MOLECULAR AND CELLULAR BIOLOGY, Jan. 2010, p. 68–77 Vol. 30, No. 1
0270-7306/10/$12.00 doi:10.1128/MCB.00913-09

Tagged Mutagenesis by Efficient Minos-Based Germ Line Transposition▽†

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Received 14 July 2009/Returned for modification 1 September 2009/Accepted 24 October 2009

Germ line gene transposition technology has been used to generate "libraries" of flies and worms carrying genomewide mutations. Phenotypic screening and DNA sequencing of such libraries provide functional information resulting from insertional events in target genes. There is also a great need to have a fast and efficient way to generate mouse mutants in vivo to model developmental defects and human diseases. Here we describe an optimized mammalian germ line transposition system active during early mouse spermatogenesis using the Minos transposon. Transposon-positive progeny carry on average more than 2 new transpositions, and 45 to 100% of the progeny carry an insertion in a gene. The optimized Minos-based system was tested in a small rapid dominant functional screen to identify mutated genes likely to cause measurable cardiovascular "disease" phenotypes in progeny/embryos. Importantly this system allows rapid screening for modifier genes.

Transposons are mobile genetic elements that form a major fraction of eukaryotic genomes (32). The vast majority of these are defective and immobile. An intact DNA transposon codes for a transposase that excises the transposon via its inverted repeats and inserts it into a new site in the genome, potentially creating a mutation. Members of the TC1/*Mariner* family represent an excellent tool for insertional mutagenesis using a simple "cut and paste" mechanism for transposition integrating into TA targets. They have several advantages over other methods. Chemical mutagenesis with *N*-ethyl-*N*-nitrosourea (ENU) to identify genes affected by point mutations is laborious, time-consuming, and very difficult in light of the many single nucleotide polymorphisms (SNPs) in genomes. Generating mutations in embryonic stem (ES) cells (with retrovirus, recombination, or transposons) is time-consuming and labor-intensive due to the requirement to generate mice from ES cells. In contrast, if transposition could be carried out efficiently in the germ line and hence only requires breeding, the transposon would provide a tag to easily identify the insertion site. The added advantage would be that there is no particular preference for sites of integration, as seen with retroviruses (2). Importantly, transposons without transposase coding capacity can be mobilized by the enzyme in *trans*. Thus, transposons can be provided with sequences to abrogate or enhance transcription to mutagenize the genome. Transposons have been used successfully for in vivo mutagenic screens in yeast (*Saccharomyces cerevisiae*), *Caenorhabditis elegans*, and flies (14, 20, 26, 33), but not mammals due to the inefficiency of transposition in the mouse germ line (6, 12). Modification has largely been restricted to transposition in embryonic stem cells (19, 23) and to tumorigenesis through continuous transposition in somatic tissues (8). Here we describe an efficient germ line-based Minos transposon system in mammals suited to generate a "library" of mutant animals and a subsequent small screen for cardiovascular phenotypes. The system was engineered in C57BL6 mice, the strain that has traditionally been used most for mouse phenotypic studies.

MATERIALS AND METHODS

Optimization of codon usage and synthesis of a new "mammalianized" Minos transposase gene. We calculated a relative frequency of each synonymous codon and compared it to data deposited for flies and mice in the codon usage database at http://www.kazusa.or.jp/codon/. The three stop codons were not taken into account, because they appear only once in a coding sequence, nor did we consider the ATG and TGG codons, which encode methionine and tryptophan and are not degenerated. We designed a new transposase DNA sequence in order to incorporate codons, which are more frequently used in mammals, especially mice. The new "mammalianized" transposase gene (~1 kb) was constructed by ligation of 3 DNA fragments, namely linkers A, B, and C, as schematically represented in Fig. 1, top. Each of the 3 linkers was made from 4 to 6 oligonucleotides. Linker A also included the Kozak sequence. The following oligonucleotides were used: for linker A, A1 (5′-CCCCGACGTCCCACCATG GTGCGCGGTAAGCCTATCTCTAAGGAGATCAGAGTACTGATCAGGGA CTATTTTAAGTCTGGGAAGACACTC-3′), A2 (5′-CCCATTTTTCTTGAA AATCTGTATCACCCCATGCACAGAGCTCTTAGGCAAGTTTAACTGCT TGCTTATCTCAGTGAGTGTCTTCCCAGA-3′), A3 (5′-TTCAAGAAAAAT GGGAACATTGAGAATAACATCGCGAATAGAGGCCGAACATCCGCAA TAACCCCCCGCGACAAGAGACAGCTGGCCAAAATTGT-3′), and A4 (5′-GGGGACGAGGCCAATTGTCTGCGACCACTTGGAAGCCAAGTTTCTC AGGGATTGGCGGCGGTCAGCCTTCACAATTTTGGCCAGC-3′); for linker B, B1 (5′-GGGGCAATTGGCAAGACTGTCAAGCGGGAGTGGACCCGG CAGCAATTAAAGAGTATTGGCTACGGTTTTTATAAGTCCAAGGAAA AACCCCTGCTTAC-3′), B2 (5′-TGAAGATGATGGTATCCCACTGCCTTT GAGTCCAAGACATCCTTTCCCGAGCCCATTGCAGACGCTTCTTTTTTT TGCCGAAGCGTAAGCAGGGGTTT-3′), B3 (5′-ATACCATCATCTTCAG CGATGAGGCTAAATTTGATGTGAGTGTCGGCGACACGAGAAAACG CGTCATCCGTAAGAGGTCCGAGACATACCATAAGGA-3′), and B4 (5′-CCCCATGCATCCCCAAGCTTTCCGAGTCCTTTGGCAGACATACATCC CCATACCATAGTGCTTGCAGGAAACTTGGTTGTTCTTTTCAGGCAGT

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† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 2 November 2009.

