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A human cell-based reporter detects microhomology-mediated end joining

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Abstract

DNA double-strand breaks (DSBs) are most often repaired by two pathways in mammalian cells, homologous recombination or non-homologous end joining. Biochemical and genetic studies showed that DSBs can also be joined via microhomology-mediated end joining (MHEJ), which is always mutagenic and may result in diseases, such as cancer. In this study we established a human cell-based reporter system to determine the prevalence of MHEJ events and factors that modulate MHEJ. A nonfunctional puromycin acetyltransferase (*Pac*) gene, disrupted by an insertion flanked by two microhomologous repeats, was integrated into chromosomes of human HT1080 cells. Repair of DSBs via MHEJ using the repeats resulted in deletion of the insertion and restoration of the *Pac* gene function, thus rendering the cells puromycin resistant. Our results showed that MHEJ spontaneously occurs at the reporter locus (loci), manifested by formation of puromycin resistant (*puro*^r) colonies after culturing reporter cells in medium containing puromycin. The frequency of *puro*^r cells can be greatly increased by site-directed DSB inside the insertion. Our results also demonstrated that the frequency of *puro*^r cells is affected by the length of the repeat and by the size of the intervening sequence. Thus, this cell-based assay provides a platform for evaluating factors modulating in vivo MHEJ.

DNA double-strand breaks (DSBs) can occur as intermediates in various biological events, such as V(D)J recombination [1] and meiosis [2], or be induced by ionizing radiation, UV light, topoisomerase poisons or radiomimetic drugs [3]. As one of the most serious forms of DNA damage, a single unrepaired DSB can lead to cell death [4]. In mammalian cells, there are four enzymatically distinct pathways for the repair of DSBs, homologous recombination (HR), non-homologous end joining (NHEJ), single-strand annealing (SSA) and microhomology-mediated end joining (MHEJ or MMEJ) [5, 6]. While HR uses a homologous template (most frequently the sister chromatid) to carry out DSB repair and NHEJ joins two ends without the requirement for a homologous sequence, SSA and MHEJ rejoin DNA ends via homologous repeat sequences flanking the break point. Consequently, whereas HR is usually error-free when using the sister chromatid as the homologous template, NHEJ may occasionally cause small deletions/insertions. Like SSA which creates

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deletions via annealing at directly repeated sequences of more than 30 bp, MHEJ also leads to deletions that span one of the two microhomologous sequences (5-25 bp) and the intervening sequence, if any, and thus is a mutagenic repair pathway [5]. Indeed, MHEJ is frequently associated with deletions and chromosome abnormalities, such as translocations, in mammalian cells [7-9]. Furthermore, several studies showed that genomic alterations as a result of repair of DSBs via MHEJ may underlie tumorigenesis in human and mouse models [9-11]. Moreover, direct repeats are known to contribute to deletion formation in mitochondrial DNA (mtDNA), which is associated with aging and neurodegeneration [12, 13], implying that MHEJ may play a role in repair of mtDNA DSBs.

Compared to HR and NHEJ, MHEJ is the least characterized DSB repair pathway and most of our knowledge of MHEJ is based on studies in yeast. It was proposed that the MHEJ pathway may consist of several steps, such as end resection to reveal microhomologous sequences, annealing at microhomologies, and flap cleavage before fill-in synthesis, culminating in the sealing of DNA nicks by the action of a DNA ligase [5]. Several protein factors were shown to be involved in MHEJ. For instance, in fission yeast, Decottingnies showed that Exo1 exonuclease and Pol4 were required for MHEJ, probably for the resection of DNA ends and fill-in synthesis, respectively [14]. In *S. cerevisiae*, MHEJ is strongly dependent on the Mre11/Rad50 complex, which is probably required in the end resection step to expose microhomologies, and Rad1 flap endonuclease activity [15]. In mammalian cells, overexpression of Mre11 stimulates the resection of single-stranded DNA and facilitates MHEJ through a mechanism that requires Mre11 nuclease activity, but in an ATM/H2A.X-independent manner [16, 17]. We previously established a cell-free assay to evaluate factors involved in the MHEJ pathway [18]. By using this assay, we showed that the nuclear proteins that protect DNA ends from nucleases, including Ku and histone H1, contribute to the inhibition of MHEJ, and that flap endonuclease 1 (FEN1) can facilitate MHEJ [18]. We also found that siRNA-mediated down-regulation of DNA ligase I or ligase III in human cells impaired end joining that was mediated by microhomologous repeats, indicating that DNA ligases I and III are required in MHEJ [19]. DNA ligase IV, on the contrary, is not required in MHEJ, while facilitating Ku-dependent NHEJ [19].

