Gene conversion is strongly induced in human cells by double-strand breaks and is modulated by the expression of BCL-x_L[1]

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Running Title: Homology-directed DNA repair in human cells.

Key words: double-strand break repair, homologous recombination, BCL-x_L, apoptosis, human cells.

[1]This work was supported by NIH CA 73966 and NASA T-964W grants to A. Kronenberg, NIH GM54688 grant to M. Jasin and the NASA NSCORT in Radiation Health (A. Chatterjee, Principal Investigator). Additional support for this work is acknowledged from the Office of Biological and Physical Research, Office of Energy Research, U.S. Department of Energy under contract number DE-AC03-76SF00098 at LBNL.

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[4]The abbreviations used are: PCD, programmed cell death; DSB, DNA double-strand break; TK1, thymidine kinase; HPRT, hypoxanthine phosphoribosyl transferase; HDR, homology-directed repair; NHEJ, non-homologous end-joining, GFP, green fluorescent protein; FACS, fluorescence activated cell sorting; LOH, loss of heterozygosity.

Abstract

Homology-directed repair (HDR) of DNA double-strand breaks (DSBs) contributes to the maintenance of genomic stability in rodent cells, and it has been assumed that HDR is of similar importance in DSB repair in human cells. However, some outcomes of homologous recombination can be deleterious, suggesting that factors exist to regulate HDR. We previously demonstrated that overexpression of BCL-2 or BCL-x_L enhanced the frequency of X-ray-induced *TK1* mutations, including loss of heterozygosity (LOH) events presumed to arise by mitotic recombination. The present study was designed to test whether HDR is a prominent DSB repair pathway in human cells, and to determine whether ectopic expression of BCL-x_L affects HDR. Using TK6-neo cells, we find that a single DSB in an integrated HDR reporter stimulates gene conversion 40- to 50-fold demonstrating efficient DSB repair by gene conversion in human cells. Significantly, DSB-induced gene conversion events are 3- to 4-fold more frequent in TK6 cells that stably overexpress the anti-apoptotic protein BCL-X_L. Thus, HDR plays an important role in maintaining genomic integrity in human cells and ectopic expression of BCL-x_L enhances HDR of DSBs. This is the first study to highlight a function for BCL-x_L in modulating DSB repair in human cells.

Introduction

Cellular responses to DNA damage include removal or bypass of specific DNA lesions, checkpoint functions to delay cell cycle progression, and the programmed death of damaged cells. When PCD[4] is suppressed, new mutations in critical genomic loci (e.g. tumor suppressor genes) are maintained in the affected cells and their progeny, and this is likely to be important in malignant progression. One DNA lesion that is particularly critical to repair is the DSB. Mammalian cells have robust non-homologous DSB repair pathways, which primarily involve nonhomologous end-joining (NHEJ) (1). NHEJ proteins form a complex (DNA-PK) that binds to DNA ends (2). Although DNA-PK is conserved between rodents and humans, its activity has been estimated to be 50-fold more abundant in human cells (3), raising the possibility that DSB repair may differ in some regards between mammalian species. In addition to NHEJ, HDR is a major DSB repair pathway in rodent cells, wherein DSBs are potent inducers of recombination (4). The expectation is that individual DSBs should be potent inducers of recombination in human cells. Nevertheless, the difference in NHEJ raises doubts about this assumption.

Ionizing radiation, which causes DSBs, induces apoptosis in many cell types. Apoptosis is controlled, in part, by proteins of the BCL-2 family. Members of this family share some structural homology but function either to promote cell death or inhibit cell death. Anti-apoptotic proteins include BCL-2 and BCL-x_L (5). The relative abundance of pro- and anti-apoptotic members of this protein family determines whether a cell will live or die (6). As might be expected, dysregulation of the network of BCL-2 related proteins contributes to human carcinogenesis. BCL-2 is highly expressed in B-cell follicular lymphoma due to its juxtaposition with the IgH enhancer, and BCL-x_L is upregulated in a variety of cancers wherein high

expression is associated with advanced disease, resistance to genotoxic agents, and poor prognosis (7-10).

