Evaluation of an Hprt-Luciferase Reporter Gene on a Mammalian Artificial Chromosome in Response to Cytotoxicity

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ABSTRACT

Background Hypoxanthine guanine phosphoribosyltransferase (Hprt) is known as a house-keeping gene, and has been used as an internal control for real-time quantitative RT-PCR and various other methods of gene expression analysis. To evaluate the Hprt mRNA levels as a reference standard, we engineered a luciferase reporter driven by a long Hprt promoter and measured its response to cytotoxicity.

Methods We constructed a reporter vector that harbored a phiC31 integrase recognition site and a mouse Hprt promoter fused with green-emitting luciferase (SLG) coding sequence. The Hprt-SLG vector was loaded onto a mouse artificial chromosome containing a multi-integrase platform using phiC31 integrase in mouse A9 cells. We established three independent clones.

Results The established cell lines had similar levels of expression of the Hprt-SLG reporter gene. Hprt-SLG activity increased proportionately under growth conditions and decreased under cytotoxic conditions after blasticidin or cisplatin administration. Similar increases and decreases in the SLG luminescent were observed under growth and cytotoxic conditions, respectively, to those in the fluorescent obtained using the commercially available reagent, alamarBlue.

Conclusion By employing a reliable and stable expression system in a mammalian artificial chromosome, the activity of an Hprt-SLG reporter can reflect cell numbers under cell growth condition and cell viability in the evaluation of cytotoxic conditions.

Key words gene reporter; hypoxanthine phosphoribosyltransferase; luciferase; mouse artificial chromosome; reference standards

House-keeping genes have been routinely used as internal controls for normalization in gene expression analysis.[1–3] Hypoxanthine guanine phosphoribosyltransferase (Hprt), a nucleotide metabolizing enzyme, is such a house-keeping gene, and has been utilized in many studies of gene expression as a reference standard. As we compared the gene expression inductions by the compounds (mostly drugs) of internal control genes, using the Open TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System), HPRT-gene presented the least variation.[4] Although it has been widely used as a standard in real-time quantitative reverse transcription PCR (RT-qPCR), the Hprt promoter/enhancer has not been extensively analysed.[5, 6]

Luciferase assay systems enable the real-time monitoring of gene expression in living cells. We used the green-emitting luciferase (SLG) from Rhagophthalmus ohbai as a reporter gene in this study.[7] Reliable expression systems are needed for the evaluation of in vitro gene analysis, but transgene expression in various cell lines established by random genomic integration using conventional methods can be unstable or non-uniform due to gene silencing. Mammalian artificial chromosome technology has been developed to overcome this problem. It has been demonstrated that human artificial chromosomes (HACs) and mouse artificial chromosomes (MACs) are independently retained in host cells and provide stable expression of transgenes.[8, 9] In addition to these characteristics, features that allow cell-to-cell transfer of HACs and MACs by microcell-mediated chromosome transfer (MMCT) has shown potential for

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Abbreviations: BAC, bacterial artificial chromosome; CAG, cytomegalovirus IE enhancer and chicken beta-actin promoter; HAC, human artificial chromosome; Hprt, hypoxanthine guanine phosphoribosyltransferase; HS4, DNase I hypersensitive site 4; MAC, mouse artificial chromosome; MI, multiple integrases; MI-MAC, multiple integration site-containing mouse artificial chromosome; MMCT, microcell-mediated chromosome transfer; PAC, P1 artificial chromosome; PGK, phosphoglycerate kinase; SLG, green-emitting luciferase.

gene therapy application as well as for gene analyses in various situations.[10–13] A mouse A9 cell line, derivative of mouse fibroblast L cells used for toxicity testing, was useful for a donor cell of MMCT.[14]

The construction of larger promoter/reporter vectors requires the handling of large DNA regions, which is difficult with common cloning approaches. The bacterial artificial chromosome (BAC) recombineering method is a powerful tool for the manipulation of long DNA fragments.[15, 16] Because recombination takes place in bacteria by employing intrinsic bacterial/phage machinery, large vector construction can be achieved without complicated cloning steps. It has been reported that a mouse CD40L gene vector constructed using BAC recombination showed functional expression from a HAC.[17]

Phage integrases, enzymes that integrate DNA into a bacterial host genome, have been reported to work in mammalian cells.[18, 19] PhiC31, R4, TP901 and Bxb1 integrases mediate efficient site-specific recombination in mammalian cells, and transgenesis in mice was also reported by pronuclear injection of phiC31 integrase.[20–24] The multiple integrases (MI) system on an artificial chromosome (MI-HAC/MI-MAC), an application of mammalian artificial chromosome technology, was developed for loading gene(s) onto HACs and MACs. The MI platform has five gene loading sites for distinct recombinase/phage integrases.[25] By using this recombinase-mediated MI system, targeted recombinant cells can be obtained at high efficiency and these recombinants retain stable transgene expression compared with the random integration method.[25] Recently, it has been shown that transchromosomic mice were generated in fewer steps by direct use of mouse embryonic stem cells harboring MI-MAC.[26]

Here, we constructed a long Hprt-promoter/luciferase reporter vector using a BAC recombineering method and loaded it onto the MI-MAC system. We confirmed luciferase activity that was proportionate with cell numbers in established Hprt-luciferase cell lines.

