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Comparison of gene-trapping efficiency between retroviral and lentiviral vectors in mouse embryonic stem cells

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ABSTRACT

Gene trapping is a method of inserting DNA into the genome at random, generating insertional mutations throughout the genome. The efficiency of retroviral gene trapping is not sufficient in part because of a strong preference for retroviral integration near transcription start sites. In contrast, lentiviral vectors strongly favor integration in the entire region of highly active genes, suggesting that lentiviral vectors would improve the efficiency of gene trapping. In this study, we constructed both lentiviral and retroviral gene-trap vectors and analyzed integration sites in mouse embryonic stem (ES) cells. The frequency of false-positive gene-trap events was about 12-fold higher for the retroviral vector compared to the lentiviral vector. Within intragenic regions, most of the retroviral vector integration sites were found in the 5′ untranslated region, while the lentiviral vector integrated uniformly throughout transcriptional units. The trapping efficiency of unique genes was significantly higher for the lentiviral vector (~83%) than for the retroviral vector (~51%). Our data demonstrate that the lentiviral vector can trap the active genes more efficiently than the retroviral vector and will facilitate efficient generation of gene-trap libraries not only in ES cells but also in a wide variety of cell lines and primary cells.

1. Introduction

In the post-genome sequencing era, attention has turned towards the functional characterization of all protein-coding genes. One of the most efficient ways to elucidate the functions of a gene is to analyze the phenotypes of a mutant for the gene. Several technologies are being used to produce genome-wide mutations, including gene trapping, transposon insertion, and chemical mutagenesis [1]. Gene trapping is an efficient high-throughput technology to generate insertional mutations throughout the genome and to subsequently identify the insertion sites. In the mouse genome, a large-scale international knockout mouse project is currently underway to mutate all protein-coding genes using a combination of gene trapping and gene targeting in mouse embryonic stem (ES) cells [2]. Libraries of mutant ES cells provide a powerful tool for studying the functions of a gene in vivo by production of mutant mice. At present large-scale gene-trap screens have successfully generated insertional mutations in more than half of the mouse genes. However, gene-trap insertion is non-random in practice, and only genes expressed in ES cells can be trapped. Therefore, this approach reaches a plateau without achieving gene-trap insertions in all genes. To generate mutations in the remaining genes, the technology of gene targeting by homologous recombination has been applied and successfully targeted more than 9000 genes to date [3]. Nevertheless, gene-targeting approach is laborious, time-consuming, and expensive. Thus, alternative gene-trap methods with different insertion characteristics would be valuable to increase the number of newly trapped genes.

Conventional gene-trap vectors comprise a promoterless reporter gene and/or selectable marker gene flanked by an upstream splice acceptor site and a downstream polyadenylation (polyA) signal sequence (i.e. promoter trap) [1,4]. The integration of promoter-trap vector into an intron results in the production of a spliced fusion transcript between the endogenous gene and the reporter gene. However, this strategy can only trap genes that are transcriptionally active in the target cells. To overcome this problem, it has been proposed to use polyA-trap vectors that contain a constitutive promoter followed by a reporter gene and a splice donor, but lack a polyA signal sequence [1,4]. The reporter gene is expressed only when the trap vector inserts within a gene in the correct orientation and captures downstream exons and a polyA signal of the endogenous gene. Since polyA trapping occurs independently of target gene expression, all genes should theoretically be trapped equally. However, the mutagenicity of polyA trap approach is negligible in part because of the degradation of polyA-trapped mRNAs by an mRNA-surveillance mechanism,

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nonsense-mediated mRNA decay [5]. It has been reported that the addition of an internal ribosomal entry site (IRES) sequence prevents this decay [5]. Nevertheless, the polyA trap approach is not commonly used for large-scale screens.

Moloney murine leukemia virus (MLV)-based retroviral vectors have been the most commonly used vectors for gene trapping. It has been demonstrated that MLV and MLV-based retroviral vectors have a strong preference for integration near transcription start sites, with a relatively weak preference for active gene [6–10]. Due to these integration site preferences, the efficiency of retroviral gene trapping is not sufficient [11]. In contrast, human immunodeficiency virus (HIV) and lentiviral vectors strongly favor integration in actively transcribed genes [6–8,12,13]. There is no preference in the location of lentiviral integration sites along the length of transcription units. These data suggest that a bias may be apparent when using retroviral or lentiviral vectors for gene trapping. In this study, we tested this assumption and describe a comparison of gene trapping efficiency and integration site preferences between retroviral and lentiviral gene-trap vectors.

