

Homologous recombination in human telomerase-positive and ALT cells occurs with the same frequency

Oliver E. Bechter, Ying Zou, Jerry W. Shay & Woodring E. Wright⁺

Department of Cell Biology, Southwestern Medical Center, The University of Texas, Dallas, Texas, USA

Homologous recombination is thought to be the molecular mechanism for maintaining telomere length in alternative lengthening of telomeres (ALT) cells. We used a recombination reporter system to show that the frequency of homologous recombination is the same for ALT- and telomerase-positive cells, suggesting that if ALT cells have a recombination defect it specifically involves telomeric sequences. We compared internal and telomere-adjacent positions of our reporter construct to investigate if telomeric sequences near an induced double-strand break alter the frequency of recombination between nontelomeric sequences, and found no differences among the different cell lines analysed. Our results indicate that the underlying defect in homologous recombination in ALT cells does not affect sequences independent of their chromosomal location but is likely to be primarily a specific telomeric defect.

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INTRODUCTION

Chromosome ends contain repetitive hexameric sequences (TTAGGG in mammals) known as telomeres (reviewed in Collins, 2000). Telomeres serve as an expendable DNA buffer for the end-replication problem (Olovnikov, 1996). During lagging-strand synthesis, DNA polymerase is unable to replicate the very end of the chromosome, resulting in the loss of telomeric DNA with each cell division if compensatory mechanisms are not present.

Two mechanisms to overcome the end-replication problem have been described in human cells. One pathway involves telomerase, a multi-subunit cellular ribonucleoprotein that adds telomeric repeats onto chromosomal ends (Greider & Blackburn, 1987). Although telomerase is the preferred pathway of telomere homeostasis in immortalized human cells, the alternative

lengthening of telomeres (ALT) pathway is also capable of maintaining telomeres (Bryan *et al.*, 1995).

ALT is characterized by the lack of detectable telomerase activity and a heterogeneous pattern of telomere length, usually ranging from very short (<1 kb) to abnormally long (>20 kb) (Rogan *et al.*, 1995). ALT cells also contain ALT-associated PML bodies (APBs), complexes consisting of promyelocytic leukaemia (PML) protein plus telomeric DNA, telomere binding proteins such as TRF1 and TRF2, and proteins involved in DNA recombination (for example, RAD50, RAD51, RAD52, MRE11, NBS1, BLM and WRN) (Henson *et al.*, 2002). Approximately 25% of all *in vitro* simian virus 40 (SV40) large T-antigen immortalized human fibroblast cell lines use the ALT pathway while it is found in about 7% of tumour-derived cell lines or human tumour samples (Bryan *et al.*, 1997). The diagnosis of ALT is hampered by our lack of understanding of its molecular basis. For example, it is still an outstanding question as to whether telomerase-positive tumours contain some ALT-positive cells (Henson *et al.*, 2002).

There is good evidence that homologous recombination is involved in the ALT pathway in yeast and increasing evidence in human cells. In telomerase-negative mutant yeast cells, telomere maintenance requires the homologous recombination factor RAD52 (Teng & Zakian, 1999). Telomerase-negative *Kluyveromyces lactis* with two types of marked telomeres exhibit recombinational telomere elongation, generating a repeating pattern within most telomeres. Transformation of these cells with marked DNA circles containing telomeric repeats created long repeating arrays formed at chromosome ends, suggesting that rolling circle gene conversion may be a mechanism for telomere elongation in telomerase-negative *K. lactis* cells (Natarajan & McEachern, 2002). Reddel and co-workers demonstrated that tagged telomeric ends in human ALT cells show copy switching of the marker to other telomeres, which did not occur in telomerase-positive cell lines (Dunham *et al.*, 2000).

It is currently unknown whether human ALT cells inherit a general versus telomere-specific recombination defect compared to telomerase-positive cells, or if the frequency of recombination is altered near a telomeric sequence or within the subtelomeric region. The present study uses a reporter system that quantitates the capacity of ALT- versus telomerase-positive cells to initiate an

Department of Cell Biology, Southwestern Medical Center, The University of Texas, 5323 Harry Hines Blvd, Dallas, Texas 75390-9039, USA

⁺Corresponding author. Tel: +1 214 648 8694; Fax: +1 214 648 8694;

E-mail: WWRIGH@mednet.swmed.edu

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active recombination process (Pierce *et al.*, 1999). Our results demonstrate that the frequency of homologous recombination for an intrachromosomal location is identical in telomerase-positive and ALT cells derived from the same parental cell strain. Recombination near telomeric sequences and in a subtelomeric location is not increased in human ALT cells. Thus, ALT is likely to represent a specific intertelomeric recombination defect.