CCTTATGGTATGTC-3′); and for linker C, C1 (5′-CCCCAAGCTTCACTTC ATCGAAGGGACCGTTAATGCGAAAAATACATTAACATTCTCCAGGA TAGTTTGCTGCCCTCAATACCAA-3′), C2 (5′-TTTGGTCCGCTTGGCGG TGTGCGATGATGCTCCGTCCTGCTGAAAAGTGAATTCACCACAATC GGATAGTTTTGGTATTGAGGGCA-3′), C3 (5′-GCCAAGCGGACCAAAA ACTGGCTGCAGTACAATCAGATGGAGGTGCTCGATTGGCCCTCAAA TAGTCCGGATCTAAGCCCAATCGAA-3′), C4 (5′-GCTTGATTTTCAAGT CGGAAATGTTCCTCTGTGGCTCGTTTCGCAGCTGGTTTTTCATTAGC CACCAGATATTTTCGATTGGGCTTAG-3′), C5 (5′-ACTTGAAAATCAA GCTGCAAGAGATGTGGGACTCAATCTCTCAGGAGCACTGCAAAAA CCTGCTCAGCAGCATGCCTAAACGAGTGA-3′), and C6 (5′-GGGGATG CATCCTCTAGAGGGGCCCCGGATCCTCAGAACTGTGTAACGTCGCC CTTGGCCTGCATCACGCATTTCACTCGTTTAGGCA-3′).

Basically, 20 pmol of each oligonucleotide was phosphorylated in a buffer containing 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 20 mM ATP for 30 min at 37°C. The appropriate oligonucleotide mix was annealed (3 min at 85°C and allowed to cool down to room temperature). The gaps were filled in by Klenow polymerase, using 50 μM deoxynucleoside triphosphates (dNTPs) and 1 U of Klenow fragment (Roche Diagnostics GmbH, Indianapolis, IN), for 30 min at 37°C. After heat inactivation of the enzyme at 75°C for 20 min, nicks were ligated overnight at room temperature by adding 1 U T4 DNA ligase (Promega, Madison, WI). After heat inactivation of the ligase at 65°C for 10 min, each linker was amplified. One microliter from the appropriate ligation mix was used as a template in a 50-μl PCR. Linkers were amplified (15 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) using the following primers: Linker-A-s (5′-CCCCGACGTCCCACCATGGT-3′) in combination with Linker-A-as (5′-GGGGACTAGTCCAATTGTCT-3′), Linker-B-s (5′-GGGGC AATTGGCAAGACTGT-3′) in combination with Linker-B-as (5′-CCCCATG CATAAGCTTTCCG-3′) and Linker-C-s (5′-CCCCAAGCTTCACTTCATCG-3′) in combination with Linker-C-as (5′-GGGGATGCATCCTCTAGAGG-3′). Obtained PCR products were gel purified, subcloned into pGEM-T Easy (Promega), and sequenced by BaseClear (Leiden, The Netherlands).

The final transposase gene was obtained by cloning Linker B as an MfeI/HindIII fragment into pGEM-T Easy plasmid already containing Linker A and subsequent cloning of Linker C as a HindIII/NsiI fragment, resulting in the pGEM-MM vector.

Comparative analysis of transposition efficiency in ES cells. In order to compare the activity of the modified transposase genes with the one previously used (6, 35), we cloned the mammalianized-Minos (MM) transposase into the same pJG-3 vector as the fragment represented by the AatII blunt site plus XbaI blunt site (hereafter, AatII blunt/XbaI blunt), resulting in plasmid pJG3-MM. A total of 5×10^6 ES (Tdw129/B6) cells were cotransfected with 10 μg of pMiLRNeo plasmid DNA (35) and 10 μg of either pJGD/ILMi (35) or pJG-MM plasmid DNA. Ten micrograms of pMiLRNeo alone was used as a transfection control. Cells were plated at densities of 2.5×10^5, 1×10^6, and 3.75×10^6 per 10-cm dish, and integrants were selected on G418 (0.5 μg/ml). Colonies were counted after staining with methylene blue.

Amplification of 5′ and 3′ H1t homology and construction of a targeting construct. Genomic DNA from 129 ES cells was used to amplify the H1t flanking regions.

The 5′ H1t flanking region was amplified (35 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 3 min), using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies). The following set of primers was used: 5H1T-S (5′-GGGG ATCCGGCGCCGAGCTCCGGCAGTAAAGGAC-3′) and 5H1T-as (5′-GGG ATATCGGTCAAGAGCTGGACAAGAACTACACC-3′).