We previously reported that human B-cell lines suppressed for apoptosis by overexpression of BCL-2 or BCL-x_L showed significantly elevated levels of radiation-induced *TK1* mutations (11). *TK1* mutations can arise by several mechanisms, including LOH by mitotic interchromosomal recombination (12). Mitotic recombination between homologous chromosomes is presumed to involve homology at the point of crossover, although the fidelity of repair at the breakpoint junctions is usually not assessed. To determine if human cells can efficiently repair DSBs by HDR, and also to determine the effect of BCL-x_L overexpression on HDR, we stably transfected TK6-neo and TK6-bcl-x_L cells with a direct repeat green fluorescence protein (DR-GFP) recombination reporter (13). A single DSB is introduced into this reporter via transient expression of the I-*Sce*I endonuclease and when this DSB is repaired by gene conversion, expression of a functional *GFP* gene results. Expression of I-*Sce*I greatly stimulated gene conversion in both cell types. In TK6-neo cells, HDR was stimulated 40- to 50-fold, demonstrating conclusively that DSBs are potent inducers of homologous recombination in wild-type human cells. In addition, DSB-induction led to a 3- to 4-fold increase in the number of recombinants in TK6-bcl-x_L cells as compared to TK6-neo cells, suggesting a novel role for BCL-x_L in modulating DSB repair in human cells.

Materials and Methods

Cell culture

TK6-neo and TK6-bcl-x_L cells are human B-lymphoblasts that have been described previously (11). Cells were grown at 37˚C in suspension cultures and were maintained in exponential growth at densities of 1×10^5 to 1×10^6 cells/ml.

Transfection and recombination assays

Stable cell lines containing the *pDR-GFP* reporter construct (13) were generated by electroporation (625 V/cm, 950 μF) using the Bio-Rad Gene Pulser II apparatus (Bio-Rad, Hercules, CA). One million TK6-neo cells or TK6-bcl-x_L cells were transfected in 400 μl *Cytomix* buffer using 10 μg of circular plasmid (14). Cells were seeded at 500 cells/well in 96-well plates in standard growth medium supplemented with 0.2 μg/ml puromycin. Puromycin-resistant cell lines were subjected to Southern analysis using *Sal*I*/Hind*III digests to confirm intact integration of the *pDR-GFP* reporter and single copy integration was verified using *Pst*I with a cocktail of restriction enzymes that do not cut within the vector (13). The stable cell lines derived from TK6-neo cells are designated TK6-neo-DR-GFP and the stable cell lines derived from TK6-bcl-x_L cells are designated TK6-bcl-x_L-DR-GFP.

The construct *pNZE-CAG*, used to monitor plasmid uptake and the response to electroporation in TK6-neo and TK6-bcl-x_L cells, was described elsewhere (13). Transfectants were monitored by flow cytometry for transient expression of GFP 40 to 48 h post electroporation.

I-*Sce*I was expressed in transient transfections from the *pCβASce* expression vector using the same promoter as described for *pNZE-CAG* (15). One hundred μg of the construct were used

to transfect 1×10^6 TK6-neo-DR-GFP or TK6-bcl-x_L-DR-GFP cells. Transfected cells were analyzed by flow cytometry two to seven days after electroporation to measure the percentage of cells expressing GFP.

Flow cytometry and FACS

Flow cytometry was performed with a Beckman-Coulter EPICS XL flow cytometer (Beckman-Coulter, Fullerton, CA) using an argon ion air cooled laser (emission at 488 nm/15 mW power) and XL Data Acquisition and Display software (version 1.5). Green (FL1) versus orange (FL2) fluorescence plots were performed as described (13). GFP-positive cells (GFP+) are shifted green-ward off the green/orange diagonal in the direction of increasing FL1. Typically, 5×10^4 to 1×10^5 cells were analyzed in each experiment for a given cell line.

