Published in final edited form as:

Mutat Res. 2010 January 5; 683(1-2): 115–122. doi:10.1016/j.mrfmmm.2009.10.016.

Efficient repair of DNA double-strand breaks in malignant cells with structural instability

Yue Cheng, Zhenhua Zhang, Bridget Keenan, Anna V. Roschke, Kenneth Nakahara, and Peter D. Aplan

Genetics Branch, National Cancer Institute, Navy 8, Room 5101, 8901 Wisconsin Ave., Bethesda, MD 20889-5105

Abstract

Aberrant repair of DNA double strand breaks (DSBs) is thought to be important in the generation of gross chromosomal rearrangements (GCRs). To examine how DNA DSBs might lead to GCRs, we investigated the repair of a single DNA DSB in a structurally unstable cell line. An *I-SceI* recognition site was introduced into OVCAR-8 cells between a constitutive promoter (EF1 α) and the *Herpes simplex* virus thymidine kinase (TK) gene, which confers sensitivity to gancyclovir (GCV). Expression of *I-SceI* in these cells caused a single DSB. Clones with aberrant repair could acquire resistance to GCV by separation of the EF1 α promoter from the TK gene, or deletion of either the EF1 α promoter or the TK gene. All mutations that we identified were interstitial deletions. Treatment of cells with etoposide or bleomycin, agents known to produce DNA DSBs following expression of *I-SceI* also did not generate GCRs. Because we identified solely interstitial deletions using the aforementioned negative selection system, we developed a positive selection system to produce GCR. A construct containing an *I-SceI* restriction site immediately followed by a hygromycin phosphotransferase cDNA, with no promoter, was stably integrated into OVCAR-8 cells. DNA DSBs were produced by an *I-SceI* expression vector. None of the hygromycin resistant clones recovered had linked the hygromycin phosphotransferase cDNA to an endogenous promoter, but had instead captured a portion of the *I-SceI* expression vector. These results indicate that even in a structurally unstable malignant cell line, the majority of DNA DSBs are repaired by religation of the two broken chromosome ends, without the introduction of a GCR.

Keywords

DNA double strand break (DSB); chromosomal rearrangement; *I-SceI*; ovarian cancer

1. Introduction

Mammalian cells have developed effective systems to respond to and repair DNA double strand breaks (DSBs). Unfaithful repair of these breaks can lead to cell death or gross chromosomal rearrangements (GCR), including deletions, amplifications, inversions, and translocations

Address correspondence to: Dr Peter D. Aplan, Navy 8, Room 5101, 8901 Wisconsin Ave, Bethesda MD 20889, USA, aplanp@mail.nih.gov, Tel: +1 301 435 5005, Fax: +1 301 496 0047.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure of Potential Conflicts of Interest

The authors declare that there are no conflicts of interest.

(1-4). GCRs, in turn, can lead to amplification of proto-oncogenes, or generation of novel, chimeric fusion oncoproteins (5-7).

Given that these oncogenic GCRs are important causes of malignant transformation, it is therefore important to understand the mechanisms that lead to these GCRs and generation of oncogenic fusion genes. Two basic DNA repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) have been identified in human cells (8-11). Since most oncogenic GCRs are thought to be mediated via improper NHEJ-mediated repair of two DNA DSBs, the study of improper NHEJ repair should yield insights into the mechanisms that lead to GCRs.

There are limited techniques available that can be used to produce a specific DNA DSB in living cells for these studies. Using a system to assay DNA DSB repair by HR in embryonic stem (ES) cells, investigators have previously demonstrated that two induced DNA DSBs were required to produce a GCR (12). We wondered whether those results might be specific for ES cells, or DNA DSB repair via HR, and hypothesized that in non-embryonic, hematopoietic cells a single induced break might recombine with a spontaneous DNA DSB, since it has been estimated that approximately 50 DNA DSBs occur per cell cycle (13). We used the yeast *I-SceI* endonuclease to develop a loss-of function reporter system in which a DNA DSB was introduced in a predetermined region. In this system, the 18-bp *I-SceI* recognition site was inserted between a constitutive promoter (EF1 α) and the Herpes simplex virus thymidine kinase (*TK*) gene, which confers sensitivity to gancyclovir (GCV). The ensuing chromosomal changes flanking the breakage site can then be studied after transfection of an *I-SceI* expression vector into these cells. Using hematopoietic U937 cells, we found that approximately 50% of the cells transfected by an *I-SceI* expression vector showed clear evidence of *I-SceI* cleavage, suggesting that *I-SceI* mediated cleavage of genomic DNA was reasonably efficient. The most common mutation that occurred in the cells that survived GCV selection was an interstitial deletion. More complex rearrangements, such as insertions or duplications, were also noted. At the breakpoint junction, many clones showed hallmarks of NHEJ, such as direct or inverted repeats or micro-homology (14). However, we did not detect any clear evidence of GCRs in over 100 independent clones analyzed.

