Efficient Integration of Short Interspersed Element-flanked Foreign DNA via Homologous Recombination*

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We investigated whether mouse short interspersed elements (SINEs) could influence the recombination frequency of foreign DNA. Vectors harboring a reporter gene in combinations of SINEs B1 and/or B2 or a portion of long interspersed element-1 were prepared and tested in vitro by a colony assay using HC11 murine mammary epithelial cells and in vivo by microinjection into fertilized mouse eggs. In transfected HC11 cells, the number of colonies surviving G418 selection increased by 3.5-fold compared with control when the reporter was flanked by fused B1-B2 sequences. Similar results were obtained from microinjection study; in fetuses 11.5 days post coitum, transgene positives in control and SINE-flanked vectors were 16 and 53%, respectively. Individual B1- and B2-harboring vectors showed equivalent activities with each other, as determined by the colony assay (2.8-fold versus 3.2-fold compared with control). We determined the contribution of homologous recombination to the SINE-mediated increase in integration frequency through a polymerase chain reaction-based strategy; in more than half of embryos transgenes underwent homologous recombinations involving B1 sequences. These results demonstrate that the SINE sequences can increase the integration rate of foreign DNA and that such an increase is most likely due to the enhancement of homologous recombination.

Mammalian genomes contain both unique and repetitive DNA sequences. In addition to the highly abundant satellite DNA, which occurs in tandem arrays mostly clustered in heterochromatic regions, the genome also contains repeated sequences that are interspersed among single-copy sequences of euchromatic regions, such as the short interspersed repeated DNA elements (SINEs)1 and long interspersed repeated DNA elements. Many of these repetitive DNA family members are present in extremely high copy numbers, more than 106 copies/haploid genome (reviewed in Refs. 1 and 2).

Both B1 (130 bp) and B2 (190 bp) sequences are murine SINEs with unknown functions (3). From 130,000 to 180,000 copies of the B1 repeat and from 80,000 to 120,000 copies of the B2 repeat are present in the mouse genome scattered on all chromosomes (4). Kramerov et al. (5) reported that members of the B1 family were nearly identical with one another. Individual members of the B2 family also show a high degree of homology, displaying only 3–5% deviations from the B2 consensus sequence (3). The presence of so many short homologous sequences throughout the mammalian and higher eukaryotic genomes has implications in genetic recombinations (1, 6).

The introduction of exogenous DNA into mammalian cells has become an increasingly important procedure. An efficient integration of delivered DNA into the host genome is the most critical step in the whole procedure of many biological applications, especially in areas such as transgenesis and gene therapy. However, little information is available about the actual molecular events involved in the integration process. The integration phenomenon appears to be predominantly random and nonhomologous (11, 12); thus, as far as there is no additional machinery or modulatory elements to accelerate the process, the integration of a foreign sequence into the genome might occur nearly by chance. Such a passive integration pattern may also limit the integration frequency. Therefore, the development of strategies that could lead to an efficient, nonrandom integration of foreign DNA could have a far reaching impact on areas where efficient gene transfer is a crucial step.

To design a vector that can mediate a highly efficient integration of foreign DNA, we searched for sequences that have a potential of increasing the recombination frequency between the input DNA and the host genomic loci. Because the SINE sequences such as B1 and B2 and the long interspersed repeated DNA element sequences such as L1 exist in high copy numbers dispersed throughout the mouse genome, we examined the possibility that these interspersed repetitive sequences could efficiently guide an exogenous DNA into genomic
Efficient Integration of SINE-flanked Foreign DNA