A Beckman-Coulter Elite ESP sorter (Fullerton, CA) was used to sort GFP+ cells, using a 525 (± 20) nm band pass filter (Omega Optica). Ten thousand to thirty thousand GFP+ cells were collected. The sorted cell population was 95-98% GFP+. Sorted cells were expanded and reanalyzed for GFP expression. An aliquot of the expanded culture was frozen and the remaining 3×10^7 cells were subjected to DNA extraction for analysis of structural changes leading to gene conversion using restriction digests and Southern analysis.

Southern analysis of gene conversion in GFP+ cells

Restriction analysis was performed using standard methods to confirm that gene conversion had occurred in GFP+ cells containing DR-GFP (13). The *SceGFP* is mutated through the incorporation of the I-*Sce*I site at a *Bcg*I restriction site by substituting 11 bp of wild-type GFP sequence with those of the I-*Sce*I site. These substituted bases also introduce two in-

frame stop codons. The 5' and 3' truncated GFP gene, *iGFP*, can be used to correct the mutation in the *SceGFP* gene to result in a *GFP+* gene with a restored *Bcg*I site. Molecular analysis confirms that GFP+ cells are generated by non-crossover gene conversion events within DR-GFP while deletional recombination events result in a 3'-truncated *GFP* gene that cannot express GFP (13).

Results

DSBs induce HDR by gene conversion >50-fold in human cells

TK6-neo cells are TK6 cells that contain an integrated copy of the *pSFFV-neo* vector (11). To test whether human cells undergo homologous recombination at elevated levels upon introduction of a chromosomal DSB, we integrated the recombination reporter substrate DR-GFP into the genome of TK6-neo cells (Fig.1A). Two independent clones containing a single copy of the substrate were used for subsequent analysis.

Clones were examined for both spontaneous and DSB-induced gene conversion within the DR-GFP substrate. When these clones were examined for green fluorescence using flow cytometry, few fluorescent cells were obtained (e.g., 0.01% for mock-transfected cells; Fig. 1B), indicating that spontaneous gene conversion is rare. To examine DSB-promoted gene conversion, the I-*Sce*I endonuclease was transiently expressed from *pCβASce*, to introduce a DSB in the upstream *SceGFP* gene. GFP+ cells were readily detected upon I-*Sce*I expression. For example, 0.54% of cells were GFP+ in the clone shown in Fig. 1B (middle panel), indicating more than a 50-fold increase over mock-transfected cells.

To prove that GFP+ cells arose by gene conversion, GFP+ cells were separated from GFP- cells in populations electroporated with the I-*Sce*I expression vector. FACS-sorted GFP+ cells were expanded in culture for DNA extraction and Southern blot analysis. Prior to sorting, 0.33-1% of the TK6-neo cells in a given population transfected with I-*Sce*I were GFP+ (e.g., Fig. 1B, middle panel). Generally, sorted cultures had 96-98% GFP+ cells upon immediate re-analysis. After several weeks of growth the fraction of GFP+ cells in some sorted cultures decreased slightly to 83-96% (e.g., Fig. 1B, right panel), while in others the fraction of GFP+ cells did not change at all. The reason for the slight decrease in the fraction of GFP+ cells in

some of the sorted cultures during expansion is unclear, although it might be the result of the low number of GFP+ cells in the starting population or occasional epigenetic silencing of the *GFP* gene. Following expansion, the level of fluorescence of each population was generally stable for several weeks in culture.

Genomic DNA from the sorted populations was subjected to Southern analysis using the *GFP* coding sequence as a probe (13). If a gene conversion event occurs at the I-*Sce*I site, the *GFP* wild-type sequence is restored along with its *Bcg*I site, which is detected by restriction digest in conjunction with *Sal*I and *Hin*dIII digests. The gene conversion product was observed in each of the GFP+ sorted populations (Fig. 1C), i.e., the *Sal*I/*Hin*dIII band of 3021 bp is not cleaved by I-*Sce*I (Fig. 1C, lanes 3 and 6), but is cleaved by *Bcg*I to two bands of 2140 bp and 881 bp (Fig. 1C, lanes 2 and 5). (Note: Because *Bcg*I is not a robust restriction enzyme, we frequently do not obtain complete cleavage.) These results demonstrate that the expression of GFP resulted from gene conversion restoring the wild-type *GFP* sequence by HDR.