In order to determine whether the above findings were unique to hematopoietic U937 cells, or were more generalizable to other cell types, we extended this system to an epithelial cell line. In an effort to produce GCRs, we chose the OVCAR-8 ovarian cancer cell line for these studies, since this cell line displayed a high degree of ongoing structural chromosomal instability (15).

2. Materials and methods

2.1 Cell line, DNA transfection and drug treatment

OVCAR-8 cells were obtained from the NCI-60 anticancer drug discovery panel (15,16). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Transfections of linearized vector pEF1 α TK, containing *I-SceI* recognition sequences, were performed using electroporation as previously described (14). Transfections of the *I-SceI* expression vector, pCBASce, were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's recommended protocol. OVCAR-8 cells were at approximately 75–90% confluence before the transfection. The next day cells were split into ten 100mm tissue culture dishes from one 75 cm² flask. On the third day following the transfection, 500 μ g/ml G418 (Invitrogen, Carlsbad, CA), 40 μ M GCV (InvivoGen, San Diego,

CA) or 200µg/ml Hygromycin B (Invitrogen, Carlsbad, CA) were added, depending on the selection used. All resistant clones were isolated and expanded for further analysis.

For etoposide treatment, the A15 cells were transfected with I-*SceI* expression vectors, cultured for 48 hours, and incubated with etoposide at 100µM for one hour, 10µM for 4 hours or 5µM for 4 hours. For bleomycin treatment, A15 cells were transfected with I-*SceI* expression vectors, cultured for 48 hours, and subsequently treated with bleomycin at 5µg/ml or 20µg/ml for 30 min. Following treatment with either etoposide or bleomycin, the cells were washed with PBS and expanded for 3 days. The cells were then split into eight 100 mm tissue culture dishes and incubated for 24 hours before addition of GCV (40µM).

2.2 Vector construction

Both pEF1αTK (containing I-*SceI* recognition site) and I-*SceI* expression vectors (PCBASce) were previously described by Varga, et al (14) and Richardson et al (17). A vector (named pTBGHygro) containing the hygromycin phosphotransferase cDNA (Hygro^R; confers resistance to hygromycin) fused in frame to human β-globin exon 3, preceded by β-globin intron 2, exon 2, and an I-*SceI* recognition sequence was generated as follows. A *Bam*HI/*Eco*RI fragment containing the I-*SceI* recognition sequence, β-globin exon 2, intron 2 and Exon 3 was generated by PCR and ligated into the *Bam*HI and *Eco*RI sites of pcDNA3 (Invitrogen). This plasmid was then digested with *Bam*HI and *Bgl*II and religated to delete the CMV promoter from the pcDNA3 backbone. Finally, this plasmid was digested with *Eco*RV and *Not*I, and a PCR-generated hygromycin phosphotransferase cDNA was ligated in-frame into β-globin exon 3 to generate the pTBGHygro vector.

2.3 Conventional and Inverse PCR

All PCR amplifications, unless otherwise indicated, were performed using PCR SuperMix High Fidelity enzyme and buffers (Invitrogen, Carlsbad, CA). DNA extraction, digestion and ligation were as previously reported (14). To verify DNA quality, we routinely amplified the *SVCT* locus for all clones. For inverse PCR, DNA was digested with either *Hind*III or *Mbo*I, and re-ligated with T4 Ligase (Promega, Madison, WI). Long PCR fragments for either conventional or inverse PCR were amplified using the TaKaRa LA Taq kit (TaKaRa Bio, Otsu, Shiga, Japan), following the manufacturer's recommended protocol. Details of primer sequences and locations in the vector are listed in Supplemental Table 1.

2.4 Fluorescence in situ hybridization and chromosome painting

Chromosome preparations were made from OVCAR-8 cell line and clones OV-11 and A-15 by conventional methods. Slides were pretreated and denatured as described elsewhere (18). The pEF1αTK and pTBGHygro plasmids were labeled by nick translation with digoxigenin-11-dUTP (Roche, Indianapolis, IN), precipitated in ethanol with 50x excess of human Cot-1 DNA (Roche, Indianapolis, IN), and resuspended to a final concentration of 50 ng/µl in Hybrizol solution (Qbiogene, Montréal, Canada). Whole chromosome painting probes for chromosomes 2 and 9 (labeled in green) were obtained from Applied Spectral Imaging (Vista, CA). After denaturing at 80°C for 10 min and preannealing at 37°C for 60 min, 10 µl of probe mixtures were applied under 22 × 22 mm coverslips. Slides were incubated in a moist chamber overnight at 37°C. After detection with anti-digoxigenin antibodies labeled with rhodamine (Roche, Indianapolis, IN) they were mounted in antifade solution (Vector Laboratories, Burlingame, CA) containing DAPI. Leica microscope equipped with DAPI, FITC and rhodamine filters (Chroma Technology, Rockingham, VT) and Sensys CCD camera (Photometrics, Tucson, AZ) connected with Q-FISH software (Leica Microsystems Imaging Solutions, Cambridge, UK) were used for image acquisitions.