2. Materials and methods

2.1. Gene-trap vector construction

Lentiviral and retroviral gene-trap vectors were derived from the self-inactivating (SIN) lentiviral vector CS-CDF-CG-PRE [14] and retroviral vector pCLBabe-puro-SIN GFP [15], respectively. The Venus fluorescent protein gene [16] and the blasticidin resistant (Bsd) gene linked by the Thosea asigna virus 2A (T2A) peptide sequence (Venus-T2A-Bsd) was cloned into pIRES–EGFP (Clontech), which contains an IRES sequence of the encephalomyocarditis virus, and the Venus-T2A-Bsd-IRES cassette was polymerase chain reaction (PCR)-amplified using the forward primer (5′-CTCGAGATGGTGAGCAAGGGCGAGGA-3′) and the reverse primer (5′-ACGATGATAATATGGCCACAAGCTAGC-3′), in which the NheI site just downstream of IRES contains a translational stop codon to prevent translation of 3′ portion of trapped gene initiated from the IRES. A splice donor sequence of the human bcl-2 gene intron 2/exon 3 was PCR-amplified using the forward primer (5′-GCTAGCACTTCAGGGATTTGAATG-3′) and the reverse primer (5′-GAATTCCCAGTTCTAAGGACGTCTG-3′). A splice acceptor sequence of the mouse Engrailed 2 (EN2) gene (2454–4536 bp) was PCR-amplified using the forward primer (5′-GTTAACCGACTGGCCTTGAGTCGCG-3′) and the reverse primer (5′-GCTGAGTTTCAGACCAACCTCGAG-3′). An XhoI–NheI fragment of the Venus-T2A-Bsd-IRES cassette and an NheI–EcoRI fragment containing the bcl-2 splice donor sequence were cloned into the XhoI and EcoRI sites of CS-CDF-CG-PRE, resulting in CS-CDF-VBsd. An HpaI–XhoI fragment containing the EN2 splice acceptor sequence was cloned into the HpaI and XhoI sites of CSII-EF-MCS, resulting in CSII-EF-EN2SA. An XhoI–PmeI fragment of CSII-EF-EN2SA was cloned into the XhoI and PmeI sites of CS-CDF-VBsd, resulting in LTV-VBsd. An HpaI–EcoRI fragment of LVT-VBsd was cloned into the HpaI and EcoRI sites of pBabe-puro-SIN, resulting in RTV-VBsd. LTV-VBsd and RTV-VBsd are called the lentiviral and retroviral gene-trap vectors in this paper, respectively.

2.2. Viral vector production

Lentiviral and retroviral gene-trap vectors pseudotyped with the vesicular stomatitis virus G glycoprotein were produced as described previously [14]. The copy number of integrated vectors present in a population of infected cells was determined by real-time genomic quantitative PCR. B6;129-Gt(ROSA)26Sor$^{tm2Sho/J}$ mouse [17] that carry one copy of the GFP gene was used as a control of the vector (Venus transgene) copy number. Briefly, genomic DNA isolated from infected cells (200 ng) or from tail biopsies of B6;129-Gt(ROSA)26Sor$^{tm2Sho/J}$ mouse (50, 100, 150, or 200 ng) was added to 2 μl of LightCycler FastStart DNA Master HybProbe Master Mix (Roche Diagnostics), 1.6 μl of 25 mM $MgCl_2$, 10 pmol each of Forward primer (Venus-F: 5′-CACATGAAGCAGCACGACT-3′), Reverse primer (Venus-R: 5′-CTTCAGCTCGATGCGGTT-3′), 3′ Fluorescein probe (Venus-Flu: 5′-GCTACGTCCAGGAGCGCACCATCTTCT-3′), and 5′ LCRED640 probe (Venus-LC: 5′-CAAGGACGACGGCAACTACAAGACCCG-3′), and water was added to a 10-μl final volume. PCR amplification was performed in a LightCycler (Roche Diagnostics) with a 10-min pre-incubation at 95 °C followed by 45 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 1 min. Gene-trapping efficiency in infected cells was determined by flow cytometry for Venus expression.

2.3. Cell culture

E14 Mouse ES cells [18] were cultured on a 0.1% gelatin-coated dish in E14 proliferative medium containing Knockout DMEM/15% Knockout Serum Replacement (Invitrogen), 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and ESGRO (1000 units/ml) (Millipore) without a feeder layer.