RESULTS

Intrachromosome homologous recombination

Individual telomerase-positive SW39 and ALT-positive SW26 cell clones transfected with pDR-GFP were tested for intact recombination cassettes by analysing GFP expression after transient transfection with the I Sce-I plasmid. This plasmid has two inactive GFP genes, so that a functional gene is present only after homologous recombination initiated by digestion of the I Sce-I site in the 5' GFP cassette (Pierce *et al.*, 1999) (Fig. 1A). Four of nine SW39 clones and 5 of 15 SW26 clones showed GFP expression after double-strand break (DSB) repair. Recombination competent clones were analysed for copy number by Southern blot analysis. *HindIII* excises an 812 bp internal band and one of variable size depending on the next *HindIII* site (Fig. 1). The number of variable-size bands indicates the number of inserted copies/sites. Most clones had two copies of the transgene (Fig. 2A and Table 1). Recombination assays were performed at least twice for each individual clone in independent experiments. The frequency of recombination (calculated as outlined in Fig. 3) in telomerase-positive SW39 clones (2.2–5%, average 3.5%) was the same as in ALT pathway SW26 clones (2–6.5%, average 3.4%) (Table 1). There was no correlation between copy number and the frequency of recombination.

Recombination next to telomeres

A chromosomal healing vector with 1.6 kb of telomeric repeats downstream of the iGFP gene (Fig. 1) was linearized with *ClaI* and transfected into SW39 and SW26 cells. Eight of 20 SW39 and 8 of 10 SW26 clones showed a recombination signal after DSB induction. Molecular analysis to determine the position and copy number was carried out as described in Fig. 1. Only clones with single copy insertions were analysed further (Fig. 2B,C). A chromosomal healing event occurred in three SW39 clones (#B, #C, #E) whereas two clones showed an internal integration site (#A, #F). Four SW26 clones showed a chromosomal healing event (#F, #B, #D, #G) and two exhibited an internal integration (#E, #C). *Bal31* digestion confirmed that the appearance of a single band in *EcoRV* and *PstI* digests reflected a NextTel position. As shown in Fig. 2D, *Bal31* treatment of a clone with internal position did not change the presence of the two GFP bands. However, in a clone with NextTel position of the reporter system, the GFP-specific telomeric smear showed progressive reduction in size with *Bal31* treatment. We also performed fluorescence *in situ* hybridization (FISH) analysis (Fig. 2E) with a GFP-specific probe in a subset of SW39 and SW26 clones to validate further the results of the Southern analysis.

The original chromosome healing vector contained 1.6 kb of telomeric repeats. Analysis of the size of the smears containing the telomeric GFP insertions on overexposed gels showed that the telomeric sequence had been elongated to between 2 and 3 kb in the telomerase-positive SW39 cells, consistent with the average telomere length in those cells. Similarly, the GFP telomeric smear was very heterogeneous in size in the ALT pathway SW26 clones, ranging from <3.5 to >19 kb in one clone (Fig. 2D, SW26 #G) and from 2.6 to 7.7 kb in another.