2.5 Southern blot analysis

Duplicate samples of 10 µg of genomic DNA were isolated from the indicated clones, digested with *Hind*III, and size fractionated using a 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose membranes and hybridized to ³²P-labeled probes as previously described (14).

2.6 Nucleotide sequence analysis

PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and transformed into DH-5α cells. Plasmid DNA was extracted (Qiagen, Valencia, CA) and sequenced (NAPCORE, The Children's Hospital of Philadelphia or Retrogen, San Diego, CA). Nucleotide sequences were compared to the human genome assembly based on NCBI Human Genome Build 34.

3. Results

3.1 Creation of a reporter cell line

In an attempt to produce GCRs, we generated a cell line which could serve as a reporter for unfaithful repair of a DNA DSB. A plasmid that expresses the TK gene under the control of the EF1α promoter, with the 18-bp recognition sequence for the *I-Sce*I restriction enzyme placed between the EF1α promoter and TK gene (Fig. 1A, Supplementary Figure 1), was linearized and introduced into OVCAR-8 cells. Stable transfectants were selected with G418, and several clones that had integrated a single copy of the construct were identified by Southern blot hybridization. These clones were then treated with 40µM GCV to verify expression of *Hsv-tk* and sensitivity to GCV. One clone, named A15, had integrated a single copy of the EF1αTK vector and was shown to be sensitive to GCV. We used inverse PCR to clone the integration sites, and determined that the construct was integrated on chromosome 2, between nucleotides 24378945 and 24378974 (Fig. 1B). To confirm this integration event, we synthesized a probe (named CHR2; nuc 24378079–24378704) immediately downstream of the *Hind*III site shown in figure 1B, and within an intron of the *C2orf84* gene. This probe was hybridized to a Southern blot of genomic DNA from the parental OVCAR-8 cell line and the A15 clone. As shown in figure 1C, a novel, rearranged fragment generated by the insertion is seen in the A15 subclone, as well as the endogenous band from the remaining intact allele. To further characterize the insertion, we co-hybridized metaphase chromosomes to a chromosome 2 paint and the EF1αTK vector. Comparison of the parental OVCAR-8 cell line with the A15 cell line demonstrates that both the parental and daughter cell lines had one morphologically normal copy of chromosome 2, and 3 additional chromosomes with a portion of chromosome 2. Figure 1D demonstrates that the vector is integrated on the telomeric portion of a derivative chromosome 2.

3.2 Generation and analysis of GCV^R clones

The A15 clone was transfected with the *I-Sce*I expression vector pCBASce (12), and incubated without GCV selection for 3 days. GCV resistant (GCV^R) clones were then selected by the addition of GCV to a final concentration of 40µM. After three weeks selection in GCV, no GCV^R clones were obtained from the control (vector alone) transfectants. However, 5–10 GCV^R clones/10 cm dish were detected. We isolated 59 GCV^R clones and attempted to expand them. Thirty-six clones were successfully expanded and subjected to DNA analysis; the remainder either did not expand or became contaminated and were discarded. Following PCR and sequence analysis, two clones were determined to be duplicates (C23 and C35). Four clones (C1, C27, C34, and C36) had evidence of *I-Sce*I cleavage, as evidenced by inability to amplify across the *I-Sce*I site, and loss of exogenous EF1α sequences on Southern blot, but were unable

to be characterized further. Finally, thirty-one independent clones were analyzed by Southern blot and sequencing analyses.

The GCV^R clones were screened with PCR primers that flanked the I-SceI cleavage site (primers IPCREF2 and INVALU5R in Fig. 2A and Supplemental Table 1). Based on our previous observations in U937 cells (14), we anticipated that small interstitial deletions and insertions would be detected by this assay, as shown in Fig. 2C. The parental cell line A15 showed a PCR product of the expected size (1204bp), whereas clones C30, C33, C35, C40, C44, and C53 all generated variable-sized, smaller PCR products, suggesting an interstitial deletion. Additional clones (for example, C34, C43, C47, C54 of figure 2C) did not produce a PCR product, suggesting that either one or both primer binding sites had been deleted, or that the primer binding sites had been separated by a large distance (for instance, by a large insertion or GCR). Direct sequencing of PCR products showed that 7 clones (C4, C9, C23, C30, C33, C44 and C53) had small (less than 1 kb) interstitial deletions (Table 1).

We used Southern blot analysis to further evaluate clones that did not generate PCR products with primers IPCREF2 and INVALU5R. Since the EF1aTk vector does not contain any *HindIII* sites, EF1a and Neo probes hybridize to the same *HindIII* fragment in the parental A15 clone (see Figure 1B for location of probes). Furthermore, if the A15 clone is cleaved at the I-SceI site, and undergoes an interstitial deletion during DNA DSB repair, the resulting daughter clone will also contain EF1a and G418 sequences on the same *HindIII* fragment (which is smaller than the *HindIII* fragment in the parental A15 clone). As demonstrated in figure 2D, clones C17, C18, C23, C24, C28, and C29 had variably sized *HindIII* fragments, different from that of the parental A15 clone, which hybridized to the Neo probe. In each case, the same novel (i.e., smaller than the fragment from the parental A15 clone) *HindIII* fragments hybridized to the EF1a probe, suggesting that these clones had undergone an interstitial deletion.