2.4. Analysis of integration sites and trapped transcripts

Gene-trap vector integration sites and trapped transcripts were analyzed by inverse PCR and 5′ Rapid Amplification of cDNA Ends (5′ RACE).

Inverse PCR: Genomic DNA (0.5–2 μg) was digested with ApoI, treated with T4 DNA ligase, and purified by PCR purification kit (Qiagen). The purified DNA was used as a template for inverse PCR as described previously [19]. All of the reagents for PCR were obtained from Takara Bio. For the first-round PCR, a 50-μl reaction mixture was prepared by adding 38.5 μl of sterile distilled water, 5 μl of 10× Ex Taq buffer, 4 μl of dNTP mixture (2.5 mM each), 1 μl (33 pmol each) of oligonucleotide primers (5′-CCCTGGGGATCTTGCATGATGGC-3′ and 5′-GGACTCACTCGCTGGCTCCTCAC-3′ for the retroviral vector or 5′-GCAGCATTGGTAGCTGCTGTGTTGC-3′ and 5′-GCCTGGCTAGAAGCACAAGAGGAGGA-3′ for the lentiviral vector), and 0.5 μl of Ex Taq DNA polymerase (5 units/μl) (Takara Bio). Then, 35 cycles of PCR were carried out, with each cycle consisting of 94 °C for 30 s and 68 °C for 1 min. Using 1 μl of the first-round PCR product as a template, 35 cycles of second-round PCR were carried with another set of primers (5′-CCGCGTGGTTCGCCTCACAGCTTTCC-3′ and 5′-GCCAGTCGGTTAACATCGAGGGAT-3′ for the retroviral vector or 5′-CTTGTGATTGCTCCATGTTTTTCCAGG-3′ and 5′-CCAGTCACACCTCAGGTACCTTTAAGACC-3′ for the lentiviral vector). Only PCR products with a single band were purified and cloned into the pT7Blue vector (Takara Bio), and then clones were sequenced.

5′ RACE: Total RNA samples (5 μg) were reverse transcribed using 2.5 pmol of oligonucleotide (TTCAGCTCGATGCGGTTC), 1 μl of 100 mM dNTP, 40 units of RNaseOUT (Invitrogen), 1 μl of 0.1 M DTT and 200 units of Superscript III (Invitrogen) in a 50-μl reaction mixture at 45 °C for 2 h. RNA was subsequently removed by incubation with 2 units of RNase H at 37 °C for 1 h. cDNA was purified by PCR purification kit and 10 μl of purified cDNA was used for 5′ RACE. 5′ RACE was performed using the 5′ RACE system for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) according to the manufacturer's instructions. Oligonucleotide primers used were: 5′-GAAGTCGTGCTGCTTCATGTGGTCGGG-3′ for the first round of PCR and 5′-CGTCCAGCTCGACCAGGATGGGCAC-3′ for the second round of PCR. Purified 5′ RACE products were cloned into the pT7Blue vector and sequenced. Statistic analysis was performed by chi-square test. Sequence analysis

was performed using the NCBI Blast search and searches of the FANTOM consortium database.

3. Results

3.1. Construction of lentiviral and retroviral vectors for gene trapping

To compare the efficiency of gene trapping and integration site preferences between retroviral and lentiviral vectors, a gene-trap cassette was engineered into both vectors (Fig. 1). The gene-trap cassette comprises a splice acceptor sequence of the EN2 gene, the Venus fluorescent protein gene and the Bsd gene linked by the T2A peptide sequence, an IRES sequence, and a splice donor sequence of the Bcl2 gene. The end of the Bsd gene and three bases downstream of the IRES harbor a stop codon. The gene-trap cassette is flanked by long terminal repeats (LTRs) in reverse orientation. Both gene-trap vectors are SIN vectors in which enhancer and promoter regions in the U3 region of the 3′ LTR are deleted, and therefore the U3 region including this deletion is copied to the 5′ LTR during reverse transcription and integration. To facilitate integration site analysis (5′ RACE or inverse PCR) and to avoid difficulties due to retroviral integration site preference (i.e. GC-rich regions), both vectors contain a polyA-trap module for 3′ RACE analysis. Although double tagging vectors previously reported [20] contain a strong internal promoter for the polyA-trap module, our vectors do not contain a strong internal promoter to avoid induction of the expression of any downstream genes. Since previous report has demonstrated that an IRES sequence prevents degradation of polyA-trapped mRNAs by nonsense-mediated mRNA decay [5], an IRES sequence was engineered into our vectors for polyA traps of longer mRNAs. When the gene-trap vector integrates into an intron of transcriptionally active endogenous gene, a fusion transcript is generated between the upstream and downstream exons from the endogenous gene and the Venus-T2A-Bsd reporter gene. After translation of the fusion transcript, the T2A peptide sequence self-cleaves the fusion protein. Any translation initiated from the IRES is terminated by the stop codon three bases downstream of the IRES sequence.