EXPERIMENTAL PROCEDURES

Vectors

The inspB4ins2 vector is described elsewhere.[26] The vector has two insulator cassettes consisting of repetitive 5′-DNaseI hypersensitive site 4 (HS4) elements from chicken beta-globin to prevent promoter interference from neighboring regions. The PPAC ori km vector was modified from the pPAC4 vector (Children's Hospital Oakland Research Institute, Oakland, CA). The BstEII/AscI region was replaced by a linker sequence containing a multiple cloning site (McaTI, FseI, PmeI and AvrII). pCAG-phiC31 expresses a bacterial phiC31 integrase optimized for mammalian codon usage and is driven by the CAG promoter.

Hprt promoter cloning by BAC recombination

A detailed flow diagram for the construction of phiC31neoHprt-SLG is described in Fig. 1 and Table 1. Briefly, the locus-specific homology arm (white boxes in Fig. 2A) of the Hprt promoter region was synthesized for the retrieving 20 kb BAC fragment. The arm was ligated into the inspB4ins3 vector, and then the coding sequences of the *Rhagophthalmus ohbai* luciferase gene, SLG (pSLG-test vector, Toyobo, Osaka, Japan) was inserted. Additionally, a phiC31neo module (the phiC31 integrase attB site and a neomycin resistance gene cassette) was also ligated into the inspB4ins3 vector. The vector was digested by AscI and AvrII and then ligated into PPAC ori km. The Hprt gene promoter region was retrieved by gap-repair from a BAC clone (B6Ng01-126E09; Riken, Tokyo, Japan) into the P1 artificial chromosome (PAC) vector using *E. coli* strain DY380.[13, 14] Clones were selected at 32 ℃ on LB agar containing kanamycin. To check whether the clones were precisely retrieved by gap-repair of the promoter arms, the clones were amplified by PCR, and the vector was confirmed by restriction enzyme digestion.

Cell culture and compounds

Mouse A9 (MI-MAC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37 ºC.[26] Blasticidin (InvivoGen, San Diego, CA) and cisplatin (Wako, Osaka, Japan) were diluted with culture medium at the time of use.

Establishment of Hprt-SLG cells

Principles for recombinase-mediated integration using the MI-MAC system are described elsewhere.[25, 26] The phiC31neoHprt-SLG PAC vector was purified using a Large Construction Kit (Qiagen, Hilden, Germany). A9 MI-MAC cells were co-transfected with phiC31neoHprt-SLG and pCAG-phiC31 using Lipofectamine 2000 (Invitrogen) for 6 h (Fig. 2B). Twenty-four hours after transfection, cells were expanded for 24 h and then selected with 600 μg/mL G418 (Invitrogen). Surviving colonies were picked and recombination checked by genomic PCR analyses.

PCR analyses

Amplified regions and primer sequences for genomic PCR are described in Fig. 2C and Table 2, respectively. All PCR reactions were performed with KOD FX neo polymerase (Toyobo) under the following conditions.

For junction PCR (Fig. 2C; JP-5, JP-3); 95 °C for 2 min for 1 cycle, 98 °C for 10 sec and 68 °C for 1–1.5 min for 35 cycles. For long PCR (Fig. 2C; LP-6, LP-14, LP-10 and LP-15); 95 °C for 2 min for 1 cycle, 98 °C for 10 sec and 74 °C for 6–15 min for 5 cycles, 98 °C for 10 sec and 72 °C for 6–15 min for 5 cycles, 98 °C for 10 sec and 70 °C for 6–15 min for 5 cycles, 98 °C for 10 sec and 68 °C for 6–15 min for 20 cycles, 68 °C for 10 min for 1 cycle. Extension time was modulated according to target size. Long PCR products were digested with appropriate restriction enzymes to confirm amplification of the target.