If the clone had undergone a GCR, the size of the *HindIII* fragments that hybridized to the EF1a and Neo probes would depend on the location of endogenous *HindIII* sites. Therefore, if the clone had undergone a GCR, we would expect the EF1a and Neo probes to hybridize to *HindIII* fragments that were not the size of the wild-type fragment, and not of the same size. This situation is in contrast to the interstitial deletions, in which the non-wild-type fragments that hybridized to the EF1a and Neo probes were the same size.

As shown in Fig. 2D, 5 clones (C16, C19, C20, C21 and C26) lost the EF1a region, but had variable, non-wild-type sized fragments that hybridized to the Neo probe, suggesting that these clones had either a larger interstitial deletion (which had deleted EF1a sequences), or had a GCR that had deleted EF1a sequences. Using *HindIII* or *MboI* digested DNA samples, we performed inverse PCR to clone the rearrangement from 12 clones (Fig. 2B, Table 1). All of these clones analyzed by inverse PCR represented larger interstitial deletions. In sum, all 31 clones analyzed, including 12 that were analyzed by Southern blot alone and 19 that were sequenced, represented interstitial deletions (Table 1). Thirteen of the 19 sequenced clones were simple interstitial deletions, and six clones represented more complex rearrangements, due to DNA insertion and inversion events. Two clones had insertions that were derived from the I-SceI expression vector, and two other clones had small insertions of undetermined origin. Clone C19 had an inverted duplication at the I-SceI site, and clone C26 had a 357 bp insertion that matched sequences, from a gene encoding a hypothetical protein (LOC730045) located on chromosome 18. Thirteen clones showed micro-homology (1 to 8 bp) at the junction sites (Table 1).

3.3 Characterization of clones induced by I-SceI and genotoxic agents

Since we were unable to produce GCRs by induction of a single DNA DSB, we reasoned that we might be able to produce GCRs by generating two or more simultaneous DNA DSBs.

Therefore, we used etoposide or bleomycin to generate random DNA DSBs, and I-*SceI* to produce a specific DNA DSB. Following transfection of the A15 cell line with an I-*SceI* expression vector, cells were treated with 100 μ M etoposide for one hour or 10 μ M etoposide for four hours, and then allowed to recover for four days before GCV selection. A total of seven GCV^R clones were identified; one clone (V1) from the 100 μ M/ one hr treatment, the other six clones from the 10 μ M/ 4 hr treatment. The A15 clone was treated with bleomycin in a similar fashion, for 30 min with either 5 or 20 μ g/mL bleomycin. A total of 15 GCV^R clones were identified, four from the 5 μ g/ml treatment, and 11 from the 20 μ g/ml treatment.

Compared to clones generated by expression of I-*SceI* only, cells treated with both I-*SceI* and etoposide or bleomycin had a lower yield of GCV^R clones, several times less than that of the I-*SceI* only group. However, analysis of the GCV^R clones from all three groups demonstrated only interstitial deletions; no GCRs were identified (Table 1). The size of the deletions was similar in all three groups, with the maximum deletion identified being 3.2 kb (Table 2). All three groups showed similar frequencies of microhomology and insertions at the breakpoint junctions (Table 1).

3.4 Use of a positive selection system to produce GCR

Given that the negative selection (ie, detection of clones missing a gene product) approach described above produced only clones with interstitial deletions leading to loss of TK expression, we devised a positive selection approach. This positive selection approach would detect the presence of a gene product, as opposed to the absence of a gene product, and would not detect clones with a simple interstitial deletion. A vector (pTBGHygro) containing the hygromycin phosphotransferase cDNA (Hygro^R; confers resistance to hygromycin) fused in frame to human β -globin exon 3, preceded by β -globin intron 2, exon 2, and an I-*SceI* recognition sequence was generated (Fig. 3, Supplementary Figure 2). The vector also contains a G418^R expression cassette to allow for selection of cells that have integrated the vector. We transfected this vector into OVCAR-8 cells, and identified a clone (named OVCAR8-11) that had integrated a single copy of the vector. As anticipated, since the Hygro^R cDNA lacks a promoter, these cells were hygromycin sensitive. As described above, we used inverse PCR to determine that the insertion site for the OVCAR8-11 clone was chromosome 9, nucleotide 65632493, 20 kb telomeric of AK094938. We used Southern blot analysis with a PCR-generated probe (CHR9 nuc. 65631975–65632438, see Figure 3) to verify the insertion site (Figure 3B); multiple cross-hybridizing bands are seen since this integration site is within a low copy number repeat region. We further characterized the insertion by chromosome painting and FISH. Neither the parental OVCAR-8 nor the daughter OVCAR8-11 clone had an intact chromosome 9; instead, there were 3 chromosomes in both cell lines that contained chromosome 9 material (Figure 3B and C). The pTBGHygro vector hybridized to a subcentromeric region of chromosome 9 (Figure 3C).