In this study, we have established a cell-based reporter system to determine prevalence of in vivo MHEJ events and conditions that modulate in vivo MHEJ. The MHEJ reporter contains an impaired puromycin acetyltransferase (*Pac*) gene with an insertion that is flanked by two direct microhomologous repeats. When MHEJ utilizes the repeats to repair a DSB that occurs within the insertion, deletion of one of the repeats and the insertion is generated, and consequently, the *Pac* gene function would be restored, rendering the cell puromycin resistant. Our results showed that this reporter can detect MHEJ. The spontaneous frequency of puromycin resistant (*puro*^r) cells was quite low, likely due to the rarity of a DSB spontaneously occurring in the sequence between two repeats. When a DSB was created in the region between two repeats by an endonuclease, I-SceI, the frequency of *puro*^r cells was significantly increased as compared to the spontaneous frequency. Our results also demonstrated that repair of DSBs mediated by MHEJ was more efficient with 10-bp repeats than with 5-bp repeats. Furthermore, the length and composition of intervening sequence between the microhomologous sequences could significantly affect the efficiency of MHEJ.

MATERIALS AND METHODS

Cells and cell culture

Human fibrosarcoma cell line HT1080 and its stably transfected derivatives were grown in DMEM supplemented with 1× MEM nonessential amino acids and 10% fetal bovine serum (FBS) in a humidified 10% CO₂ incubator at 37 °C.

Construction of MHEJ reporter

The plasmid vector pIRESHyg3 that contains a hygromycin B gene (hygromycin-resistant gene or *hyg^r* gene) was purchased from Clontech Laboratories, Inc. (Mountain View, CA). The puromycin acetyltransferase (*Pac*, confers puromycin resistance) gene was PCR amplified using pIRESpuo3 (from Clontech Laboratories) as the template and using primers: 5'-ATT GCC TAG TGA GTT GCT AGC GAC CTT CCA TGA CCG AGT ACA-3' and 5'-ATC ACA ATC GGA CTT GGA TCC GGG TCG TGC GCT CCT TTC-3'. This DNA fragment was digested with *NheI* and *BamHI* and inserted into the *NheI*-*BamHI* site of pIRESHyg3. The *Pac* gene was then disrupted by an insertion at the *BstEII* site. This insertion contains an 18-bp *I-SceI* recognition site (TAGGGATAACAGGGTAAT) and an *AscI* site (GGCGCGCC), flanked by two direct repeats (5-bp, GTCAC, or 10-bp, AGCGGGTCAC), one of which is also a part the insertion and the other is a part of *Pac* (Figure 1). The length of the intervening sequence between two direct repeats in this vector was 26-bp. To investigate the effect of intervening sequences on MHEJ a 1-kb DNA fragment (1025-bp) was inserted at the *AscI* site in the vector harboring 10-bp repeats.

SspI-linearized pIRESHyg3 harboring the impaired *Pac* was electroporated into HT1080 cells using BioRad GenePulser Xcell in 0.4 cm cuvettes at 250 μ F, 350 V. Two days after electroporation hygromycin B (400 μ g/ml) was added into medium and cells were cultured for about 14 days and then hygromycin-resistant colonies were picked up to establish MHEJ reporter lines. MHEJ reporter lines that harbor the 26-bp intervening sequence flanked by 5-bp or 10-bp microhomologous repeats were designated as 5bpMH-26bpIS (5-bp microhomology-26-bp intervening sequence), or 10bpMH-26bpIS lines, respectively. Similarly, MHEJ reporter lines that harbor the 1-kb intervening sequence flanked by 10-bp repeats were designated as 10bpMH-1kbIS lines.