3.2. Gene-trapping efficiency in HeLa cells

To examine the gene-trapping efficiency of retroviral and lentivial vectors, HeLa cells were infected with both gene-trap vectors at 0.5 copies/cell, which were determined by real-time genomic quantitative PCR of infected cells. Assuming that Venus expression results from a gene-trap event, the gene-trapping efficiency was 28.8% for the retroviral vector and 15.6% for the lentiviral vector (Fig. 2A). Next we analyzed integration sites of the gene-trap vectors. HeLa cells were infected with both gene-trap vectors at 0.05 copies/cell and selected with blasticidin. Genomic DNA was isolated from 45 blasticidin resistant clones for each vectors, and genomic regions flanking integration sites were retrieved by inverse PCR, sequenced, and mapped to the human genome using NCBI BLAST search (Supplementary Table 1). Within intragenic regions, the frequency of integration occurred in the 5′ untranslated region (UTR) of a gene was 26.3% (5 of 19) for the retroviral vector and 6.5% (2 of 31) for the lentiviral vector (Fig. 2B). Consistent with previous studies, our results demonstrate that the retroviral vector integration is favored near transcription start sites. Insertions into intergenic regions occurred in 57.8% (26 of 45) of the retroviral vector and 31.1% (14 of 45) of the lentiviral vector integration events.

3.3. Gene-trapping efficiency in mouse ES cells

We next analyzed in more detail the gene-trapping efficiency and integration sites in mouse ES cells. Mouse ES cells were infected with the retroviral vector at 0.66 copies/cell and with the lentiviral vector at 0.5 copies/cell. Based on the percentage of Venus-positive cells, the gene-trapping efficiency was 19.7% for the retroviral vector and 3.7% for the lentiviral vector (Fig. 3A). As is the case in HeLa cells, a higher gene-trapping efficiency was obtained with the retroviral vector compared to the lentiviral vector. To examine the integration site preferences, mouse ES cells were infected with both gene-trap vectors at 0.05 copies/cell and selected with blasticidin. A total of 176 clones for the retroviral vector and 182 clones for the lentiviral vector were isolated, and vector integration sites were mapped to the mouse genome (Supplementary Table 2). Sequencing analysis revealed that 160 of 176 (90.9%) clones for the retroviral vector and 170 of 182 (93.4%) clones for the lentiviral vector were independent clones. Within intragenic regions, 63.9% (69 of 108) of the retroviral vector integration sites were found in the 5′ UTR (54 of 108) and the first 10% of the coding region of the genes (15 of 108) (Fig. 3B). In contrast, the lentiviral vector integrated uniformly throughout transcriptional units. Some genes were trapped multiple times, and hot spots for integration were observed more frequently with the retroviral vector. Hnrpf and Ptbp1 genes were trapped more than three times with both vectors, suggesting common integration hot spots which may be defined by chromatin structure. The number of trapped unique genes was 64 for the retroviral vector and 132 for the lentiviral vector. The expression of expected fusion transcripts was confirmed by 5′ RACE and RT-PCR, and