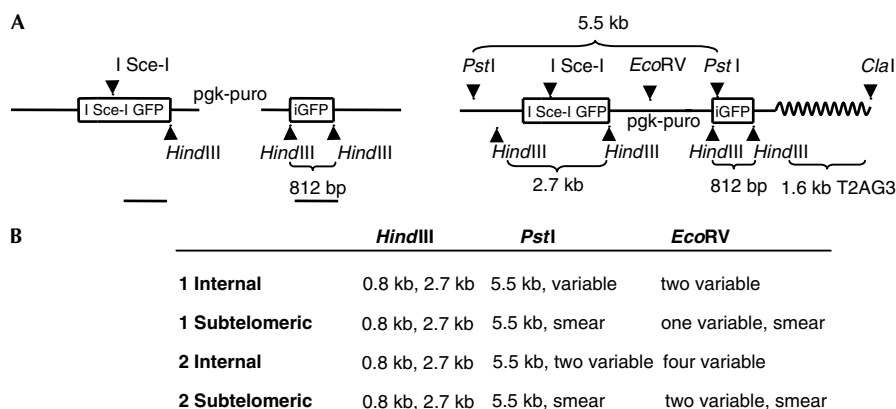


Fig. 1 | Recombination reporter systems. (A) Schematic illustration of the pDR-GFP recombination reporter system (left) and its modification into a chromosomal healing vector (right). *HindIII* cuts three times in the pDR-GFP recombination reporter system, excising an 812 bp internal band as well as a second band of variable size, depending on the genomic integration site, and therefore allowing determination of copy number. For the chromosomal healing vector, a 1.6 kb telomeric sequence was cloned 482 bp downstream of the iGFP gene. Due to cloning, additional *PstI* and *HindIII* sites were introduced into the plasmid giving rise to bands of fixed size following *HindIII* digestion. In order to increase the possibility of a chromosomal healing event, the vector was linearized with *ClaI* before transfection. All Southern blots except for those after *Bal31* treatment were probed with a *HindIII*-excised 812 bp GFP probe. (B) Diagnostic algorithm for determination of internal versus subtelomeric position after transfection with the chromosomal healing vector. The pattern of bands expected after digestion with *PstI* and *EcoRV* helps to distinguish between one and more than one integrated subtelomeric copy. Since all clones contained a complete cassette of the reporter construct, the lack of a second band is decisive to distinguish between the internal and NextTel position. The left-hand *HindIII* site is not present in the basic recombination reporter vector shown in (A), so the 2.7 kb band becomes a band of variable size dependent on the integration site.