The OVCAR8-11 subclone was transfected with an I-*SceI* expression vector, and selected with hygromycin, in the hopes of recovering rare clones that had undergone a GCR, and juxtaposed gene regulatory sequences from a distant genomic region to the Hygro^R gene, thus allowing expression of the Hygro^R gene, leading to hygromycin resistance. No Hygro^R clones were recovered from control experiments with an empty vector (pBluescript II) transfection, and we recovered few clones following transfection with the I-*SceI* expression vector. All 14 of the clones analyzed were vector capture events, in which a portion of SV40 regulatory sequences derived from the I-*SceI* expression vector became juxtaposed to *beta-globin* exon 2 (Fig. 3, Table 3), leading to hygromycin resistance.

4. Discussion

Aberrant repair of DNA DSBs is likely to play a role in generating the GCRs that are hallmarks of human malignancies. We previously reported that induction of a single DNA DSB in a hematopoietic cell line did not generate GCRs, but instead only small interstitial deletions (14). However, given that solid tumors typically display more GCR, and genetic instability than do hematopoietic malignancies (6,⁷,¹⁹,20), we wished to determine if GCRs could be induced by a single DNA DSB in an epithelial cell line. For these experiments, we used the OVCAR-8 cell line, which has previously been reported to have a high degree of ongoing chromosomal instability (15).

We induced DNA DSBs in OVCAR-8 cells by first introducing the recognition sequence for the *I-SceI* restriction enzyme, followed by transfection with an *I-SceI* expression vector. Clones that had been cleaved by *I-SceI* were analyzed using a combination of PCR assays and Southern blot hybridizations. The simplest form of repair for a DNA DSB induced by *I-SceI* would be a perfect religation event that recreates an *I-SceI* recognition sequence which would remain susceptible to *I-SceI* cleavage. Although our experiment was not designed to detect perfect repair of *I-SceI* cleavage, other studies have utilized a substrate that includes sequences flanked by *I-SceI* sites (21). In those studies, cleavage by *I-SceI* and religation at the *I-SceI* sites could be detected by loss of intervening sequence. The investigators noted that 33–65% of the clones recovered had been repaired by a precise religation event (21). If the cleaved recognition sequence is repaired by an imprecise mechanism, such as non-homologous end-joining, clones can be isolated due to loss of TK function.

We characterized 31 clones that sustained mutations following the repair of a single *I-SceI*-induced DNA DSB in OVCAR-8 cells. Similar to previous reports (10,¹⁴,²²-28), the most common DNA DSB repair leading to loss of TK expression was an interstitial deletion, often accompanied by additional mutations, such as insertions and inversions. Many of these clones showed hallmarks of NHEJ, such as microhomology at the site of DNA DSB repair in these cells. Several clones displayed more complex DNA DSB repair events. One clone contained 357 bp of sequence derived from LOC730045, located on chromosome 18q, and two clones captured *I-SceI* expression vector sequences similar to previous findings (14,23). However, none of the clones analyzed showed evidence of a GCR. It remains possible that cleavage at the *I-SceI* site induced a GCR at a site distant from the *I-SceI* site; a distant GCR would not have been identified by the Southern blot or PCR assays, which focused on aberrant repair events at the *I-SceI* site.

Prior studies have demonstrated that balanced chromosomal translocations are extraordinarily uncommon events when a single DNA DSB is induced by *I-SceI* in murine embryonic stem cells (12). However, if two simultaneous DNA DSB breaks are introduced, the frequency of balanced translocations increases more than 2,000 fold (12). A special case exists at telomeric DNA, where integration of a vector containing an *I-SceI* recognition site near a telomere greatly increases the frequency of clone that have undergone either a large (~300 bp or greater) deletion or GCR (26).

To determine if simultaneous production of a specific DNA DSB (by expression of *I-SceI*) and a non-specific DNA DSB would lead to production of a GCR, we treated cells with etoposide or bleomycin. We characterized 22 clones that had been transfected with an *I-SceI* expression vector and subsequently treated with either etoposide or bleomycin. Similar to the *I-SceI*-induced cells, none of these clones showed evidence of a GCR. However, it remains possible that the timing which was employed for this experiment (transfection of the *I-SceI* plasmid 48 hours prior to treatment with etoposide or bleomycin) was sub-optimal.

A potential disadvantage of the above approach is that it utilizes a negative selection system. Results obtained by us (14) and others (23,26) with this type of approach (a negative selection based on TK and GCV selection) has yielded primarily interstitial deletions of 5 kb or less. Therefore, we developed a complementary vector that allowed for positive selection. However, as described above, all of the clones Hygro^R clones identified were vector capture events, in which a portion of SV40 regulatory sequences derived from the I-SceI expression vector became juxtaposed to β -globin exon 2 or intron 2. These vector capture events at I-SceI cleavage sites have been described previously (14,23), and are presumably due to the ability of large amounts of excess plasmid DNA in the nucleus to serve as “patches” at the DNA DSB site.