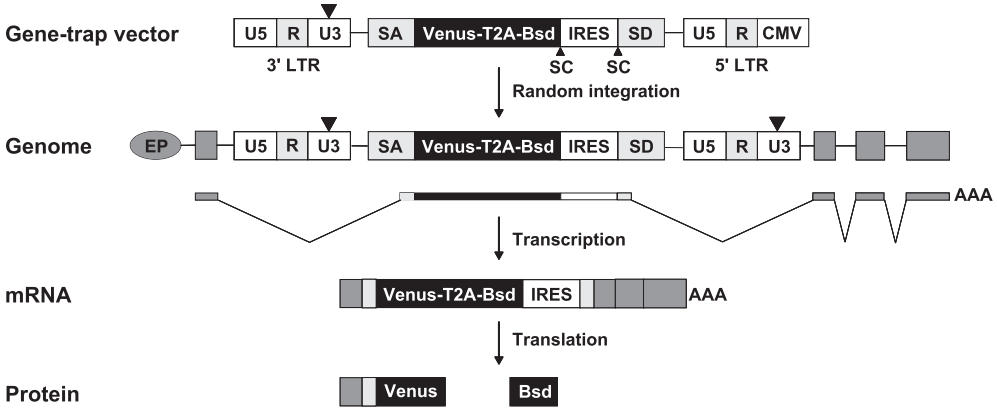

Fig. 1. Lentiviral and retroviral gene-trap vectors. The gene-trap cassette containing the Venus-T2A-Bsd reporter gene is inserted between two LTRs of SIN (inverted triangle) lentiviral and retroviral vector backbones in reverse orientation. SA, splice acceptor sequence; SD, splice donor sequence; SC, stop codon; EP, endogenous promoter.

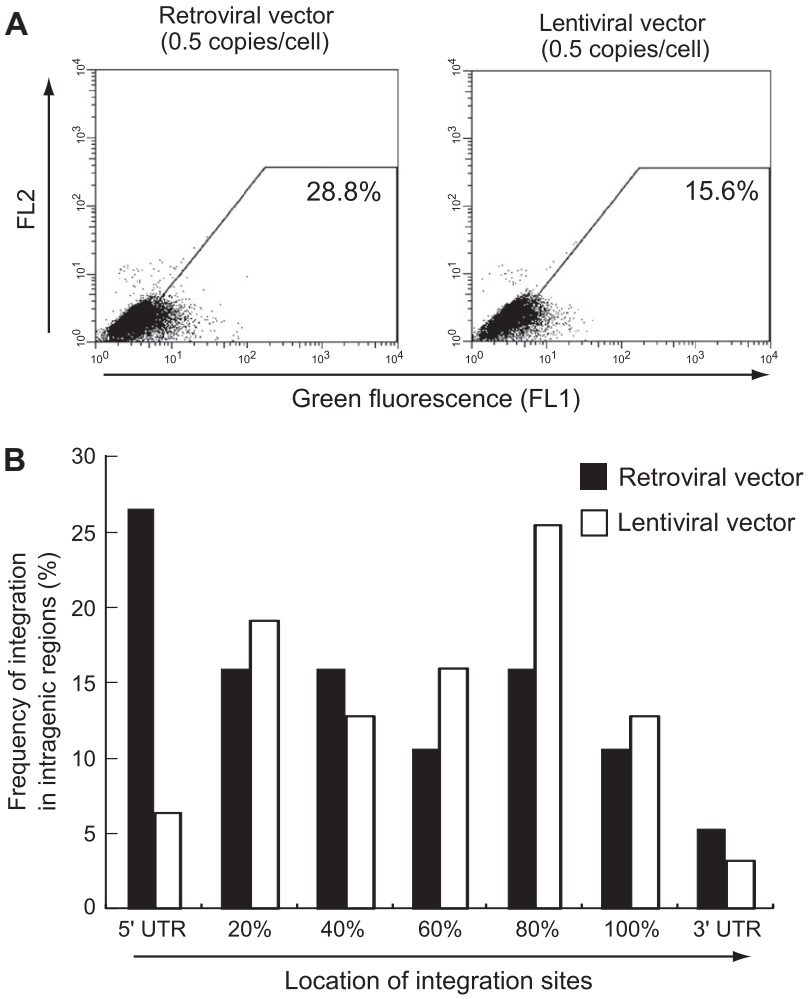

Fig. 2. Gene-trapping efficiency and integration site preferences in HeLa cells. (A) HeLa cells were infected with the lentiviral and retroviral gene-trap vectors at 0.5 copies/cell. Venus expression was analyzed by flow cytometry 72 h after infection. The percentage of Venus-positive cells is shown. (B) The location of integration sites within intragenic regions is divided into seven sections: 5′ UTR, 3′ UTR, and five sections in the coding region on the basis of the distance from the start codon, expressed as a percentage relative to the entire length of the coding region. The percentage of gene-trap clones in each section is shown.

representative data are illustrated in Supplementary Fig. 1. The frequency of integration into intergenic regions was about 5-fold higher for the retroviral vector (52 of 160) than for the lentiviral vector (12 of 170).