Ectopic expression of BCL-x_L enhances HDR of a single, site-specific DSB in human cells

We then investigated whether ectopic expression of BCL-x_L affects the ability of human lymphoid cells to undergo HDR. TK6-bcl-x_L cells are TK6 cells containing an integrated copy of *pSFFVneo-bcl-x_L* and express approximately 80- to 100–fold higher levels of functional BCL-x_L than TK6-neo cells (11). We constructed four independent clones of TK6-bcl-x_L cells containing a single integrated copy of DR-GFP for use in gene conversion studies.

In mock transfection experiments, very few GFP+ cells were detected in either TK6-neo-DR-GFP or TK6-bcl-x_L-DR-GFP cells (Fig. 2A). The combined results for two TK6-neo-DR-GFP cell lines were 0.016% (±0.003) GFP+ cells (mean ± 1 S.E.M.), and for four TK6-bcl-x_L-

DR-GFP cell lines were 0.010% (±0.002) GFP+ cells (mean ± 1 S.E.M.). Gene conversion was rare in each cell type, indicating that high levels of BCL-x_L did not noticeably enhance gene conversion in the absence of DSB induction. It is not clear if the very small difference in the number of GFP+ cells detected in the different genetic backgrounds is meaningful, and it is unlikely that fluctuation analysis would clarify this point given the low spontaneous frequency.

DSB-mediated gene conversion was measured in all cell lines after transient expression of I-*Sce*I. To exclude position effects of the integrated DR-GFP, each DR-GFP derivative of TK6-neo and TK6-bcl-x_L was analyzed individually. I-*Sce*I-induced DSBs stimulated gene conversion in TK6-neo-DR-GFP cell lines resulting in 0.56% (±0.12) and 0.61% (±0.08) GFP+ cells for clone 1-7 and clone 2-2, respectively (mean ± 1 S.E.M; Fig. 2B). Ectopic expression of BCL-x_L strongly enhanced the fraction of GFP+ cells following the induction of the single site-specific DSB. Expression of I-*Sce*I in four independent TK6-bcl-x_L-DR-GFP cell lines resulted in 2.32% (±0.14), 3.10% (±0.56), 2.87% (±0.31) and 3.01% (±0.25) GFP+ cells (mean ± 1 S.E.M.; Fig. 2B). Three conclusions may be drawn from these data: 1) a single DSB stimulates HDR 40- to 50-fold in wild-type TK6 cells, 2) one DSB stimulates HDR 200- to 300-fold in TK6-bcl-x_L cells and, 3) ectopic expression of BCL-x_L enhances HDR when compared to isogenic control cell lines.

To ensure that the increase in DSB-stimulated HDR in TK6-bcl-x_L cells was not an artifact attributable to electroporation and plasmid uptake, we measured the transfection efficiencies in TK6-neo and TK6-bcl-x_L cells using the GFP expression vector *pNZE-CAG*. We monitored both the fraction of GFP+ cells and the intensity of GFP fluorescence by flow cytometry 40 to 48 h post transfection. The mean fluorescence intensities for GFP+ cells were similar in each genetic background. Plasmid uptake was slightly higher (1.4-fold) in the TK6-

bcl-x_L derived DR-GFP cell lines (Figure 2C, $p<0.001$, *t*-test). We detected 9.18% (±0.64) (mean ± 1 S.E.M) GFP+ cells for TK6-neo clones, and 13.07%(±0.72) (mean ± 1 S.E.M.) for TK6-bcl-x_L derived DR-GFP cell lines. When we correct for the small difference in plasmid uptake, ectopic expression of BCL-x_L results in a 3- to-4-fold enhancement of HDR.