Fluorescence in situ hybridization (FISH) mapping

FISH analysis was performed using a standard protocol.[11, 26] Metaphase nuclei from established cell lines (A9 Hprt-SLG cells) were spread on slides. Biotin-labeled Hprt-SLG vector and digoxigenin-labeled mouse minor satellite DNA were prepared as hybridization probes. To suppress background signals, a fifty-fold amount of non-labeled mouse Cot-1 DNA was added during hybridization. Chromosomal DNA was counter-stained with DAPI-Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Fluorescence images were captured by Metafer, and analyzed with ISIS (Carl Zeiss, Oberkochen, Germany).

Luciferase and cell viability assays

A9 Hprt-SLG cells were seeded at 5×10^4 cells per well in a 96-well micro-clear bottom black plate (Greiner, Kremsmünster, Austria) 24 h prior to compound addition. After culture for 72 h with blasticidin (0–10 μg/mL) or cisplatin (0–20 μM), cells were washed twice with PBS and then subjected to the following analyses. Luciferase activity was measured with a Phelios luminometer (Atto, Tokyo, Japan) using Tripluc assay reagent (Toyobo). A cell viability assay was performed using alamarBlue (AbDSerotec, Oxford, UK) according to the manufacturer's instructions and an Infinite F500 fluorescent plate reader (Tecan, Männedorf, Switzerland). Three independent wells were used to determine the Luciferase activity.

RESULTS

Construction of the Hprt-SLG reporter vector

To develop a retrieving vector, the following components were sequentially ligated into inspB4ins2: insulator sequence (HS4), retrieving arm, SLG luciferase and phiC31neo module (Fig. 1, steps 1 to 5). Detailed operations for each step are described in Table 1. The locations of the Hprt retrieving arms were determined by RepeatMasker to avoid repetitive elements, which are known to be deleterious for subsequent BAC retrieving (Fig. 2A white boxes). The module containing all components was transferred to a PAC vector backbone (Fig. 1, steps 6 to 8), and the resulting PAC vector was used as a retrieving vector. We successfully retrieved 20 kb of Hprt promoter from the BAC clone to give phiC31neoHprt-SLG (Fig. 1 step 9). The large 20-kb promoter/enhancer region of Hprt was successfully recloned into an expression vector for Hprt gene expression using BAC recombineering method.

Vector transfection into A9 MI-MAC cells

Figure 2A and B represent a genomic map of the mouse Hprt gene and schematic map of the MI-MAC and phiC31neoHprt-SLG vector array, respectively. The retrieved phiC31neoHprt-SLG vector was co-transfected into A9 MI-MAC cells with a phiC31 integrase expression vector (Fig. 2B), and 32 candidate colonies were isolated. We performed sequential PCR analyses of these clones to clarify the relationship between total isolated colony numbers and accurate integration in each step. Figure 2C shows a post-integration map at the phiC31 site on the MI-MAC and the regions amplified by PCR using the primer sets in Table 2. All PCR analysis results are summarized in Table 3. First, 26 of 32 clones were 5′-junction-PCR-positive (#2–11, #13–15, #17, #19–23 and #25–31). Among 16 of these clones (#2, 5, 6, 9, 10, 13, 15, 17, 19, 21, 22, 26–28, 30 and #31) 10 were positive for the 3′-junction PCR (#2, 10, 13, 15, 17, 21, 22, 26, 27 and #30). Subsequently, only four clones (#2, 17, 21 and #30) were positive for the 3′-6 kb PCR that included the SLG reporter element, and then #21 clone was excluded by failure to amplify the 3′-15 kb fragment (LP-15 in Table 3 and Fig. 2C). We, therefore, obtained three cell lines (#2, #17 and #30; Hprt-SLG cells) that integrated the entire vector region from the 16 clones examined. These clones produced almost the same levels of luciferase activity (Fig. 3A), and FISH analysis showed Hprt-SLG signals on the MAC (Fig. 3B). Thus, we presumed that these lines had uniform reporter gene expression from the MI-MAC.

Evaluation of Hprt-SLG luminescence compared with cell numbers

To verify luminescence linearity of Hprt-SLG cells, we seeded clone #17 at various densities from 1,000 to 40,000 cells in a 96-well black plate and performed a luciferase assay after 2 h. Luciferase activity showed linear luminescence (Fig. 4A, upper panel, green line). This luminescence profile was mostly consistent with cell viability measured with an alamarBlue cell viability assay (Fig. 4A upper panel, blue line). Similar to the MTT as-

say, the Alamar Blue reagent detects reduced substrates in response to metabolism in living cells. [27] We also seeded 10,000 cells, and measured luciferase activity every 24 h up to 72 h. The correlation of luminescence and living cells showed an almost linear ratio through the incubation period (Fig. 4A lower panel). According to these results, we considered that luminescence intensity of Hprt-SLG reflected living cell numbers under conditions of growth. To assess the reporter response under toxic conditions, we administrated cytotoxic compounds to clone #17. Treatment with blasticidin (Fig. 4B upper panel) or cisplatin (Fig. 4B lower panel) for 72 h decreased SLG activity in a dose-dependent manner. Next, we estimated cell viability using the alamarBlue cell viability assay. We found a positive correlation between SLG activity and cell viability using both blasticidin and cisplatin. We also obtained similar results using clones #2 and #30 (data not shown).