Colonies—vectors. Cultured as described previously (16). Transfection was performed by Aat pGB1/2 into the which cuts the vectors only once, size-fractionated, and transferred to a Bgl digested with 100 Life Technologies, Inc.) to obtain stable transfectants. The G418-resistant colonies were selected individually using a cloning cylinder and NeoR gene, washed, and exposed to an x-ray film according to the genomic DNAs. 10 (Sigma), expanded into large populations, and harvested to prepare resistant colonies were selected individually using a cloning cylinder and 5′-TTGGATCCAGGTGTTCTCGTG3′. To amplify the B2 sequence (GenBank accession number M31441), a set of Xhol- or Bgl-II anchored primers, 5′-AAAGATCTGGTGGATGC-GCTCAG3′ and 5′-ACACCTCAGGCTTCAGCAC-3′ were used. Amplified B1 (145 bp) and B2 (152 bp) fragments were eluted from agarose gel, digested with relevant restriction enzymes, and then cloned in a fused form into Xhol sites of pGEM7zf and pSP73 (pGB1/2 and pSB1/2, respectively). The cloned repetitive sequences were identified by sequencing (Sequenase, CLONTENDED). The vector pB1/2, was constructed by subcloning the AatII/EcoRI fragment of pGB1 into the AatII/EcoRI sites of pGB1/Bgl-II and pBGL-II. The resulting pB1/2 vector was confirmed by restriction digestions with BglII/XhoI, and pB1/2 were prepared by inserting a 4.7-kb XhoI/Hind-III fragment of the pPGK/geo fragment (13) vector into the SalI and HindIII sites of the pSB1/2, pB1/2, and pSP73 vectors, respectively. A 710-bp L1 sequence spanning the 5′-untranslated region through the middle of the ORF1 of the L1 sequence (14) was amplified by PCR using a set of XhoI-anchored L1-specific primers (5′-CTGCCTCGAGCTCTTCTGA-ACA-3′ and 5′-GCTCTCGAGCAAGCTTGTT-3′) and cloned into the XhoI site of pSP73 and pGEM7zf vectors (pSL1 and pGL1, respectively). The pGL1 vector was restricted with AatII/EcoRI, and the resulting L1 fragment was subcloned into the corresponding enzyme sites of the pSL1 vector to generate pL1L1. The pL1/geo and pL1L1/geo vectors were constructed in a similar way. To make pB1/2/geo, pB2/2/geo and pB11/geo vectors, pSP73 was digested with BglII and filled with Klenow enzyme. The linearized vector was added with d(T) to their 3′ ends using T3 polymerase to prepare a kind of T-vector (15), into which a B1, B2, or deleted B1 (B14) monomer was inserted (pSB1, pSB2, or pSB14). The B14 vector was amplified like the B1 sequence but using another B1 3′ primer, 5′-AGCCCTAGCTGCTCGAGCA-ACA-3′ and 5′-GCTCTCGAGCAAGCTTGTT-3′ and cloned into the XhoI site of pSP73 and pGEM7zf vectors (pSB1 and pGL1, respectively). The pGL1 vector was restricted with AatII/EcoRI, and the resulting L1 fragment was subcloned into the corresponding enzyme sites of the pSL1 vector to generate pL1L1. The pL1/geo and pL1L1/geo vectors were constructed in a similar way.

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Construction of Repetitive Sequence-containing Plasmids and Characterization of Their Integration-mediating Activity—B1 and B2 sequences were amplified from CBA genomic DNA by PCR and fused with each other in the same orientation (named B1/2). The 3′ A-rich region that normally exists in endogenous copies was not included in B1/2. We also amplified about 710 bp of L1 sequence encompassing part of the 5′-untranslated region and ORF1, a segment of L1 reported to be present at 4 × 104 copies genome (21), and confirmed its identity and orientation by digestion with Psfl. Using these elements, we constructed a series of vectors having various combinations of the repetitive sequences (Fig. 1A). The rationale for designing a fused construct was such that if the integration rate of foreign DNA is largely determined by the frequency of collision between exogenous and endogenous DNA elements sharing high sequence homology, the fused heterodimer might be more beneficial in increasing the integration rate than either of the monomers. All the test vectors contained a PKG/geo reporter gene. We analyzed the integration frequency of these constructs by a colony assay, which measured the number of G418-resistant colonies (22, 23).