Southern blotting was performed to determine the number of integrated vectors in the individual hygromycin B-resistant lines. Briefly, 10^7 cells were harvested and genomic DNA was extracted with Zymo-spin columns (Zymo Research, CA). Twenty μ g genomic DNA was digested with *XbaI* at 37°C for overnight. After *XbaI* digestion, DNA was purified and separated in 0.8% agarose gel by electrophoresis, and transferred to nylon membranes. For reporter lines with 26-bp intervening sequence, the membranes were hybridized with [³²P]dCTP-labeled probes (735-bp) which were part of the *Pac* gene (Figure 1). For lines with 1-kb intervening sequence the probe (807-bp) was obtained by PCR amplification of a DNA fragment located in the pCMV region of pIRESHyg3 vector (Figure 1), using primers: 5'-TTC GCG TTA CAT AAC TTA CGG-3' and 5'-GCG CCA TTA CCC TGT TAT CC-3'. After 18 h of hybridization, membranes were washed and exposed to X-ray film. A single copy reporter integration would yield a single band with a length depending on the location of the adjacent *XbaI* site in the human genome. Multiple integrations would yield multiple bands. If multiple copies in tandem were integrated, the Southern blot would show a 6.4 kb or 7.4 kb (the length of the construct in 26-bp or 1-kb line, respectively) band and a band with a length that depends on the location of the adjacent *XbaI* site.

Determination of the spontaneous frequency of puromycin-resistant (*puro^r*) colonies

To determine the spontaneous occurrence of MHEJ events using the 5-bp or 10-bp repeats, HT1080 MHEJ reporter cells were plated in 100mm dishes at 2×10^6 cells per dish. One day after plating puromycin was added to medium at a final concentration of 1 μ g/ml. To determine colony forming efficiency (CFE) of MHEJ reporter cell lines, cells were seeded at 500 or 1000 cells/dish in 100 mm dishes containing puromycin-free medium. Nine to twelve days after the puromycin selection, colonies were stained with 1% crystal violet in methanol and counted. The frequency of *puro^r* colonies was measured as the number of *puro^r* colonies

divided by the total number of colony-forming units (CFU, total number of cells plated multiplied by CFE).

Evaluation of the effect of DSBs induced by X-ray on MHEJ

A 10bpMH-26bpIS line with two integrated copies of the reporter was used to evaluate the effect of X-rays on MHEJ. Cells were subjected to a single dose of 5 or 10 Gy X-irradiation delivered at the rate of 0.2 Gy/min using an aluminum filter at 100 kVp (Faxitron Cabinet X-ray System, Faxitron X-ray Corp., Wheeling, IL). Following irradiation, cells were fed with fresh medium. Treated cells were plated at 2×10^6 cells/dish for puromycin (1 μ g/ml) selection or plated at 500 or 2000 cells/dish for the CFE determination. The frequency of puro^r colonies was determined as described above. For each dose, 4–6 independent experiments were performed.

Evaluation of repair of the site-specific DSB via MHEJ

The MHEJ reporter vector contains an 18-bp endonuclease I-SceI recognition site flanked by two microhomologous repeats (Figure 1). To investigate repair of site-specific DSBs induced by I-SceI via MHEJ, MHEJ reporter cells were transfected with a plasmid construct, pCMV(3xNLS)I-SceI, that expresses I-SceI [20]. In brief, cells were seeded in 6-well plates at 4.5×10^5 cells/well 24 h before transfection and were transfected with pCMV(3xNLS)I-SceI using TransIT-LT1 transfection reagent (Mirus, Madison, USA). Twenty-four hours after transfection, cells were aspirated and plated onto 100 mm dishes at 2×10^5 cells/dish, and 1 μ g/ml puromycin was applied 24 h later for selection of puro^r colonies.

Statistics

The mean frequencies of puro^r colonies were compared between MHEJ lines with different MHEJ constructs and between the irradiated and unirradiated MHEJ lines using the Student's t-test. Significance levels were determined using MINITAB software (Minitab, Inc., State College, PA).

RESULTS AND DISCUSSION

We developed a cell-based reporter system that allows us to quantitatively evaluate in vivo MHEJ. The reporter contains a nonfunctional puromycin acetyltransferase (*Pac*) gene due to a disruptive insertion. This insertion contained two endonuclease (I-SceI and AscI) recognition sites, flanked by two direct repeats (5-bp or 10-bp), one of which is also a part the insertion and the other is a part of *Pac* (Figure 1). Human HT1080 fibrosarcoma cells were transfected with constructs, and clones that were resistant to hygromycin were selected. When a DNA DSB occurring at the region between two microhomologous repeats is repaired by using the repeats, a deletion of one of the repeats and the intervening sequence is produced, leading to the restoration of the *Pac* gene function, and consequently rendering the cell resistant to puromycin.