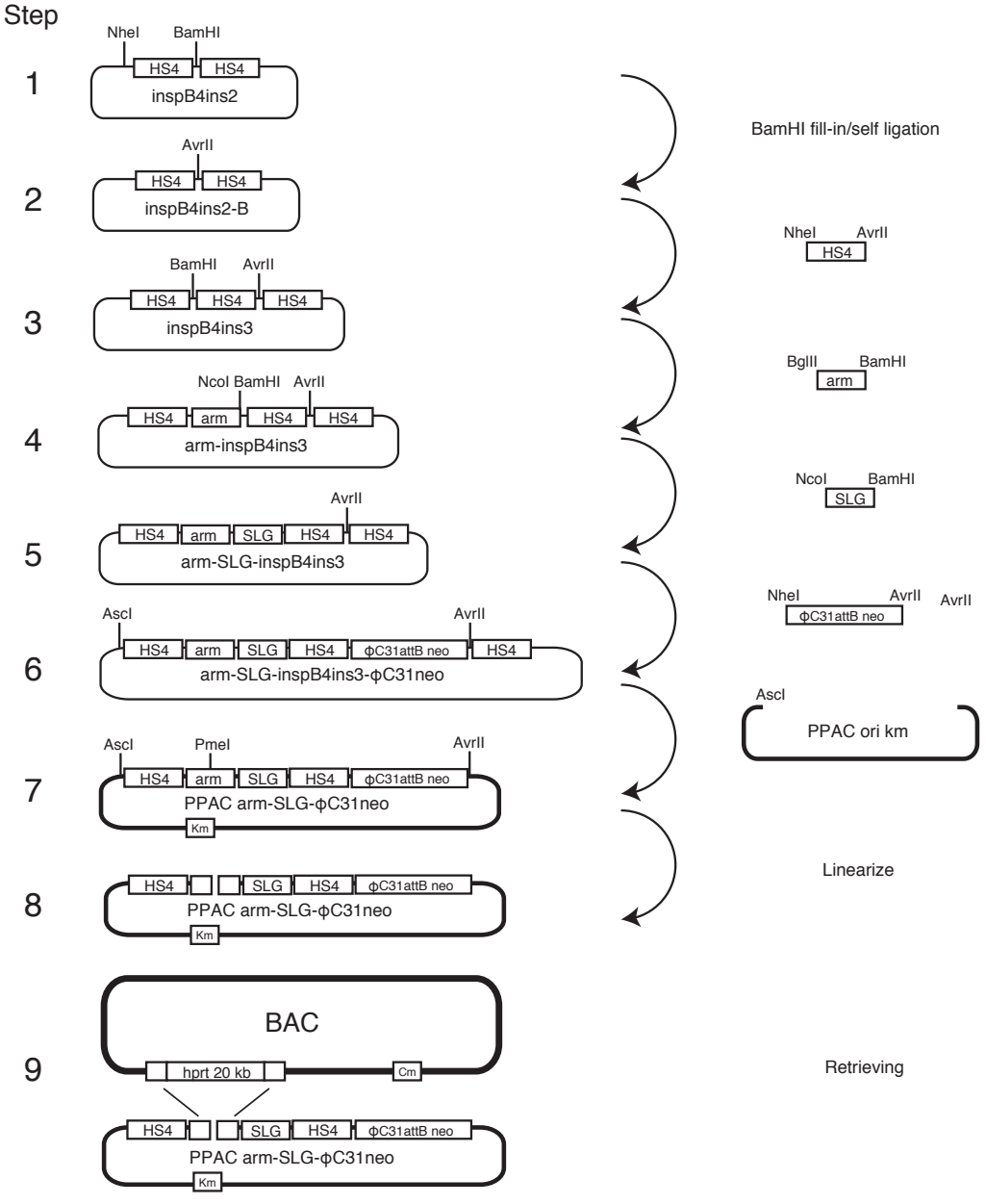

Fig. 1. Construction of phiC31neoHprt-SLG.
Flow chart of PAC vector construction for BAC retrieving. Arrows indicate the sequential steps via the operations described in Table 1. The retrieving vector is composed of an Hprt homologous arm-SLG sequence flanked by HS4 insulators and a phiC31neo cassette.
BAC, bacterial artificial chromosome; Hprt, hypoxanthine guanine phosphoribosyltransferase; HS4, DNase I hypersensitive site 4; PAC, P1 artificial chromosome; SLG, green-emitting luciferase.