In summary, we have established two complementary systems to generate a specific, single DNA DSB in an epithelial cancer cell line, which may be useful for elaborating DNA DSB repair mechanisms. Despite examination of over 65 mutant clones, PCR and Southern blot analysis revealed no evidence for a GCR in any of the clones analyzed. Instead, we detected principally small deletions or insertions, with features suggesting repair by NHEJ. These findings indicate that GCRs are likely to be a rare consequence of DNA DSB repair, even in a cell line such as OVCAR-8, which is prone to structural instability. It is possible that use of a cell line that is deficient in proteins required for conventional NHEJ will be needed to produce GCRs following a single induced DNA DSB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tamas Varga, Ilan R Kirsch and Michael Kuehl for suggestions and helpful advice, and Maria Jasin for the gift of the pCBASce expression vector. This work was supported by the Intramural Research Program of the NIH, NCI.

Abbreviations

DSB	double-strand break
GCRs	gross chromosomal rearrangements
NHEJ	non-homologous end joining

References

- (1). Agarwal S, Tafel AA, Kanaar R. DNA double-strand break repair and chromosome translocations. *DNA Repair* 2006;5:1075–1081. [PubMed: 16798112]
- (2). Aplan PD. Chromosomal translocations involving the MLL gene: Molecular mechanisms. *DNA Repair* 2006;5:1265–1272. [PubMed: 16797254]
- (3). Rowley JD. Chromosome translocations: dangerous liaisons revisited. *Nature Reviews Cancer* 2001;1:245–250.
- (4). Deveney R, Chervinsky DS, Jani-Sait SN, Grossi M, Aplan PD. Insertion of *MLL* sequences into chromosome band 5q31 results in an *MLL-AF5Q31* fusion and is rare but recurrent abnormality associated with infant leukemia. *Genes Chromosomes & Cancer* 2003;37:326–331. [PubMed: 12759932]
- (5). Aplan PD. Causes of oncogenic chromosomal translocation. *TRENDS Genetics* 2006;22:46–55.
- (6). Tonon G, Modi S, Wu L, Kubo A, Coxon AB, Komiyama T, O’Neil K, Stover K, El-Naggar A, Griffin JD, Kirsch IR, Kaye FJ. t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a

- novel fusion product that disrupts a Notch signaling pathway. *Nature Genet* 2003;33:208–213. [PubMed: 12539049]
- (7). Tognon C, Knezevich SR, Huntsman D, Roskelley CD, Melnyk N, Mathers JA, Becker L, Carneiro F, MacPherson N, Horsman D, Poremba C, Sorensen PH. Expression of the ETV6-NTRK3 gene fusion as a primary event in human secretory breast carcinoma. *Cancer Cell* 2002;2:367–376. [PubMed: 12450792]
 - (8). Valerie K, Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* 2003;22:5792–5812. [PubMed: 12947387]
 - (9). Betti CJ, Villalobos MJ, Diaz MO, Vaughan AT. Apoptotic triggers initiate translocation within the *MLL* gene involving the nonhomologous end joining repair system. *Cancer Res* 2001;61:4550–4555. [PubMed: 11389089]
 - (10). Donoho G, Jasin M, Berg P. Analysis of gene targeting and intrachromosomal homologous recombination stimulated by genomic double-strand breaks in mouse embryonic stem cells. *Mol. Cell. Biol* 1998;18:4070–4078. [PubMed: 9632791]
 - (11). Weterings E, van Gent DC. The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair* 2004;3:1425–1435. [PubMed: 15380098]
 - (12). Richardson C, Jasin M. Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature* 2000;405:697–700. [PubMed: 10864328]
 - (13). Vilenchik MM, Knudson AG. Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. *Proc. Natl. Acad. Sci. USA* 2003;100:12871–12876. [PubMed: 14566050]
 - (14). Varga T, Aplan PD. Chromosomal aberrations induced by double strand DNA breaks. *DNA Repair* 2005;4:1038–1046. [PubMed: 15935739]
 - (15). Roschke AV, Tonon G, Gehlhaus KS, McTyre N, Bussey KJ, Lababidi S, Scudiero DA, Weinstein JN, Kirsch IR. Karyotypic complexity of the NCI-60 drug-screening panel. *Cancer Res* 2003;63:8634–8647. [PubMed: 14695175]
 - (16). Alper O, De Santis ML, Stromberg K, Hacker NF, Cho-Chung YS, Salomon DS. Anti-sense suppression of epidermal growth factor receptor expression alters cellular proliferation, cell-adhesion and tumorigenicity in ovarian cancer cells. *Int. J. Cancer* 2000;88:566–574. [PubMed: 11058872]
 - (17). Richardson C, Moynahan ME, Jasin M. Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev* 1998;12:3831–3842. [PubMed: 9869637]
 - (18). Roschke AV, Stover K, Tonon G, Schäffer AA, Kirsch IR. Stable karyotypes in epithelial cancer cell lines despite high rates of ongoing structural and numerical chromosomal instability. *Neoplasia* 2002;4:19–31. [PubMed: 11922387]
 - (19). Yu VP, Koehler M, Steinlein C, Schmid M, Hanakahi LA, van Gool AJ, West SC, Venkitaraman AR. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev* 2000;14:1400–1406. [PubMed: 10837032]
 - (20). Mitelman F, Johansson B, Mertens F. Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. *Nat. Genet* 2004;36:331–334. [PubMed: 15054488]
 - (21). Lin Y, Lukacsovich T, Waldman AS. Multiple Pathways for Repair of DNA Double-Strand Breaks in Mammalian Chromosomes. *Mol. Cell. Biol* 1999;19:8353–8360. [PubMed: 10567560]
 - (22). Honma M, Lzumi M, Sakuraba M, Tadokoro S, Sakamoto H, Wang W, Yatagai F, Hayashi M. Deletion, rearrangement, and gene conversion; genetic consequences of chromosomal double-strand breaks in human cells. *Environ. Mol. Mutagen* 2003;42:288–298. [PubMed: 14673874]
 - (23). Lin Y, Waldman AS. Capture of DNA sequences at Double-strand breaks in mammalian chromosomes. *Genetics* 2001;158:1665–1674. [PubMed: 11514454]
 - (24). Phillips JW, Morgan WF. Illegitimate recombination induced by DNA double-strand breaks in a mammalian chromosome. *Mol. Cell. Biol* 1994;14:5794–5803. [PubMed: 8065314]
 - (25). Sargent RG, Brenneman MA, Wilson JH. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol. Cell. Biol* 1997;17:267–277. [PubMed: 8972207]