HC11 murine mammary epithelial cell line was transfected with these vectors and allowed to form stable transformants without the drug selection. A later calibration of a Rous sarcoma virus-driven luciferase vector was included as an internal standard. After 10–14 days of selection, the surviving colonies were stained with X-gal or Giemsa, and the number of colonies was counted. As shown in Fig. 1B, the number of colonies was reproducibly higher in repetitive sequence-containing vectors. Compared with the pPKG promoter, the colony number increased

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by about 1.8-fold when the βgeo sequence was flanked with a single copy of a fused B1/2 sequence and by about 3.5-fold when the βgeo sequence was entrapped within a pair of B1/2. A significant level of increase in integration frequency was also observed when one or two copies of the L1 sequence were used to represent the repetitive sequences. These results suggest that the repetitive sequences, especially the B1/2 sequence, positively influenced the integration rate of a flanking transgene. The B1 and B2 sequences used to prepare the B1/2 in this experiment were sequenced and compared with the previously reported sequences. The 1.9-kb p.neo fragment used as the probe for Southern blot analysis is indicated. B, colony assay with five kinds of vectors using HC11 cells. After 12 days of selection the surviving colonies were counted after Giemsa staining. Values shown have been corrected for the transfection efficiency of the luciferase control vector.

To determine which of the two SINE members played a dominant role in increasing the integration rates of foreign DNA, two vectors containing either monomeric B1 or B2 sequence at both sides of the βgeo reporter gene were constructed (Fig. 4A). In the newly prepared p(B1/2)βgeo vectors, the orientation of the B1 or B2 sequences relative to the reporter gene was reversed compared with that of p(B1/2)βgeo to examine whether the orientation of the repetitive sequences could affect the expression of the reporter gene and

Detection of Transgenes in 11.5-dpc Mouse Fetuses Microinjected with pβgeo and p(B1/2)βgeo Vectors—The results of colony assay represent the combined consequences of the integration and subsequent expression of the exogenous gene. However, there could be colonies that did not survive the drug selection because of inefficient expression of the Neo5 gene, even though they had the transgenes integrated in the genome. To assess the effect of B1/2 on the integration efficiency alone and to clarify whether the SINE-mediated integration and expression is maintained through the later stage of mouse development, we evaluated the integration frequency mediated by B1/2 in an in vivo system by microinjecting the B1/2-containing recombinant DNA into the pronuclei of fertilized mouse one-cell embryos.

BCF1 mouse eggs were microinjected with linearized p(B1/2)βgeo or pβgeo DNA and transferred to pseudo-pregnant ICR mice. At 11.5 dpc the fetuses were isolated, stained with X-gal, and analyzed for the presence of transgenes by PCR and Southern blot analysis. The stage of 11.5 dpc was chosen because it is one of the most critical stages in the murine development that is associated with major changes in the developmental pattern and thus the gene expression pattern (25). As shown in Table I, 4 of 25 fetuses (16%) from pβgeo were identified as transgene-positive, while 17 of 32 (53.3%) from p(B1/2)βgeo turned out to carry the transgene. Among the 17 fetuses carrying the p(B1/2)βgeo sequence, 8 stained positive for β-gal in isolated regions of the body, 2 showed an unrestricted expression pattern over the whole body, and the remaining 7 (Fig. 3) did not show any β-gal expression. In the case of the pβgeo control, two of the four fetuses expressed β-gal. Thus, the integration rates, not only in cultured HC11 cells but also in fetuses, were shown to be significantly higher in the SINE-containing vector than in the control.

Contribution of Individual Components of the Fused B1/B2 Sequence to the Increase in Integration Frequency—Although both B1 and B2 sequences belong to the mouse SINE family, they apparently do not share any evolutionary relationships. Because they are not physically associated with each other in the mouse genome, the contribution of each element to the enhancement of flanking DNA integration could be significantly different if the process is dependent on the DNA sequence rather than on the copy number of the repetitive sequence in the genome.