Gene conversion is associated with loss of the I-SceI site in human cells expressing high levels of BCL-x_L

To confirm that HDR is associated with loss of the I-*Sce*I site in the DR-GFP reporter, genomic DNA was prepared for Southern analysis from sorted GFP+ (e.g. Figure 3A, right panel) and GFP- TK6-bcl-x_L-DR-GFP cells (13). In cells that remained GFP- after transfection with the I-*Sce*I expression vector, the non-recombined *Sce*GFP gene gives bands of 2140 bp and 881 bp when the *Sal*I/*Hin*dIII band is digested with I-*Sce*I (Fig. 3B, lane 9) but maintains the 3021 bp band when digested by *Bcg*I (Fig. 3B, lane 8). This indicates that the GFP- cell population maintained the I-*Sce*I site, presumably because only a fraction of the cells were successfully transfected. By contrast, in GFP+ cells, the 3021 bp band is not cleaved by I-*Sce*I (Fig. 3B, lanes 3 and 6), but is cleaved instead by *Bcg*I to produce two bands of 2140 bp and 881 bp (Fig. 3B, lanes 2 and 5; note: because *Bcg*I is not a robust restriction enzyme, we frequently did not obtain complete cleavage). Thus, this analysis confirmed that gene conversion had occurred in GFP+ cells.

Discussion

Our results provide evidence that HDR is an important mechanism of DSB repair in human cells. We examined HDR and its induction by DSBs in TK6 cells that carry wild-type TP53 and manifest an apoptotic response to X-rays that is typical of lymphocytes (16). HDR was stimulated 40- to 50-fold following the induction of a single site-directed DSB**.** These results are consistent with earlier work in human cells that suggested that clastogens could elicit gene conversion, albeit at lower frequencies (17, 18). The present study shows that HDR is a significant DSB repair pathway in human cells, as has been well documented in yeast, chicken and rodent cells {for review see (19)}.

In mammalian cells, HDR is presumed to require the activity of RAD51 to promote strand exchange, and is facilitated by interacting proteins that include XRCC2 and XRCC3 (reviewed in (19)). Hamster cells deficient in either XRCC2 or XRCC3 are defective in error-free HDR of DSBs (13, 20). A deficiency in one of the proteins known to participate in HDR can impair this type of DNA repair by up to two orders of magnitude. Several other proteins regulate HDR, presumably through direct or indirect interactions with RAD51. Included among these is BRCA1 (21). Mouse embryonic stem cells containing a hypomorphic *Brca1* allele have 5- to 6-fold lower levels of HDR of an I-*Sce*I-induced chromosomal DSB (21, 22). Other cells carrying *BRCA2* mutations also have diminished capacities (5- to >100-fold) to repair a chromosomal DSB by gene conversion (22). Thus, regulation of HDR appears to involve a complex network of proteins, including known tumor suppressors.

Here we tested derivatives of TK6-bcl-x_L and TK6-neo cells for their ability to undergo error-free HDR. We previously showed that ectopic expression of BCL-x_L or BCL-2 enhanced radiation-induced *TK1* mutagenesis in TK6 cells (11). The enhanced *TK1* mutagenesis is

associated with a higher proportion of mitotic recombination (manuscript in preparation). However, because of the difficulty in analyzing breakpoint junctions, we were not able to investigate the fidelity of the recombination events in our previous systems. The integrated reporter DR-GFP used in the present study specifically addresses this point and indicates 3- to 4-fold higher levels of error-free HDR in TK6-bcl-x_L cells as compared to TK6-neo cells. One way radiation-induced *TK1* mutagenesis might be enhanced in cells expressing high levels of BCL-x_L or BCL-2 is through the upregulation of HDR. Although HDR is generally an error-free process, it can occasionally result in a loss of function at a heterozygous locus when the repair event leads to homozygosity of a mutant allele.