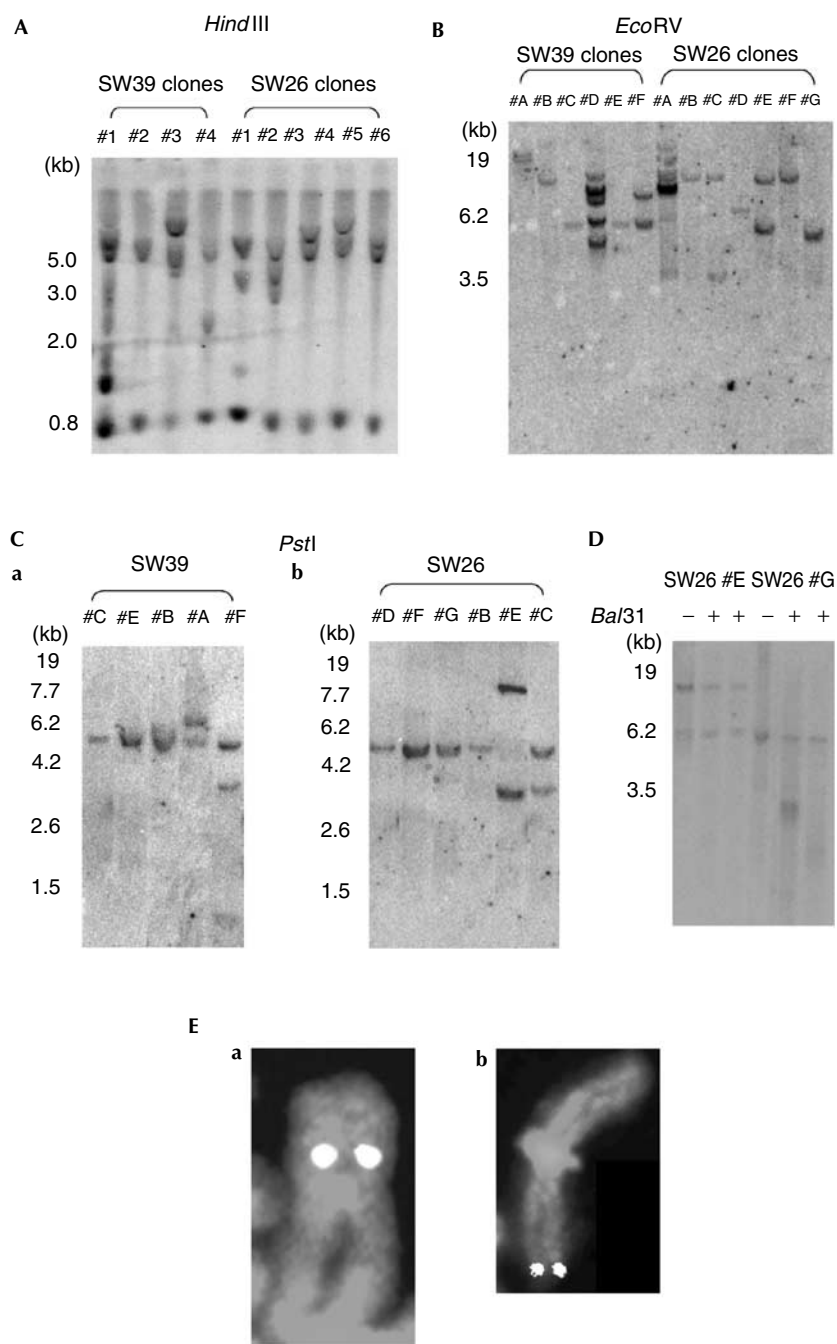


Fig. 2 | Molecular analysis for determining the copy number and position of the recombination reporter system. (A) Southern blot analysis of all recombinogenic SW39 and SW26 clones with the reporter system in an intrachromosomal position (20 µg *Hind*III-digested genomic DNA per lane). (B) *Eco*RV Southern analysis of SW39 and SW26 clones transfected with the chromosomal healing vector. All clones further analysed for their frequency of recombination had one copy either next to the healed telomere (SW39 #B, #C, #E; SW26 #B, #D, #F, #G) or at an internal location (SW39 #A, #F; SW26 #C, #E). Clones SW39 #D and SW26 #A are examples of clones that were excluded due to multiple copies inserted. (C) *Pst*I Southern analysis of SW39 and SW26 clones after transfection with a chromosomal healing vector. The presence of one single band represents a clone with the recombination reporter system in the NextTel position, whereas two bands indicate an internal + location. (D) *Bal*31 digestion of SW26 clones #E and #G as an example for the accuracy of molecular analysis by conventional Southern blotting. In all, 15 µg of genomic DNA was treated with 2.5 U of *Bal*31 for 1 and 2 h (#E) or for 2 and 4 h (#G), respectively, followed by *Eco*RI digestion. In contrast to the clone with internal position, the clone with NextTel location of the reporter system shows sensitivity to *Bal*31 treatment after hybridizing with a Puro-GFP probe. (E) FISH analysis confirmed the data obtained by Southern blot analysis. (Ea) A clone with internal position. In contrast to that, (Eb) a clone with subtelomeric location of the integrated pDR-GFP reporter system is shown.

Table 1 | Percentage of recombination (GFP-positive cells) in telomerase-positive SW39 and ALT pathway SW26 clones

SW39 clones	Position (number of copies)	GFP-positive cells (%)	SD (%)	SW26 clones	Position (number of copies)	GFP-positive cells (%)	SD (%)
#1	Internal (×3)	3.4	0.6	#1	Internal (×2)	3.0	2.0
#2	Internal (×1)	5.0	1.2	#2	Internal (×3)	3.6	0.9
#3	Internal (×2)	2.2	0.3	#3	Internal (×2)	6.5	1.2
#4	Internal (×2)	3.6	1.0	#4	Internal (×2)	2.2	0.7
				#5	Internal (×2)	2.0	1.4
#A	Internal+ (×1)	4.3	2.7	#E	Internal+ (×1)	0.3	0.1
#F	Internal+ (×1)	13.4	2.4	#C	Internal+ (×1)	4.5	0.6
#B	NextTel (×1)	3.1	1.6	#F	NextTel (×1)	5.2	3.3
#C	NextTel (×1)	0.5	0.4	#B	NextTel (×1)	1.4	0.3
#E	NextTel (×1)	2.1	1.3	#D	NextTel (×1)	2.0	1.2
				#G	NextTel (×1)	3.9	0.2

The pDR-GFP recombination reporter system is placed either internally (internal, without; internal+, with telomeric sequence adjacent) or next to a healed telomeric (NextTel).

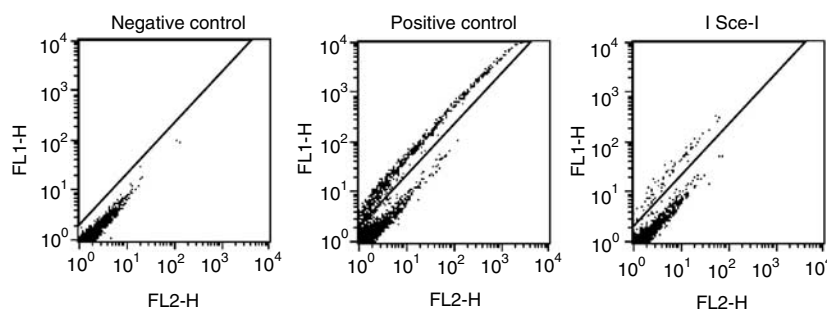


Fig. 3 | FACS analysis and calculation of the frequency of recombination after an induced homologous recombination process had occurred. Mock electroporated cells that are negative for GFP expression for most of the clones analysed show autofluorescence and fall along a diagonal between orange (x-axis) and green (y-axis). Some clones exhibit background activity, which never exceeded levels above 0.5%. In contrast, GFP-expressing cells shift towards the green axis. Positive control samples determine the transfection efficiency for each cell clone in each individual experiment. The frequency of recombination is calculated according to the following formula: frequency (%) = ((number of GFP positive cells after I Sce-I induction – background) / (number of GFP transfection – background)).