To estimate the copy number of transgenes genomic DNAs from each of independent G418-resistant clones transfected with the Sca1-linearized pβgeo or p(B1/2)βgeo vector were subjected to Southern blot analysis after digestion with HindIII, which cuts the vector only once. When hybridized with a 1.9-kb Neo5 sequence as the probe (Fig. 1A), about 49% (18/37) of the p(B1/2)βgeo clones turned out to carry only one or two copies of transgenes, whereas about 28% (5/18) of the pβgeo clones showed one or two copies of transgenes.
Moreover, the integration frequency of the B1/2 fusion sequence was only slightly higher than those with either of the monomeric sequences inserted in the reverse orientation. Thus, the three constructs carrying the repetitive modules seemed to be functionally equivalent in guiding the transgenes into the genome. Considering the results of Figs. 1B and 4B, it appears that the number of isolated repetitive sequence modules in a vector is a critical variable in increasing the chances for these sequences to collide with the endogenous homologs.

Identification of Homologous Recombination Events in (p(B1d)2)geo-injected Blastocyst Embryos by Hord-PCR—Because the only difference between the repetitive sequence-flanked vector and the control vector was the presence of the repetitive sequences, it is reasonable to infer that the repetitive sequence was responsible for the increase in integration rates of transgenes. One of the mechanisms underlying this increase could be the enormous chance of homologous recombination of the incoming B1 or B2 sequence of the vector with the corresponding, high copy numbered cellular homologs. To determine the nature of the integration events, we prepared a vector that can test the sequence-dependent (homologous) recombination events between the two homologs of B1 sequences. Basically, the construct was equal to p(B1)2geo except for a deletion of a part (20 bp of B1 3′-terminal region) of the repetitive sequence (p(B1d)2geo; Fig. 5A). Lehman et al. (9) reported that the examined recombinational breakpoints of Alu sequence were mostly centered in the middle region, especially between the two RNA polymerase III promoters. Therefore, the p(B1d)2geo construct was designed to retain the entire middle region of the B1 sequence, lacking the proximal part only, so that the inherent recombination activity would not be disturbed. The p(B1d)2geo vector has been evaluated in vitro and in vivo for the integration capacity before it is applied for the analysis of B1-mediated integration events. In a colony assay, the p(B1d)2geo was shown to be capable of integrating at a frequency about two times greater than that of the pgeo control vector (Fig. 5B). Also, 52% (36/69) of the blastocyst embryos microinjected with linearized p(B1d)2geo DNA were transgene-positive when analyzed by a combined PCR and Southern blot procedure (Fig. 5C). These results once again demonstrate the high integration-mediating capability of the B1d sequence. The extra bands in the PCR-positive lanes (3, 5, 9, 18, 19, 27, and 29) in Fig. 5C appear to be artifacts associated with the positivity because it was also shown in the positive control.

With the integration-enhancing ability of B1d proven, we next went on to analyzing the nature of the B1d-mediated integration events in developing embryos. For this, microinjection was performed with pgeo and p(B1d)2geo vectors, and the eggs were cultured into the blastocyst embryos. Individual embryos were analyzed by a PCR process designed to detect only the homologous recombination event (named Hord-PCR; see Fig. 6). When homologous recombination occurs, the B1d sequence near the reporter gene turns into an intact B1 sequence by acquiring from cellular B1 sequence the truncated 20-bp sequence with which the primer B1–3′ can anneal. The other primer was designed to anneal with the 5′ PGK promoter region, and thus, with this primer pair, PCR of the homologous recombination product should yield a 300-bp fragment consisting of a part of the PGK promoter and a part of the B1 sequence from homologous recombinants. The specific PCR product of the homologous recombinants was detected by Southern blot analysis using a 500-bp PGK promoter sequence as the probe. In Fig. 7, the extra bands associated with PCR positivity can be seen in lanes 12, 20, 21, 25, and 27 as well as in the positive control; only the results for 40 out of 75 samples are shown.