One way the apoptotic program may limit HDR is through the cleavage and inactivation of hRAD51 (23). In a preliminary study, we observed that caspase-mediated cleavage of hRad51 is significantly delayed and reduced following X-irradiation of TK6-bcl-x_L cells (unpublished results). If hRAD51 function is maintained in TK6 cells with both wild-type p53 and high levels of BCL-x_L this may contribute to enhanced recombination, as reported in other cells expressing high levels of RAD51, although a new study indicates that ectopic expression of RAD51 can downregulate HDR in p53 mutant CHO cells or in p53 null HT1080-1885 cells (23-26).

A recent report in mouse L cells expressing wild-type TP53 showed that overexpression of BCL-2 did not alter the frequency of spontaneous gene conversion (27). This agrees with our finding that overexpression of BCL-x_L did not appear to modulate gene conversion in mock-transfected human cells with wild-type TP53. While the failure to observe an effect of BCL-2 family members on spontaneous gene conversion seems surprising, given our results for DSB-promoted events, this may simply reflect an inability to measure these very rare events with sufficient precision. An alternate possibility is that the initiating events for spontaneous gene

conversion (and the proteins that transact the conversion events) differ from the endonuclease-induced DSBs used by us and by Saintigny, et al. (27). The rodent cell studies did not address the impact of BCL-2 or BCL-x_L expression on DSB-initiated HDR in cells with wild-type TP53. However, their studies using rodent cells with mutated forms of TP53 indicated that BCL-2 or BCL-x_L down-regulated the high levels of HDR in these cells. This raises the possibility of a complex feedback regulation of HDR that includes TP53 and BCL-2 family members.

The mechanism of action of BCL-x_L is not entirely understood. BCL-x_L localizes to the periphery of the mitochondria and the perinuclear membrane (28). Given what is known about the intracellular localization of BCL-x_L, its ability to modulate HDR is most likely through an indirect mechanism, not via direct interaction with the recombination machinery.

BCL-2 was the first anti-apoptotic protein implicated in human carcinogenesis. High levels of BCL-2 have been detected in the majority of follicular neoplasms, in diffuse large B-cell lymphomas and less commonly in other non-Hodgkin's lymphomas (29). The progression from indolent to aggressive disease in these lymphomas can involve LOH on selected chromosomes (30, 31). Mitotic recombination is a major mechanism for the generation of LOH in tumors (32). Our study provides direct evidence that high levels of expression of BCL-x_L elevate HDR. We speculate that this dysregulation of HDR may actively promote malignant progression.

Acknowledgements

The authors are grateful to Hector Nolla, Flow Cytometry Facility, Cancer Research Laboratory, UC Berkeley, for support with the flow cytometry and cell sorting. We would also like to thank Dr. David Schild (LBNL) for many helpful discussions.

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Figure legends:

Figure 1. (A) The recombination repair substrate DR-GFP is composed of two differentially mutated GFP genes, *SceGFP* and *iGFP* (14). When the I-*Sce*I endonuclease is expressed in cells containing DR-GFP, a DSB will be introduced at the I-*Sce*I site in the *SceGFP* gene. Repair of the DSB by non-crossover gene conversion with the downstream *iGFP* gene results in the reconstitution of the functional *GFP* gene. The nuclear localization signal (N) and the zinc finger domain (Z) aid in the nuclear retention of the protein (14). **(B)** Representative analysis of GFP fluorescence in TK6-neo-DR-GFP cells (clone 1-7). The percentage of GFP+ cells is indicated in the upper left corner of each panel. Very few GFP+ cells are found in mock-transfected cultures (left panel). In this example, I-*Sce*I expression strongly elicits gene conversion (> 50-fold) enhancing the fraction of GFP+ cells to 0.54% (middle panel). GFP+ cells were initially sorted to >96% purity (data not shown). Re-analysis of the sorted GFP+ population several weeks after transfection with I-*Sce*I indicates the stability of GFP expression (83.6% GFP+ cells, right panel). Two-color fluorescence was performed, with the percentage of green fluorescent cells (FL1) indicated above the diagonal. FL1, green fluorescence; FL2, orange fluorescence. **(C)** Physical confirmation of gene conversion following a single I-*Sce*I-induced DSB. A Southern blot is shown revealing the cleavage pattern of genomic DNA extracted from the sorted pools of TK6-neo-DR-GFP cells. In GFP+ cells, the wild-type GFP-sequence has been restored, i.e., the I-*Sce*I site has been replaced with the *Bcg*I site. GFP+ cells are resistant to cleavage by I-*Sce*I (lanes 3 and 6) and susceptible to cleavage by *Bcg*I (lanes 2 and 5). *Sal*I/*Hin*dIII: lanes 1 and 4. *Sal*I/*Hin*dIII/*Bcg*I: lanes 2 and 5. *Sal*I/*Hin*dIII/I-*Sce*I: lanes 3 and 6.