Table 1. Operations for constructing the phiC31neoHprt-SLG PAC vector

Steps in Fig. 1	Operation	Fragment/digestion (origin)	Insert site
1 to 2	Fill-in/self ligation	BamHI	
2 to 3	Add HS4 fragment	NheI-AvrII (inspB4ins2)	AvrII
3 to 4	Add arm fragment	BglII-BamHI (synthetic gene arm)	BamHI
4 to 5	Add SLG fragment	NcoI-BamHI (pSLG-test)	NcoI-BamHI
5 to 6	Add phiC31neo fragment	NheI-AvrII (phiC31neo inspB4ins2)	AvrII
6 to 7	Transfer to PAC vector	AscI-AvrII (arm-SLG-inspB4ins3-phi C31neo)	AscI-AvrII
8	Linearization	PmeI	
9	Retrieving of BAC		

BAC, bacterial artificial chromosome; HS4, DNase I hypersensitive site 4; PAC, P1 artificial chromosome; SLG, green-emitting luciferase.

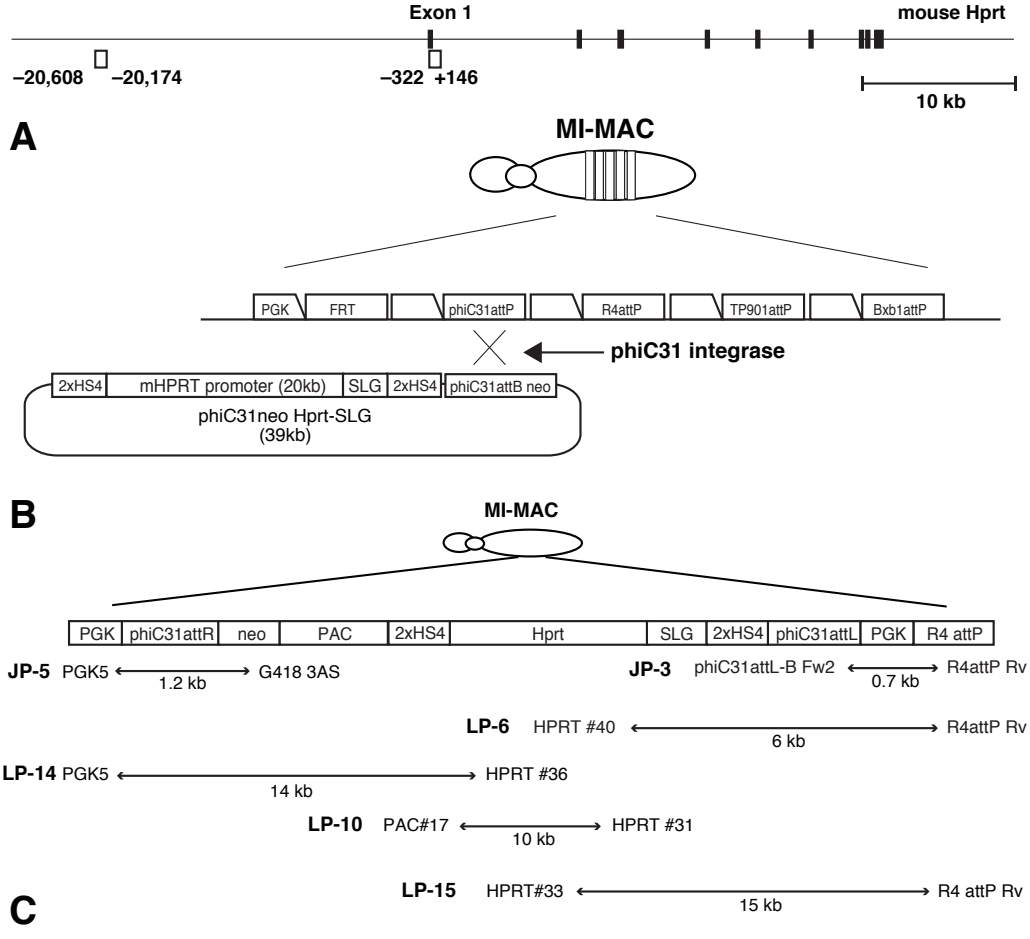

Fig. 2. Mouse Hprt promoter vector and MI-MAC integration.
A) Genomic map of the mouse Hprt gene. Vertical black lines indicate exons. Numbers beside white boxes represent nucleotide positions of Hprt retrieving arms.
B) MI-MAC integration and the retrieved Hprt-SLG PAC vector. The MI platform consists of five phosphoglycerate kinase (PGK) promoter-attP arrays. FRT, phiC31attP, R4attP, TP901attP and Bxb1attP indicate yeast recombinase FLP, phage integrase phiC31, R4, TP901 and Bxb1 recognition sequence, respectively. Recombination between phiC31attB and attP by phiC31 integrase is represented.
C) Map of PAC vector integrated into the MI-MAC at the phiC31 site. Double-headed arrows with fragment length indicate regions amplified by PCR. Primer sequences are described in Table 2. Names of PCR-amplified regions are indicated to the left of the double-headed arrows.
Hprt, hypoxanthine guanine phosphoribosyltransferase; HS4, DNase I hypersensitive site 4; MI-MAC, multiple integration site-containing mouse artificial chromosome; PAC, P1 artificial chromosome; PGK, phosphoglycerate kinase; SLG, green-emitting luciferase.

DISCUSSION

In this report, we used BAC recombination and mammalian artificial chromosome technology to engineer an Hprt-reporter whose activity reflected living/dead cell viability in response to cytotoxic compounds.

Although the house-keeping Hprt gene has been used and verified as a standard for gene expression experiments, few reports have analyzed the Hprt gene promoter. Therefore, we examined a 20 kb long region of the Hprt promoter with the expectation of achieving reliable expression. Without using PCR cloning or a standard "cut and ligation" approach, we employed a BAC recombination method to acquire the long promoter region of the Hprt gene. In this Hprt promoter retrieving experiment, the BAC recombination efficiency (successfully retrieved bacterial clones) was 1%–6%. Retrieving rates we achieved for other genes were similar (data not shown). Recombination efficiency depends on the retrieving sequence and its length, and we considered the targeted Hprt promoter region within the limits of the procedure to achieve vector construction. Using a long Hprt promoter, we