By selection with hygromycin, we obtained seven 5bpMH-26bpIS lines (5-bp microhomology-26-bp intervening sequence) and fifteen 10bpMH-26bpIS lines. We then plated 4×10^7 cells from each line for puromycin selection to determine the spontaneous frequency of puromycin-resistant (puro^r) colonies. As shown in Table 1, puro^r colonies were recovered in both 5-bp and 10-bp lines. DNA sequencing of six puro^r colonies indicated that all six colonies contained the same deletion that spans one of the two repeats and the intervening sequence (data not shown). The deletion resulted in the restoration of the *Pac* gene function, and consequently rendered cells resistant to puromycin. While there was only one puro^r colony detected in seven (14%) 5-bp lines tested, nearly half of 10-bp lines (7/15) yielded puro^r colonies (Table 1). We performed Southern blotting to determine whether or

not the copy number of reporters integrated in these lines had affected the occurrence of MHEJ. As shown in Table 1, the average of the integrated copy number in the seven 5-bp lines was 2.7, not less than the average number of 1.9 in the 10-bp lines, indicating that the lower recovery of *puro^r* cells in 5-bp lines than in 10-bp lines was not due to a shortage of integrated reporters. These results indicate that the 10-bp microhomology is more efficient for MHEJ-mediated mutant generation than the 5-bp, consistent with our previous finding in the cell-free MHEJ assay [18]. Interestingly, the only 5-bp clone from which a *puro^r* colony was isolated had 5 copies of the reporter integrated, and six of seven 10-bp lines that yielded *puro^r* colonies also had multiple copies of the reporter, indicating that the more copies of the reporter integrated, the higher the chance of *puro^r* colonies could be recovered. However, one 10-bp line with a single reporter integrated (line 11) also generated one *puro^r* colony (Table 1).

Our cell-free MHEJ assay also showed that the proximity of repeat sequences affected the MHEJ efficiency [18]. To determine whether this also holds true in cells, we constructed a reporter vector that contains a 1051-bp intervening sequence flanked by two 10-bp repeats, by inserting a 1025-bp DNA fragment at the *AscI* site (Figure 1). We obtained twelve HT1080 lines with 10-bp repeats and the 1051-bp intervening sequence, designated as 10bpMH-1kbIS lines. Each cell line had either one or two copies of the reporter integrated (Table 1). While 7 of 15 lines with the 26-bp intervening sequence generated *puro^r* colonies, none of the 1-kb cell lines produced a *puro^r* colony (Table 1), indicating that the MHEJ efficiency was greatly reduced by the longer intervening sequence.

Ionizing radiation, including X-rays, can result in many types of DNA lesions, including altered bases, single strand breaks, and double strand breaks, many of which are induced by reactive oxygen intermediates or other free radicals derived from the ionization of water [21]. To test whether repair of X-rays-induced DSBs can be detected by the MHEJ reporter, we irradiated 10bpMH-26bpIS line 20 that harbors two copies of reporter with X-rays. While a single dose of 5 or 10 Gy X-rays significantly induced DNA DSBs as indicated by formation of phosphorylated histone H2AX (data not shown), the frequency of *puro^r* colonies in neither the 5 Gy- nor 10 Gy-irradiated group was significantly different from the control (both *p*-values > 0.10, *t*-test, Table 2), suggesting that this MHEJ reporter is not very sensitive for detecting IR-induced MHEJ events that may occur.

The spontaneous frequency of *puro^r* colonies was quite low, ranging from undetectable to 10^{-5} , depending on the sizes of microhomologous repeats and the intervening sequences (Table 1). In addition, the frequency of *puro^r* colonies was not induced by X-irradiation (5 or 10 Gy). One possible reason may be that the probability of a DSB, spontaneous or X-ray-induced, occurring precisely within the 26-bp site (between two repeats, see Figure 1) is extremely low. We speculated that if we introduced a DSB in the region between two repeats, the frequency of *puro^r* colonies would be enhanced.