Figure 2. (A) Spontaneous gene conversion in TK6-neo and TK6-bcl-x_L cell lines. Cells were transfected with or without a mock plasmid (pDS267, kindly provided by Dr. D. Schild, LBNL) and analyzed for expression of functional GFP by flow cytometry. The results shown indicate the mean ± 1 S.E.M. of the combined data for two TK6-neo cell lines (8 experiments) and four TK6-bcl-x_L cell lines (27 experiments). **(B)** The induction of a DSB strongly induces gene conversion in TK6-neo cells (40- to 50-fold), and even further in TK6-bcl-x_L cells (200- to 300-fold). TK6-bcl-x_L cells have 4- to 5-fold higher levels of GFP-positive cells, indicating enhanced HDR of a DSB by gene conversion with high levels of BCL-x_L. The data represent the mean ± 1 S.E.M. of five experiments for each cell line **(C)** Plasmid uptake and/or expression is slightly elevated (1.4-fold) in TK6-bcl-x_L cells compared to TK6-neo cells. The transfection efficiency for each cell line was measured after transient transfection with pNZE-CAG. The results shown indicate the mean ± 1 S.E.M. of the combined data for two TK6-neo and four TK6-bcl-x_L DR-GFP clones. Ten experiments were carried out using two TK6-neo-DR-GFP clones, and 15 experiments using TK6-bcl-x_L-DR-GFP clones.

Figure 3. (A) Representative analysis of GFP fluorescence in TK6-bcl-x_L-DR-GFP cells (clone 2-9). The percentage of GFP+ cells is indicated in the upper left corner of each panel. No GFP+ cells were found in mock-transfected cultures (left panel). In this example, I-*Sce*I expression strongly induces gene conversion and enhances the fraction of GFP+ cells to 5.74% (middle panel). GFP+ cells were sorted to >96% purity (data not shown). Re-analysis of the sorted GFP+ population several weeks after transfection with I-*Sce*I indicates the stability of GFP expression (95.3% GFP+ cells, right panel). Data analyses were performed as described for Fig. 1B. **(B)**

Physical confirmation of gene conversion following a single I-*Sce*I-induced DSB. A Southern blot is shown revealing the cleavage pattern of genomic DNA extracted from the sorted pools of TK6-bcl-x_L-DR-GFP cells. Data analyses were performed as described for Fig. 1C. GFP+ cells are resistant to cleavage by I-*Sce*I (lanes 3 and 6) and susceptible to cleavage by *Bcg*I (lanes 2 and 5). Conversely, in GFP- cultures the *Sal*I/*Hin*dIII-fragment is cleaved by I-*Sce*I (lane 9) but not by *Bcg*I (lane 8). *Sal*I/*Hin*dIII: lanes 1, 4 and 7. *Sal*I/*Hin*dIII/*Bcg*I: lanes 2, 5 and 8. *Sal*I/*Hin*dIII/I-*Sce*I: lanes 3, 6 and 9.