constructed the phiC31neoHprt-SLG reporter vector for integration at the phiC31 site on the MI-MAC. The phiC31neoHprt-SLG and phiC31 integrase expression vectors were co-transfected into A9 MI-MAC cells, and we isolated G418 resistant colonies. Transfected A9 MI-MAC clones were mostly positive for the 5′-junction PCR assay, but we detected only three lines with full-length integration (#2, #17 and #30; Table 3). These three lines produced nearly equivalent luciferase activity [clone #21 displayed low activity in spite of containing the SLG element (data not shown)]. We presume that low luciferase activity of clone #21 resulted from deletion of the Hprt promoter region during the integration step into the MI-MAC. While we employed a 39 kb PAC vector in this study, it is considered that intact integrations will occur at lower frequencies with larger vector constructions.

Luciferase activity of the three correctly integrated cell lines exhibited linearity with respect to cell numbers. We confirmed that this correlation was retained under continuous growth conditions for up to 72 h (Fig. 4A lower panel). We also confirmed proportionate decreases in luciferase activity and cell numbers under the toxic conditions of exposure to blasticidin or cisplatin (Fig. 4B). Blasticidin is a nucleoside antibiotic generally used in mammalian or bacterial cell selection and cisplatin is an anti-neoplastic drug widely used as a chemotherapeutic drug for cancer patients. Considering these results, the Hprt-SLG reporter can report on not only living (growing) cells, but also on toxicity-induced cell death. Although, cisplatin evoked a mild cytotoxic response up to 20 μM, we confirmed similar tendencies of luciferase activity and cell viability in response to blasticidin and cisplatin, suggesting that the Hprt-SLG reporter has the potential to evaluate cytotoxicity caused by different actions. We also note that employing a 20 kb Hprt promoter contributed to reliable expression of the Hprt-reporter gene on the MAC. Now, we are verifying the usability of the Hprt-luciferase reporter in other cell lines by MAC transfer (manuscript in preparation).

Stability and uniformity of reporter gene expression are important factors and are required for reliable results in gene expression analyses. In addition, the transferrable feature of HACs and MACs by the microcell-mediated chromosome transfer method makes it possible to establish various cell lines using different recipients, including mouse embryonic stem cells.[10] Transchromosomic mice generated from such HAC/MAC-transferred mouse embryonic stem cells can pave the way for authentic in vivo analysis. Recently, a simultaneous gene-loading system for HACs has been developed and is a further multi-purpose tool for gene analysis.[28] Furthermore, an evaluation system for osteogenic differ-

Table 2. Primers used for PCR analysis to verify the reporter construct in Fig. 2C