To test this, we transfected pCMV(3xNLS)I-SceI, a plasmid construct that expresses the I-SceI endonuclease, into MHEJ reporter lines that contain one copy of reporter. I-SceI can recognize an 18-bp sequence inserted between the two direct repeats (Figure 1), and thus can generate a DSB. The number of I-SceI recognition sites in the mammalian genome is extremely small so that introduction of an I-SceI expression vector would not generate massive numbers of DSBs in the cells, which may reduce cell survival and result in fewer *puro^r* colonies. As expected, the frequency of *puro^r* colonies in I-SceI transfected lines was significantly elevated. As shown in Table 3, all transfected 5bpMH-26bpIS or 10bpMH-26bpIS lines (with one copy reporter integrated) generated *puro^r* colonies, whereas only one of their untransfected counterparts was able to generate *puro^r* colonies (10bpMH-26bpIS line 11, Table 1). Similarly, while no *puro^r* colonies were recovered in

untreated 10bpMH-1kbIS lines (Table 1), site-specific introduction of DSBs by I-SceI generated puro^r colonies in 6 out of 10 lines (Table 3). Taken together, these results clearly demonstrate that MHEJ is a DSB repair pathway in cells. Furthermore, after transfection of I-SceI, 10bpMH-26bpIS lines yielded a greater mean frequency of puro^r colonies than 5bpMH-26bpIS lines (6.4×10^{-3} vs. 1.0×10^{-3} , p-value = 0.034, t-test, Table 3), consistent with the notion that longer microhomologies are more efficient for MHEJ than shorter ones.

It should be noted that the frequency of I-SceI-induced puro^r colonies was still low (for instance, 6.4×10^{-3} , in 10bpMH-26bpIS lines), assuming that the majority of cells express the I-SceI activity and therefore have an I-SceI-generated DSB. One explanation is that NHEJ, but not MHEJ, is the predominant pathway repairing I-SceI-generated DSBs (3' overhang). MHEJ may become more utilized with blunt-ended breaks or incompatible overhangs, which are poor NHEJ substrates [22, 23]. Another explanation is that there may be other repeats with closer proximity to each other in this MHEJ reporter vector that might have “short circuited” the targeted MHEJ process mediated by the “reporter repeats.” These alternative MHEJ events do not result in puromycin resistance, but may compete with MHEJ using the reporter repeats and consequently decrease the frequency of puro^r colonies. We analyzed the DNA sequence of the 10bpMH-26bpIS construct for other possible repeats flanking the I-SceI site. Indeed, other repeats exist. For instance, a pair of GGG that flank the I-SceI-generated break lie only 6-bp apart, and another pair of TA lie 12-bp apart. These repeats may have been used for MHEJ other than the 10-bp repeats.

The fact that MHEJ is a mutagenic repair pathway that is commonly associated with human diseases, such as cancer, calls for systematic studies of MHEJ induction and the factors that are involved in MHEJ. Our cell-free MHEJ system is a quick and straightforward means for dissecting the MHEJ pathway although the conditions of reactions used in this cell-free system may not be the same as those inside the nuclei of cells. Katsura et al. developed a different system in which the linearized plasmid construct as repair substrates was transfected into CHO cells to study MHEJ [24]. However, the topological and chromatin context of linearized plasmid repair substrates might be different from DSBs occurring on chromosomes. In our cell-based reporter system the MHEJ reporter was integrated into chromosomes, allowing us to evaluate the MHEJ events that repair DSBs incurred on chromosomes. Therefore this cell-based MHEJ system can serve as a platform for genetic studies of the factors that modulate in vivo MHEJ. Future effort using this platform will shed more light on MHEJ and the mechanism involved.

Acknowledgments

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Figure 1.

A. Schematic illustration of construct for *in vivo* MHEJ reporter system. A disrupted puromycin acetyltransferase gene (*Pac*, confers puromycin resistance) which has an insertion was cloned into a pIRESHyg3 vector. The insertion contains an I-SceI recognition site (18-bp) and an AscI site (8-bp, for future insertion), flanked by two direct repeats (5-bp or 10-bp). One of repeats is a part the insertion and the other one is a part of *Pac*. Hygromycin B phosphotransferase gene (*Hyg^r*) confers hygromycin B resistance. Linearized vector was transfected into HT1080 cells, and transfected cells were cultured with medium containing hygromycin B for selection of MHEJ reporter lines. pCMV, human cytomegalovirus promoter. Horizontal bars represent Southern probes for determination of copies of reporter integrated onto chromosomes. The bar on left was used for 1-kb lines and the bar on right was for 26-bp lines. **B. Southern blotting analysis.** Lines 1 and 2 indicate 1 copy of reporter integration. Lines 3 and 4 demonstrate 5 and 2 copies of reporters integrated, respectively.