3.4. Analysis of gene-trap clones integrated in intergenic regions

Transcripts from the gene-trap vectors integrated in intergenic regions were analyzed by 5′ RACE. The result showed that transcripts in 34 of 52 clones for the retroviral vector and 3 of 12 clones for the lentiviral vector were initiated within the 3′ LTR and fusion transcripts generated by a trans-splicing event were not detected, indicating false-positive clones. Since the rest of the clones showed no sequence homology to any known transcripts using NCBI BLAST search, we further analyzed using the FANTOM consortium database that contains a more detailed transcriptome including non-coding transcripts [21,22]. Transcripts in 9 clones for the retroviral vector and 1 clone for the lentiviral vector were found in the FANTOM database, and these transcripts were not derived from protein-coding transcripts but non-coding transcripts (Supplementary Table 2). Then, we conclude that other trapped transcripts that were not found in either database are novel unannotated transcripts (Supplementary Table 3). Overall, the trapping efficiency of unique genes was significantly higher for the lentiviral vector (141 of 170 (82.9%)) than for the retroviral vector (82 of 160 (51.3%)) (Fig. 3C). The frequency of false-positive gene-trap clones was about 12-fold higher for the retroviral vector (34 of 160 (21.3%)) than for the lentiviral vector (3 of 170 (1.8%)) (Fig. 3D).

3.5. Distribution of vector integration sites in the mouse genome

The vector integration sites in ES cells were plotted on the mouse chromosomes (Fig. 4A). The genomic distribution of integration sites was found to be non-random and was not proportional to the ratio of the length of introns [23] (Fig. 4B). Both gene-trap vectors appear to have different integration site preferences. Specifically, the retroviral vector showed a highly significant bias for integration frequency in whole chromosomes compared to the lentiviral vector (Fig. 4B). Note that a strong bias for the retroviral vector integration sites on chromosomes 5 and 6 is mainly because the Rbpj gene on the chromosome 5 and the Hnrnpf gene on the chromosome 6 were trapped 9 times and 13 times, respectively. The restriction enzyme ApoI to digest genomic DNA for inverse PCR could affect the frequency of isolation of these integration sites. Although ES cells have an elevated global transcriptional activity with few hot spots of gene expression [24], the association of integration sites with gene activity was significantly observed for both gene-trap vectors (Fig. 4C). This bias