- (26). Zschenker O, Kulkarni A, Miller D, Reynolds GE, Granger-Locatelli M, Pottier G, Sabatier L, Murnane JP. Increased sensitivity of subtelomeric regions to DNA double-strand breaks in a human cancer cell line. *DNA Repair* 2009;8:886–900. [PubMed: 19540174]
- (27). Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol. Cell. Biol* 1994;14:8096–8106. [PubMed: 7969147]
- (28). Pipiras E, Coquelle A, Bieth A, Debatisse M. Interstitial deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. *EMBO J* 1998;17:325–333. [PubMed: 9427766]

Fig. 1. Integration of pEF1 α TK construct into chromosome 2 in A15 cells

A. Integration of the pEF1 α TK construct in OVCAR-8-derived clones by Southern blot analysis. DNAs were digested with *Hind*III and hybridized with probe Neo. A15 cells, with a single integration site, were used for further analysis. **B.** The integration sites on chromosome two (based on NCBI build 34) are indicated. Gray squares indicate the CHR2, EF1 α , and Neo probes used for Southern blot analyses. *I-Sce*I (I) and *Hind*III (H) sites are indicated. **C.** Genomic DNA from parental (OV8) or A15 cells, digested with *Hind*III, and hybridized to the CHR2 probe. The endogenous fragment is indicated with an asterisk, the novel fragment containing the EF1 α TK vector is indicated with an arrow. OVCAR-8 or A15 cells (Panels **D** and **E** respectively) hybridized to chromosome 2 paint (green) and EF1 α TK plasmid (red). Note the red sister chromatid signals on the magnified inset.

Fig. 2. Analyses of DNA DSB repair events in GCV resistant clones

A. Location of PCR primers used for forward PCR. **B.** Location of primers used for inverse PCR. *MboI* and *HindIII* restriction sites are indicated by M and H, respectively. **C.** Example of PCR amplification across *I-SceI* site. Parental A15 cells generated a 1.2 kb DNA fragment, clones C30, C33, C35, C40, C44, and C53 generated variable sized fragments, and clones C34, C43, C47, and C54 generated no PCR products. Bottom panel shows PCR amplification of the *SVCT* locus, used as a DNA quality control. **D.** Southern blot analysis of A15-derived clones. Genomic DNAs were digested with *HindIII* and hybridized to probes Neo (Top) and EF1α (Bottom). E indicates endogenous allele of EF1α. Note that clones C17, C18, C23, C24, C28

and C29 clones have non-wild-type sized fragments, which are detected by both the Neo and EF1 α probes. C16, C19, C20, C21 and C26 clones have lost the EF1 α portion of the vector.