Primer name	5′-sequence	Usage
PGK5	AATGGAAGTAGCACGTCTCACTAGTCTC	5′-junction/long PCR
G418 3AS	GGTAGCCAACGCTATGTCCTGATAGCGGTC	5′-junction PCR
phiC31attL-B Fw2	CTCGTCGGCCGGCTTGTCGACG	3′-junction PCR
R4attP Rv	AGTTGGGTGCACCCGCAGAGTGTA	3′-junction/long PCR
PAC#17	CTCTAGCGGGGGGATCTGCATGCAC	Long PCR
HPRT#31	GTGTATGAGGCCTCTCTGGTCATAACCTG	Long PCR
HPRT#33	GTTACTATCGAGCCTGTGACAACCACGTGG	Long PCR
HPRT#36	CTGCAGGCCCAGGTTGGTAAGCTCTCTC	Long PCR
HPRT#40	GCGGAGTGATTATCTGGGAATCCTCTGGG	Long PCR

HPRT, hypoxanthine guanine phosphoribosyltransferase; PAC, P1 artificial chromosome; PGK, phosphoglycerate kinase.

Table 3. Summary of PCR analysis of phiC31neoHprt-SLG vector-transfected MI-MAC A9 cells

#Clone	PCR regions in Fig. 2C					
	JP-5	JP-3	LP-6	LP-15	LP-10	LP-14
1	–					
2	+	+	+	+	+	+
3	+	NT				
4	+	NT				
5	+	–				
6	+	–				
7	+	NT				
8	+	NT				
9	+	–				
10	+	+	–			
11	+	NT				
12	–					
13	+	+	–			
14	+	NT				
15	+	+	–			
16	–					
17	+	+	+	+	+	+
18	–					
19	+	–				
20	+	NT				
21	+	+	+	–		
22	+	+	–			
23	+	NT				
24	–					
25	+	NT				
26	+	+	–			
27	+	+	–			
28	+	–				
29	+	NT				
30	+	+	+	+	+	+
31	+	–				
32	–					

Hprt, hypoxanthine guanine phosphoribosyltransferase; MI-MAC, multiple integration site-containing mouse artificial chromosome; NT, not tested; SLG, green-emitting luciferase.

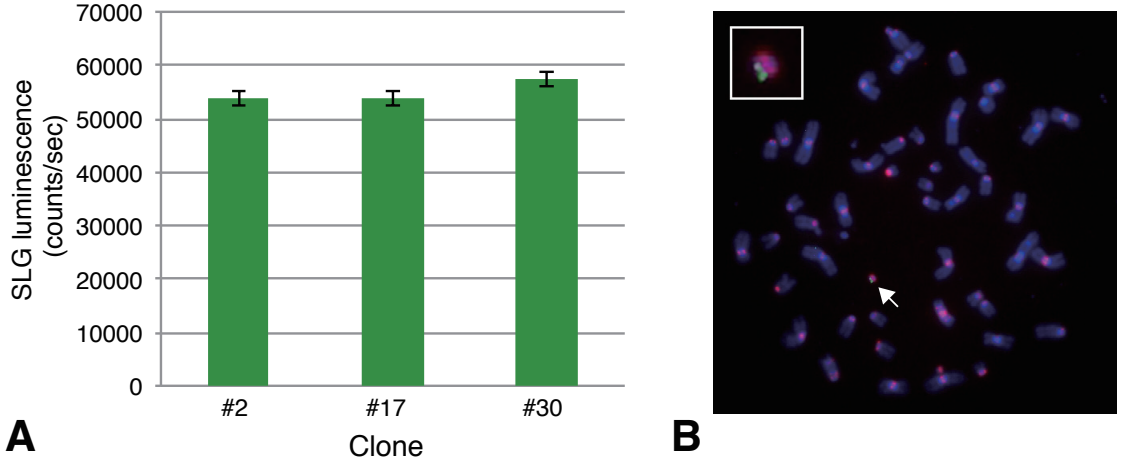

Fig. 3. Luciferase activities and FISH analyses of Hprt-SLG cells.
A) Luciferase activities of A9 Hprt-SLG cells. Each clone was seeded 24 h prior to assay. Error bars represent standard deviation ($n = 4$).
B) Representative FISH image of A9 Hprt-SLG cells. Digoxigenin-labeled mouse minor satellite (red signal) and biotin-labeled Hprt-SLG PAC vector (green signal) were used as detection probes. Arrow indicates MI-MAC and insert shows magnified image of Hprt-SLG and MI-MAC signal. Hprt, hypoxanthine guanine phosphoribosyltransferase; MI-MAC, multiple integration site-containing mouse artificial chromosome; PAC, P1 artificial chromosome; SLG, green-emitting luciferase.