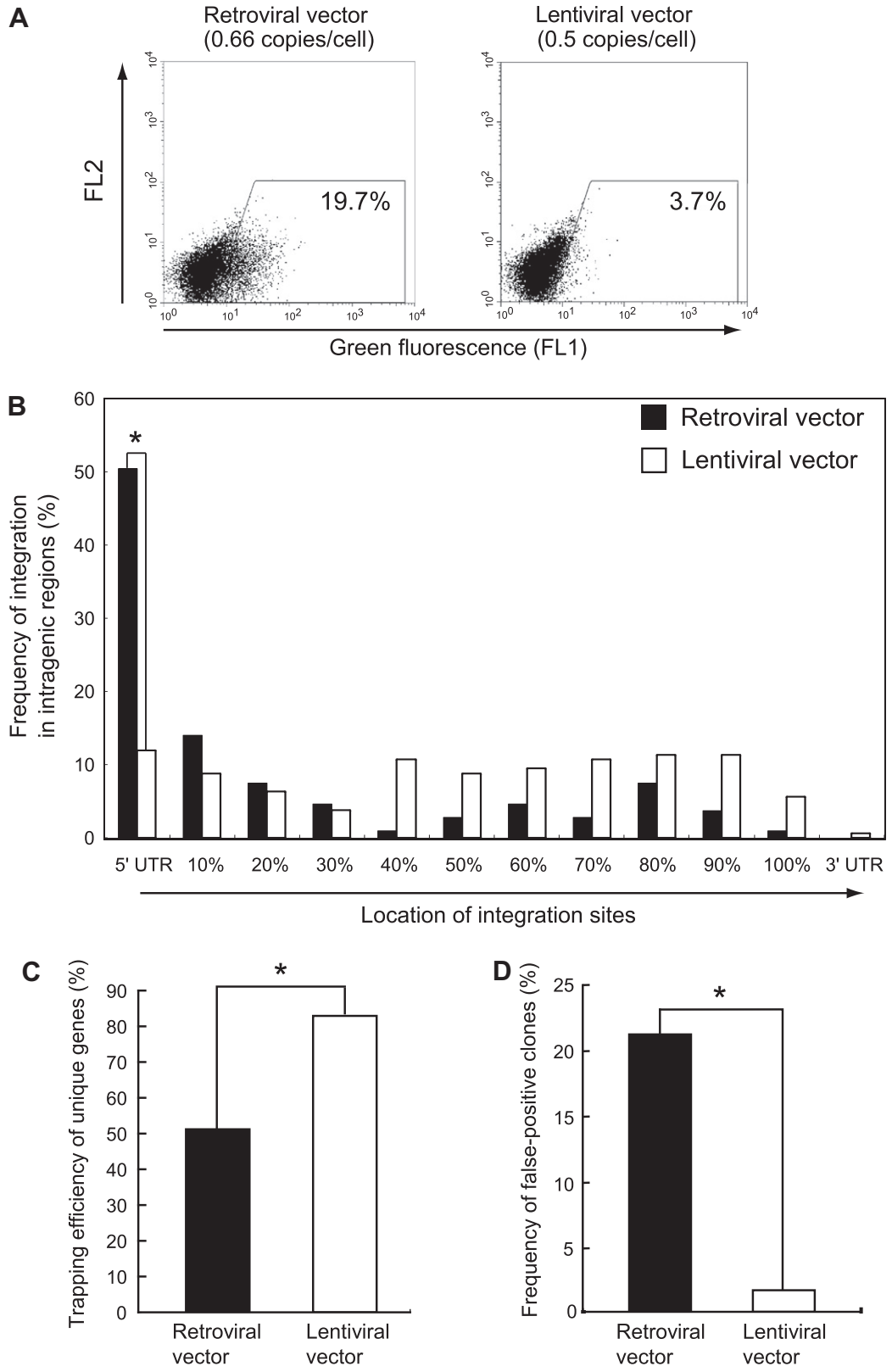

Fig. 3. Gene-trapping efficiency and integration site preferences in ES cells. (A) ES cells were infected with the retroviral vector at 0.66 copies/cell and with the lentiviral vector at 0.5 copies/cell. Venus expression was analyzed by flow cytometry 72 h after infection. The percentage of Venus-positive cells is shown. (B) The location of integration sites within intragenic regions is divided into twelve sections: 5′ UTR, 3′ UTR, and ten sections in the coding region on the basis of the distance from the start codon. The percentage of gene-trap clones in each section is shown. (C) The trapping efficiency of unique genes was calculated by dividing the number of trapped unique genes by the total number of independent gene-trap events. (D) The frequency of false-positive gene-trap clones was calculated by dividing the number of false-positive gene-trap clones by the total number of independent gene-trap events. Asterisks indicate significant differences ($P < 0.05$).