Fig. 3. I-SceI induced cleavage of the pTGHygro cassette leads to DNA capture events

A. Integration of pTGHygro construct into chromosome 9 in OVCAR8-11 cells. The *I-SceI* recognition site (I) was inserted upstream of the β -globin exons 2 and 3. HindIII (H) site and CHR9 probe are indicated. **B.** Southern blot of HindIII digested genomic DNA from OVCAR-8, A15, or OVCAR8-11 clones, hybridized to the CHR9 probe. The endogenous fragment is indicated with an asterisk, the novel fragment containing the pTGHygro vector is indicated with an arrow. Several endogenous fragments, including one that is more intense than the novel OVCAR8-11 fragment, are seen because the CHR9 probe is within a low copy number repeat region. OVCAR-8 or OVCAR8-11 cells (Panels **C** and **D** respectively) hybridized to chromosome 9 paint (green) and pTGHygro plasmid (red). Note the red signals on the magnified inset. **E.** Subclones generated by *I-SceI* expression vector resulted from cleavage at the *I-SceI* site, insertion of SV40 sequences derived from the *I-SceI* expression vector, and subsequent re-ligation between the chromosomal end and β -globin sequences from the pTGHygro construct.

Table 1
Summary of DNA break/repair events induced by I-SceI endonuclease, bleomycin and etoposide

*Clone	Result	Bp deleted from EF1a side	Bp deleted from TK side	Total Deletion**	Micro-homology 5'	Micro-homology 3',***	Comments
C2	Deletion			~1200			
C3	Deletion			~500			
C4	Deletion	62	235	315	TA		
C6	Deletion			~1400			
C9	Deletion	7	151	176	CGGG		
C11	Deletion		3060	3075			Insertion: TAGAATA
C13	Deletion			~1800			
C15	Deletion	274	539	831			
C16	Deletion	702	575	1295	A		
C17	Deletion			~1200			
C18	Deletion			~1600			
C19	Deletion	391	583	992	AG	CC	Inversion: 218 bp of I-SceI + TK
C20	Deletion	434	1535	1987			
C21	Deletion	556	884	1458	ACT		
C22	Deletion	12	2982	3012	GG		
C23/35	Deletion		568	584			Insertion: 135 bp from vector
C24	Deletion	23	367	408	GTCGA	GCCC	Insertion: 86 bp from vector
C26	Deletion	1028	305	1351	AAA	CA	Insertion: 357 bp from chro. 18
C28	Deletion			~1000			
C29	Deletion	10	854	882	C		
C30	Deletion	5	843	866			Insertion: AGGA
C31	Deletion	626	892	1536			
C33	Deletion	8	660	686	GGGCGG C		
C39	Deletion	2388	878	3284	G		
C40	Deletion			~1500			

*Clone	Result	Bp deleted from EF1 α side	Bp deleted from TK side	Total Deletion**	Micro-homology 5'	Micro-homology 3'	Comments
C43	Deletion			~1800			
C44	Deletion	208	213	439	GAAACC		
C47	Deletion			~1600			
C53	Deletion	20	662	700	C		
C54	Deletion			~1500			
C59	Deletion			~800			
B1	Deletion	100	892	1010	G		
B3	Deletion	7	2893	2918	CG		
B4	Deletion	12	460	490	CGG		
B6	Deletion	2294	972	3284			
B8	Deletion			~2100			
B9	Deletion			~2200			
B10	Deletion	25	136	179			Insertion: 22bp
B13	Deletion	426	859	1303	TAA		
B17	Deletion			~500			
B20	Deletion	822	1701	2541	AA		
B21	Deletion	1962	751	2731			Insertion: C
B23	Deletion	5	1475	1498			Insertion: 379 bp from vector
B24	Deletion		180	180	CGT		I-SceI is intact
B26	Deletion	529	830	1377			Insertion: GC
B27	Deletion			~1600			
V1	Deletion			~500			
V3	Deletion	1515	1668	3201			
V4	Deletion	10	714	742	CGGGC		
V5	Deletion	1276		1294	G	C	Insertion: 21 bp from vector
V7	Deletion	7	546	571	CCCC		
V12	Deletion	1098	395	1511			
V17	Deletion	515	1905	2438			

* C clones: I-SceI only; B clones: I-SceI + bleomycin; V clones: I-SceI + etoposide

Includes 18 bp of I-SceI sequence

Two micro-homology sites caused by two re-ligations (both 5' and 3') were only detected in some clones
~
Approximate DNA sizes detected by Southern blot analyses

Table 2
Comparison of DNA break and recombination events
occurred in A15 cells

Groups	I-SceI	I-SceI + bleomycin	I-SceI + etoposide
Characterized clones	31	15	7
Deletion ranges (bp)	176–3284	179–3284	500–3201
Median deletions (bp)	1200	1600	1294

Table 3
Hygromycin resistant clones

Clone #	Rearrangement	I-SceI fusion site	Micro-homology
8	Vector fusion	123	none
10	Vector fusion	116	A
14	Vector fusion	135	none
15	Vector fusion	113	A
17	Vector fusion	115	A
20	Vector fusion	124	none
21	Vector fusion	116	none
26	Vector fusion	124	ATT
28	Vector fusion	125	none
1A	Vector fusion	115	none
3A	Vector fusion	130	none
7A	Vector fusion	98	none
8A	Vector fusion	111	CCTA
9A	Vector fusion	112	none