This demonstrates that the chromosome-healing insertions functioned as substrates for the telomere-maintenance pathways appropriate to the host cell.

Frequency of recombination in ALT-positive SW26 cells

The frequency of recombination next to healed telomeres ranged from 0.5 to 3.1% (average 1.9%) for the three telomerase-positive SW39 clones and from 1.4 to 5.2% (average 3.1%) for ALT-positive SW26 clones, showing the same general levels as the previously analysed internal clones (Table 1). A single internal clone containing telomeric sequences exhibited a much higher rate of recombination in the telomerase-positive SW39 cells, probably due to the specific site of integration into the chromosome (Elliott & Jasin, 2001).

DISCUSSION

The best evidence that human ALT cells use homologous recombination to maintain telomeres is that tagged telomeres in an ALT cell line show dispersal of the tag to telomeric ends of different chromosomes (Dunham *et al.*, 2000). Murnane *et al.* (1994) reported that marked telomeres in ALT pathway cells undergo progressive shortening. Once these telomeres shortened they underwent sudden elongation events, in some cases when telomeres reached a critically short length (Murnane *et al.*, 1994). One possibility is that ALT cells are in a ‘chronic’ state of crisis due to a substantial number of critically short telomeres (Ford *et al.*, 2001). The presence of a ‘too short telomere’ is then recognized as a DSB, initiating a recombination process (McEachern & Blackburn, 1996). Due to the extensive homology at telomeric ends, a break-induced replication process might be engaged where the ‘broken end’ invades a donor

telomere, followed by replicative copying of the donor sequence to transfer telomeric DNA onto the invading chromosomal end (Bosco & Haber, 1998).

We hypothesized that this telomeric recombination process is specifically blocked in normal cells and that ALT cells exhibit a recombination defect in this block rather than an alteration in the general recombination machinery. We measured the frequency of recombination after an induced DSB in immortal cells derived from the same parental cell (IMR90) and that thus share the same genetic background. The frequency of intrachromosomal homologous recombination for human ALT-positive cells was indeed identical to that in telomerase-positive cells.

Our data are in agreement with those in *Saccharomyces cerevisiae*, which lack a functional telomerase gene and generate survivors that display either amplification of subtelomeric Y' elements or telomeric sequences (Lundblad & Blackburn, 1993). These two phenotypically distinct telomerase-independent pathways both require RAD52 function, and were later defined as type I and type II survivors (Teng & Zakian, 1999). Using a noninducible recombination reporter system, the frequency of spontaneous recombination in telomerase-independent survivors was the same for cells during the onset of crisis, after crisis had occurred and in normal telomerase-proficient cells. Our results confirm that the unchanged general recombination frequencies seen in yeast also apply to the human ALT pathway. In addition, we demonstrate that adjacent telomeric sequences do not alter the frequency of homologous recombination in either telomerase-positive SW39 or ALT SW26 clones, whether they integrate internally or cause a chromosomal healing event.

The intrachromosomal homologous recombination mechanism assayed by our stably integrated constructs reflects only one of many types of recombination. Extrachromosomal homologous recombination assayed following transient transfection of ALT- versus telomerase-positive cells with the pDR-GFP reporter showed no differences (unpublished observations). We found no differences in the frequency of generation of stable clones following transfection, implying that recombination involving nonhomologous sequences is the same in ALT- and telomerase-positive cells. The frequency of sister-chromatid exchange in ALT pathway cells is also the same as in other T-antigen-expressing cells (Ford *et al.*, 2001). It thus appears that a variety of recombination mechanisms are intact in ALT cells.

These results are consistent with the observation by Dunham *et al.* (2000). They showed that a tag placed in a subtelomeric location (adjacent to rather than within the telomere) was not subjected to copy switching in ALT- or in telomerase-positive human cells. Together with our data, this suggests that although recombination within telomeric repeats for critically short telomeres in ALT cells is likely to be high, the elevated frequencies do not extend into the subtelomeric region involving other homologous sequences. However, the results obtained with chromosomal healing vectors should be interpreted cautiously. Seeding new telomeres creates a new subtelomeric locus that does not contain the normal subtelomeric organization. The occurrence of TTAGGG variants such as TGAGGG at the base of the telomere, as well as blocks of sequences sharing great similarity between different chromosomes, might be hot spots for recombination in ALT cells (Allshire *et al.*, 1989; Baird & Royle, 1997; Varley *et al.*, 2002).