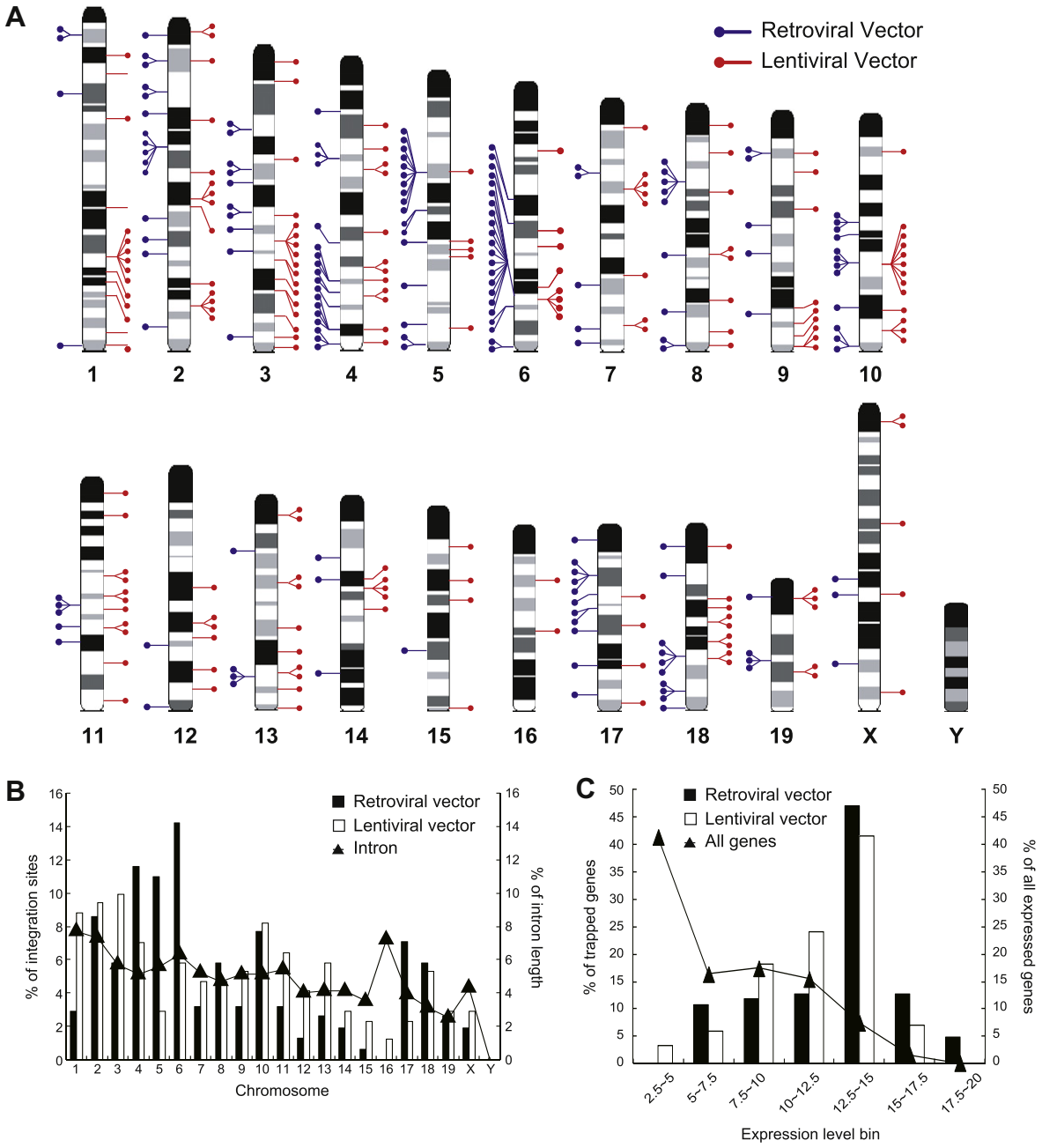

Fig. 4. Distribution of integration sites in the mouse genome. (A) Integration sites of the retroviral (blue circle) and lentiviral (red circle) vectors in ES cells are plotted on the mouse chromosomes. (B) The relationship between frequency of integration sites and intron length (black triangles) on each chromosome is shown. (C) The relationship between frequency of gene trapping and gene activity is shown. The gene expression profile of E14 ES cells was obtained from NCBI GEO (datasets GSM396240). Expression levels were analyzed by Agilent genespring software version 11.5 and classified into 7 bins. Black triangles show the percentage of all expressed genes in each expression level category.

may reflect the integration preference of both vectors for active genes and/or the selection of vectors with blasticidin.

4. Discussion

In this study, we compared the efficiency of gene trapping and integration site preferences between retroviral and lentiviral vectors in human HeLa cells and mouse ES cells. Our gene-trap vectors contain both a promoter trap module and polyA-trap module without a strong internal promoter. The gene-trapping efficiency, simply analyzed by reporter gene expression, was significantly higher for the retroviral vector in both HeLa and ES cells, which is consistent with the previous report [25]. However, detailed analysis of integration sites revealed that the trapping efficiency of unique genes was significantly higher for the lentiviral vector. This was due to a higher frequency of retroviral integration into intergenic regions, resulting in a false-positive gene trap, even though the retroviral vector strongly favored integration into the 5′ regions of genes and this feature is effective at increasing the gene-trapping efficiency. It has been shown that MLV LTR possesses bi-directional promoter activities and, even in SIN LTR, is able to aberrantly activate the expression of neighboring genes [26]. Therefore, a possible explanation for the false-positive gene trap is that the LTR of our SIN retroviral vector may transactivate neighboring genes and this could facilitate activation of a cryptic promoter in the LTR.

In contrast, the lentiviral vector integrated uniformly throughout transcriptional units with less global bias for integration site selection. Our results reflect and confirm the reported differences between retroviral and lentiviral vectors [6–10,12,13].

Overall, the features of lentiviral vectors are suitable for large-scale gene-trap screening because the noise to signal ratio is more favorable. In addition, lentiviral vectors have a wide host range and are able to transduce both dividing and non-dividing cells. Although both retroviral and lentiviral vectors have different drawbacks for gene trapping, these vectors still represent powerful tools for functional genomics analysis. Gene-trap mutagenesis is effective not only for the elucidation of gene function but also for discovering novel transcripts [27]. Our results showed that only a small number of genes trapped by both retroviral and lentiviral vectors were overlapped. Gene trapping using both vectors could be an effective strategy to mutate the majority of genes. To obtain optimal results for gene trapping, it might be necessary to reveal the mechanisms of integration for both viruses and to design hybrid vectors that draw on the strengths of each vector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.085.

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