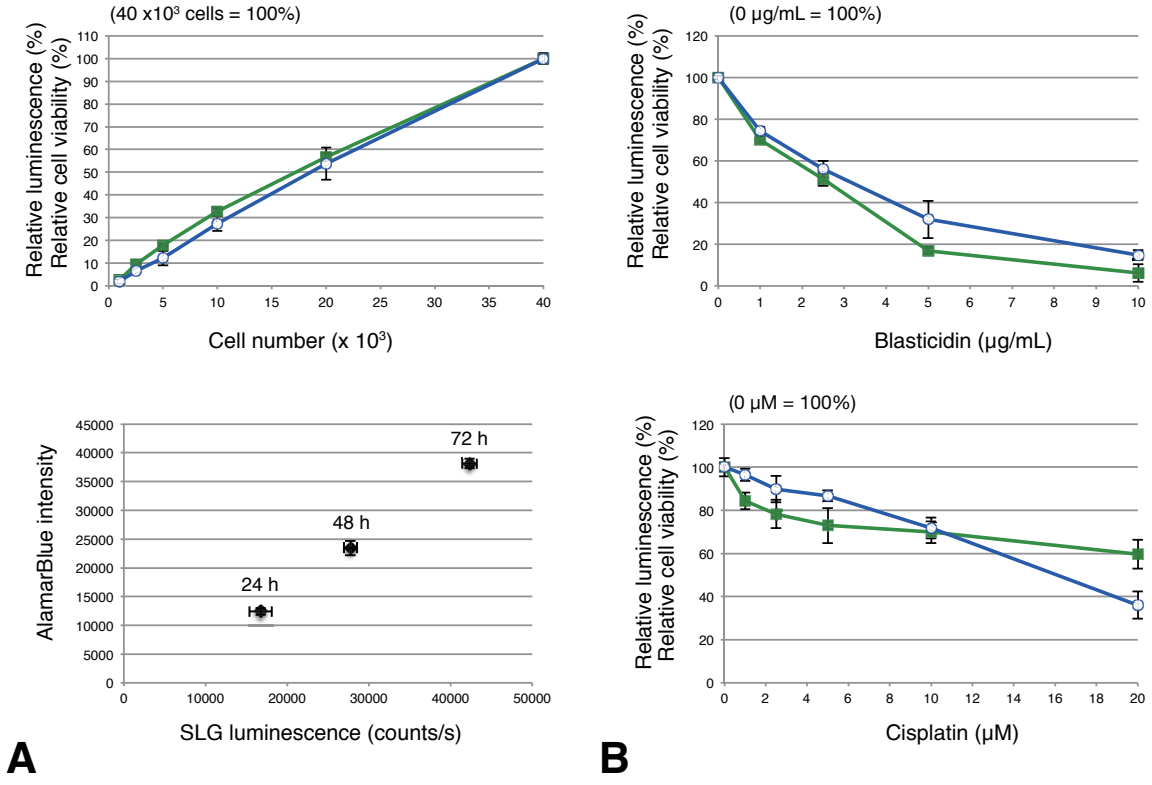

Fig. 4. Response curves of Hprt-SLG cells.
A) Hprt-SLG cells in normal culture conditions. Upper panel: Relative luciferase activity and cell viability were plotted against various cell numbers. Luciferase activities and alamarBlue signals were measured at 2 h after cell seeding. Green rectangles and blue circles indicate relative luminescence by SLG activity and relative cell viability by alamarBlue intensity, respectively. Lower panel: SLG luminescence and alamarBlue intensity were measured after continuous incubation for the indicated hours. Error bars represent standard deviation ($n = 3$).
B) Response of Hprt-SLG cells to cytotoxic compounds. Relative luciferase activity and cell viability were plotted against concentration of blasticidin (upper panel) or cisplatin (lower panel). Hprt, hypoxanthine guanine phosphoribosyltransferase; SLG, green-emitting luciferase.

entiation has been established that utilizes a luciferase reporter and a MAC.[29] The combination of multiple gene reporters, multiple gene loading and artificial chromosomes, will lead to diverse analyses of gene function and to high throughput systems that will contribute to drug development.

Noguchi et al. developed a dual-color luciferase assay system in which the expression of multiple genes can be tracked simultaneously using green- and red-emitting luciferases and this dual-color luciferase assay system was used for an in vitro test to screen skin sensitizer.[30, 31] By using green- and red-emitting luciferases as the internal control reporter and cytotoxicity specific reporter respectively, we can analysis quantitatively the cytotoxicity of chemical compounds.

In the future, I would like to develop an in-vitro nephrotoxicity test by using the dual-color luciferase assay system of Hprt-SLG reporter and nephrotoxicity marker gene- red-emitting luciferases.

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