Spontaneous frequency of MHEJ.

Table 1

Length of repeat	Length of intervening sequence	Reporter line ID	CFU _s ^a	# puro ^r colonies	Frequency of puro ^r colonies (×10 ⁸)	# reporter integrated ^b
5-bp	26-bp	2	5000000	0	0	5
		3	920000	0	0	1
		4	6192000	0	0	4
		5	3800000	0	0	1
		8	240000	0	0	1
10-bp	26-bp	9	2880000	1	34	5
		15	11600000	0	0	2
		Total: 30632000			Average: 3	
		1	360000	0	0	1
		3	20720000	0	0	1
		4	5920000	0	0	1
		5	160000	0	0	1
		6	4120000	2	49	2
		8	520000	1	192	3
		9	13240000	0	0	1
10-bp	1051-bp	11	80000	1	1250	1
		12	11640000	1	9	3
		13	560000	0	0	2
		15	11000000	1	9	4
		16	7200000	5	69	4
		18	2920000	0	0	2
		19	840000	0	0	1
		20	13960000	8	57	2
		Total: 93240000			Average: 20	
		1	10588000	0	0	1
		2	2160000	0	0	2
		3	16612000	0	0	1
		4	8908000	0	0	2

Length of repeat	Length of intervening sequence	Reporter line ID	CFU _s ^a	# puor ^r colonies	Frequency of puor ^r colonies (×10 ⁻⁸)	# reporter integrated ^b
		5	28588000	0	0	1
		6	14372000	0	0	1
		7	19492000	0	0	1
		8	8852000	0	0	1
		9	21172000	0	0	1
		10	36988000	0	0	1
		11	37388000	0	0	1
		12	29452000	0	0	1
Total: 234572000			0	Average: 0		

^aCFU_s, colony-forming units, calculated by multiplying total number of plated cells with colony-forming efficiency.

^bDetermined by Southern blotting.

Table 2X-irradiation did not increase the frequency *puro*^r colonies

Dose	Experiment	CFU ^a	# <i>puro</i> ^r colonies	Frequency of <i>puro</i> ^r colonies ($\times 10^{-8}$)
0 Gy	1	13960000	8	57
	2	3892000	0	0
	3	9812000	1	10
	4	8758800	1	11
	5	7720000	0	0
	6	9240000	1	11
				Mean $\pm$ SEM ^b : 15 $\pm$ 9
5 Gy	1	6292000	4	64
	2	5692000	0	0
	3	3832000	4	104
	4	5432000	1	18
	5	1920000	0	0
	6	1320000	0	0
				Mean $\pm$ SEM: 31 $\pm$ 18
10 Gy	1	5428000	0	0
	2	3532000	0	0
	3	2040000	0	0
	4	6560000	1	15
				Mean $\pm$ SEM: 4 $\pm$ 4

^aCFUs, colony-forming units^bSEM, standard error of the mean.

Table 3
Site-specific introduction of DSB by I-SceI increases the frequency of puro^r colonies ^a

Length of repeat	Length of intervening sequence	Reporter line ID	CFU ^b	# puro ^r colonies	Frequency of puro ^r colonies (× 10 ⁻⁷)
5-bp	26-bp	3	745904	1537	20606
		5	1414800	902	6375
		8	339800	125	3679
					Mean ± SEM ^c : 10220 ± 5251
10-bp	26-bp	1	8000	39	48750
		3	2456200	12778	52023
		4	435240	1631	37474
		9	828400	9899	119495
					Mean ± SEM: 64436 ± 18616
10-bp	1041-bp	1	2687000	0	0
		3	3053000	0	0
		5	3203000	1	3
		6	6442500	22	34
		7	4310000	16	37
		8	1413000	12	85
		9	3307000	6	18
		10	2327000	0	0
		11	6820000	29	43
		12	4497000	0	0

^a All cell lines have one copy of reporter integrated.

^b CFUs, colony-forming units

^c SEM, standard error of the mean.