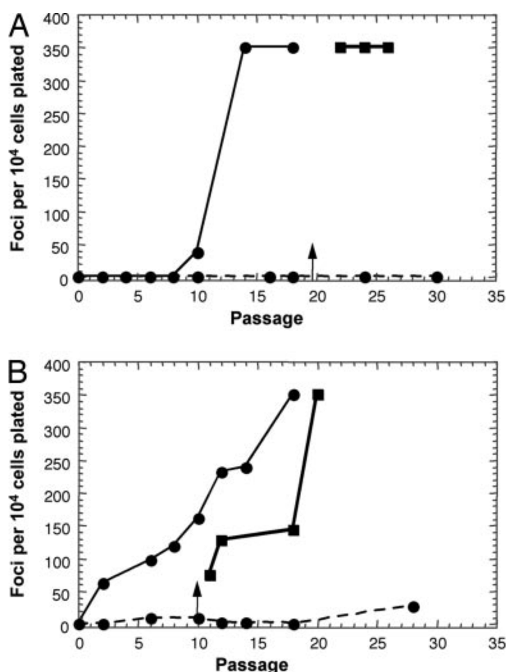

Fig. 6. Clones of I260M and K289M continue to form foci once expression has been extinguished. Individual clones of either K289M (*A*) or I260M (*B*) were expressed in C127 cells for various passages, and then expression was extinguished by addition of Tet to the medium at the passage indicated by the arrow. (*A*) K289M clone 4. ● and solid line, induced; ● and dashed line, noninduced (taken from Fig. 4); ■, extinguished. Tet was added at passage 21 to extinguish expression. (*B*) I260M clone 2. ● and solid line, induced; ● and dashed line, noninduced (taken from Fig. 4); ■, extinguished. Tet was added at passage 10 to extinguish expression.

Discussion

In this study, we show that extended expression of the I260M prostate and the K289M colon cancer-associated human pol β variants, but not WT pol β, results in cellular transformation in C127 cells as assessed by focus formation and growth in soft agar. We have also demonstrated that the transformed phenotype is maintained even when expression of either the I260M or K289M variant is extinguished. The simplest explanation for our results is that expression of I260M or K289M in C127 cells results in the induction of one or more mutations in key growth control genes that leads to cellular transformation. These results indicate that the I260M and K289M tumor-associated variants have functional phenotypes that could be associated with the onset or progression of cancer.

Mutagenic Properties of the Pol β Variants Could Play an Important Role in Cellular Transformation. In separate studies, we have shown that the I260M and K289M variants have little effect on the overall mutation frequency but that they induce different types of mutations than the WT pol β protein (24) (S.D., K. Eckert, and J.B.S., unpublished data). I260M induces expansions of TC repeats within a run of TCs, frameshift mutations within dipyrimidine sequences, and base substitutions within sequence contexts in which WT pol β does not induce mutations (S.D., K. Eckert, and J.B.S., unpublished data). The K289M variant induces transversions within interrupted runs of like residues at frequencies that are much greater than those of the WT protein (24). The molecular basis of mutation induction by the I260M and K289M proteins appears to be altered positioning of the DNA within the active site of each of these enzymes as compared with WT pol β. These results, combined with the fact that expression of these mutant pol β proteins induces cellular transformation, suggest that altering the spectrum of induced mutations in cells plays an important role in the transformation process.

Cellular Transformation May Result from Aberrant Gap Filling by Pol β During BER. The major function of pol β in cells is the removal of the dRP moiety from an incised DNA backbone and the filling of small gaps in the DNA during BER (4). In an *in vitro* assay (4), both the I260M and K289M proteins are able to function in BER with kinetics that are quite similar to the WT protein (24) (S.D., K. Eckert, and J.B.S., unpublished data). In addition, expression of I260M and K289M polymerases in C127 cells does not sensitize the cells to methylmethanesulfonate (MMS), suggesting that these proteins do not interfere with BER (T.L., S.D., and J.B.S., unpublished data). Thus, the tumor-associated pol β variant polymerases would be expected to function similarly to WT pol β in BER by excising the dRP moiety and filling in the gap in cells. Because the only known role of pol β in cells is in BER, we favor the idea that aberrant functioning of pol β in this process leads to cellular transformation. We suggest that the I260M and K289M variant polymerases will induce specific types of errors that are not normally made by WT pol β during the gap-filling step of BER, and that some of these will occur in key growth control genes. If the errors are not repaired, the resulting mutations may lead to cellular transformation. The random process of mutagenesis is consistent with our finding that different clones harboring the I260M and K289M pol β variants appear to elicit a transformed phenotype at different times during passaging. Thus, normal BER may function as a tumor-suppressor mechanism in cells.

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DNA Pol β and Cancer. We have shown that expression of variant mutator forms of pol β that arose in somatic tissue and were identified in a prostate and a colon tumor confer a transformed phenotype to cells. These results suggest that the variant polymerases confer a mutagenic phenotype to cells by introducing errors during BER, some of which may affect genes controlling cell growth. Our studies also raise the possibility that individuals harboring certain germ line polymorphisms within the pol β gene that could affect the fidelity of DNA synthesis may be at higher risk for developing cancer. Thus, the status of the pol β gene in normal tissue may be of predictive value.

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