Previous reports show that exchanges of Y' elements between telomeres occur at a low frequency in a telomerase-proficient yeast strain, which become extensively amplified in type I survivors (Horowitz & Haber, 1985; Lundblad & Blackburn, 1993). Furthermore, a subtelomeric marker gene in a telomerase-deficient strain of *K. lactis* was rapidly dispersed via recombination to other chromosomes reminiscent of amplification of Y' elements in type I survivors of *S. cerevisiae* (McEachern & Blackburn, 1996). Although there is no evidence to date that type-I-like recombination processes take place in human ALT cells, it cannot be definitely ruled out.

Our data clearly indicate that new reporter systems are needed that specifically measure recombination occurring within telomeric sequences themselves. Developing an assay for the specific alteration in recombination that is present in human ALT cells will not only provide an important criterion for diagnosing the ALT phenotype but also for determining the specific molecular changes that permit ALT telomeres to be maintained.

MATERIALS AND METHODS

Cell lines and culture. The telomerase-positive cell line SW39 and the ALT pathway line SW26 were both derived from SV40 large T-antigen-expressing IMR90 fetal lung fibroblasts and were cultured as described (Wright *et al.*, 1989).

Chromosomal healing vector. The recombination reporter system pDR-GFP and CMV/Sce 1XNLS plasmids, kindly provided by M. Jasin (Pierce *et al.*, 1999), contains a plasmid with two nonfunctional GFP genes (Fig. 1), and a plasmid encoding the restriction enzyme I Sce-I. The 5' GFP gene (SceGFP), mutated by insertion of an 18-mer restriction enzyme site I Sce-I, produces a DSB once I Sce-I is expressed (Richardson *et al.*, 1998). The truncated 3' GFP gene (iGFP) serves as a donor to the broken SceGFP gene, and short tract gene conversion usually restores an intact GFP gene.

A chromosomal healing vector was created by inserting the 1.6 kb telomeric sequence from pSXNeo1.6T2AG3 into the pDR-GFP plasmid downstream of the iGFP gene. Chromosomal healing then positions the pDR-GFP gene immediately adjacent to the newly formed telomere (Flint *et al.*, 1994; Hanish *et al.*, 1994) (Fig. 1). To generate stable clones, cells were transfected with 10 µg of plasmid using the FuGene6 transfection reagent (Roche) and selected with 500 ng ml⁻¹ of puromycin (Sigma). Individual clones were picked, expanded and analysed for GFP expression after DSB induction, and for copy number and chromosomal position of the transfected transgene.

Southern blot, *Ba*31 and GFP-FISH analysis. The copy number and location of the inserted pDR-GFP reporter system were determined by digesting 20 µg of genomic DNA with a variety of enzymes (see text), followed by standard Southern blotting procedures and hybridization with a GFP-specific probe. *Ba*31 digestion was performed with 10 µg of genomic DNA. In all, 2.5 U of *Ba*31 (New England Biolabs) was incubated for various time periods followed by *Eco*RV digestion. In order to increase the sensitivity for detecting the smear, a probe containing the puromycin resistance gene and the iGFP gene were used for hybridization. *In situ* hybridization to localize insertion sites was performed with a spectrum orange dUTP-labelled GFP probe as described (Baur *et al.*, 2001).

Electroporation and recombination assay. Log phase cells were trypsinized and washed in PBS three times. In all, 5×10^6 cells per electroporation were resuspended in 500 μ l serum-free media and incubated for 5 min with 100 μ g of plasmid DNA at 20 °C. Each experiment included a positive control (pEGFP-N3, Clontech), a negative control (pZeoSV, Invitrogen) and the plasmid encoding for I Sce-I (CMV/Sce 1XNLS). Electroporation was performed at 250 V/960 μ F for SW39 cells and at 320 V/500 μ F for SW26 cells on a BioRad gene pulser, to achieve optimal transfection efficiency for each cell line. Following electroporation, cells were cultured for 48 h, harvested, washed once, resuspended in PBS and subjected to FACS analysis in a Becton-Dickinson FACScan on an orange (FL2-H) versus green (FL1-H) fluorescence plot (Fig. 3).

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