Of the 75 blastocyst embryos injected with linearized
p(B1d)2bgeo DNA, about 21% (15/75) was clearly shown to have undergone homologous recombination (Fig. 7). Because there were two identical B1 sequences on the vector and the Hord-PCR was applied only to a single B1 sequence, this value might represent only half of the actual recombination frequencies, assuming that both B1 sequences have a similar capability of recombination. Thus, considering the frequency of the B1-mediated foreign DNA integration events as shown in Fig. 5C, it could be reasoned that more than half of the p(B1d)2bgeo-injected transgene-positive embryos had the transgenes integrated via the homologous recombinations involving the B1 sequences.

In conclusion, these results demonstrated that repetitive sequences such as B1 and B2 could substantially improve the integration frequency of flanking foreign DNA and that such an increase occurred through an enhancement in the homologous recombination events between the incoming and resident repetitive sequences.

**DISCUSSION**

In this paper we presented evidence that the B1 and/or B2 repetitive sequences can mediate highly efficient integration of flanking genes into the genome of both differentiated HC11 cells and developing mouse embryos. In an accompanying paper the integration frequency of the SINE-flanked vector has been examined in mouse preimplantation embryos.2 In that

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2 Y.-K. Kang, J. S. Park, C.-S. Lee, Y. I. Yeom, A.-S. Chung, and K.-K. Lee, submitted for publication.
report, the B1/B2-flanked vector was microinjected in a linear or covalently closed circular form, and it showed high \( \beta \)-gal expression rates (more than three times than the control) in either conformation. Further, we demonstrated in the present work that a significant proportion of the augmented integration rate is due to the homologous recombination between the endogenous and incoming repetitive sequences.

The ability of human Alu repeats to promote homologous recombination has been investigated in several cases (27–30). In those reports, the effects of Alu sequences on the homologous recombination events were shown to be controversial; some reported to be efficient and others indistinguishable from essentially random recombination. The inconsistency might, in part, derive from the absence of an assay to detect the homologous recombination events easily and directly. The homologous recombinational processes may be occurring only rarely because of the high topological constraints exerted on the cellular genome (31). In contrast, the B1 and B2 repetitive sequences placed on free-floating transgenes are free from such constraints and thus may be easily brought into close proximity to genomic cognates by diffusion, resulting in an increased chance of recombination.

The expression frequency of a transgene, in addition to the integration rate, is considered to be one of the most important factors in transgenesis. The repetitive sequences B1 and B2 may help increase the expressivity of the transgene by guiding the integration of flanking genes into a transcriptionally favorable region in the host genome. In fact, as resolved by the chromosomal painting with the B1 or B2 sequence probe, both B1 and B2 sequences are known to be preferentially clustered in the R bands (34). Genes in R bands are known to replicate early in the S phase, and virtually all of the widely expressed housekeeping genes map to these chromosomal regions (35, 36). Because the major proportion of the transgene integration mediated by the B1 and/or B2 sequences occurred through the homologous recombination at the corresponding genomic loci according to our results (Fig. 7), it is most likely that the transgenes are located at euchromatic regions and have a high probability of being expressed properly. Therefore, the mammalian germline transformation system involving the SINE sequences has dual benefits; an increased frequency of transgenesis and an improved expressivity of the transgene. In fact, as described in our another report, the p(B1₂2/geo)-injected blastocyst embryos showed much higher levels of \( \beta \)-gal expression compared with the pgeo-injected control embryos.

It is generally accepted that the frequency of random integration into a genome is highly affected by the sequences of transgene ends (26, 37). In measuring the integration efficiency of SINE-flanked vectors by the microinjection experiment we excluded such potential bias by linearizing both control and SINE-tagged vectors with the ScaI restriction enzyme, which cuts both plasmids once within the ampicillin-resistant gene leaving the repetitive sequences positioned internally in the vectors. Therefore, the free ends of both linearized vectors should be identical in the nucleotide sequence and the frequency of illegitimate recombination is expected to be similar in both vectors.

Finally, we are currently establishing an experimental pro-
of their long life cycles and high cost. The application of transgenic technology is limited mainly because domestic animals, such as goats, sheep, and cows, where the application of transgenic technology is limited mainly because of their long life cycles and high cost.

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