The 3′ H1t flanking region was amplified (3 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 4 min, following 30 cycles of 94°C for 1 min, 63°C for 1 min, and 68°C for 4 min) using the primers 3H1T-s (5′-GGTCTAGAGGGAAGGC AAAGATGGTCAT-3′) and 3H1T-as (5′-GGTCTAGACTTAAACCCGCATC GAGAC-3′). Both flanks were cloned into the pGEM-T Easy vector (Promega) and sequenced.

The 3′ H1t flanking region was subcloned as an EcoRI blunt fragment into the XbaI blunt fragment of the ppolyIIIi-LoxP vector (13), resulting in the ppoly IIIi-loxP3′H1t plasmid. The 5′ H1t flanking region was cloned into ppoly-LoxP vector as a BamHI/EcoRV fragment. Note that both restriction sites were included in primers used for the amplification. Into the HindIII blunt site of the resulting plasmid ppolyIIIi 5′H1tloxP, the mammalianized Minos (MM) transposase was cloned as an AatI blunt/XbaI blunt fragment from the pGEM-T vector (Promega).

5′H1t-LoxP-MM was subcloned as a BamHI blunt fragment into a ClaI/blunt FPPF.6 plasmid containing pgkpuro-p(A) flanked by FLP recombination target (FRT) sites (gift from M. Jaegle). LoxP3′H1t was subsequently cloned as an EcoRV blunt/NotI blunt fragment into the XhoI blunt site, resulting in the H1t-MM targeting vector.

Homologous recombination in ES cells and generation of the H1t-MM knock-in mouse line. The H1t-MM vector was linearized with SfiI and the DNA electroporated into IB10 ES cells. Genomic DNA from 175 neomycin-resistant clones was EcoRI digested and analyzed by Southern blot hybridization for the homologous recombination event. The 621-bp H1t 5′ probe, lying outside of the H1t-MM construct, was used. The probe was amplified (35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), using the primers Screen-1 (5′-GCC TGCCTAATGTGGGAATAG-3′) and Screen-2 (5′-ATCCAGCACTGATGA GTGGTA-3′).

Clone MM403 was injected into C57BL/6J blastocysts to generate chimeric mice. Male chimeras gave germ line transmission. Mice were further crossed to the FLPeR transgenic line (11). H1t-MM progeny, without the puromycin gene, were diagnosed by PCR (35 cycles of 94°C for1 min, 58°C for1 min, and 72°C for 1 min) using the primers MM159-s (5′-GAATAACATCGCGAATAGAGGC-3′) and Hit screen-as (5′-AACTCACTTCCCTGCTG-3′).

Generation of H1 t transposase transgenic mice. The mammalianized transposase gene flanked by FRT sites was inserted by homologous recombination (17) into the H1t locus of Bac clone RP23-283N14. A large ~100-kb transgenic construct was injected into C57BL/6 oocytes, and progeny were screened for insertion of the transgene by Southern blot analysis.

Histology and immunohistochemistry. Testes from both wild-type (wt) and H1tMM transgenic animals were fixed for 6 h in buffered 3.7% formaldehyde, treated with 70% alcohol, and embedded in paraffin. Ten-micrometer sections went through the following steps: xylene (2×4 min), 100% ethanol (4×2 min) and 20 min in 3% H_2O_2 in methanol. After being washed with water, they were treated for 20 min in 0.01 M citric acid, pH 6.0, in a microwave (700 W). After cooling and washing in distilled water, slides were washed 3×10 min in phosphate-buffered saline (PBS) and blocked for 20 min in a mixture of PBS, 0.5% bovine serum albumin (BSA), and 0.5% milk powder.

Rabbit polyclonal antitransposase serum at 1:300 (gift from Maria Kapetanaki) or rabbit anti-TCLFL5 antibody (31)1:100 was applied to consecutive sections for 2 h at room temperature. As a secondary step, goat anti-rabbit antibody coupled to biotin (1:200 dilution) in 2% normal goat serum was used followed by ABC Vectastain reagent at 1:200 for 30 min. 3,3′-diaminobenzidine (DAB; Vector Laboratories, Inc., Burlingame, CA) was used as a peroxidase substrate and hematoxylin as a counterstain.

Construction of the transposons. The single-trap transposon construct was made by replacing the internal ribosome entry site (IRES)-LacZ in pGT1.8Iresβgeo (27) with IRES-enhanced green fluorescent protein (EGFP). The XbaI fragment, containing IRES-LacZ, was removed from the pGT1.8Iresβgeo plasmid, and the remaining vector sequences were religated, resulting in En2-SA-SvpA vector. IRES-EGFP was subcloned into the XbaI blunt site of En2-SA-SVpA, resulting in the vector En2-SA-IRES-EGFP-p(A).

The left inverted repeat of Minos (MiL) was subcloned as a KpnI/PstI fragment and the right inverted repeat of Minos (MiR) was subcloned as a NotI/SacI fragment into modified pUC18 vector (where the regular polylinker was replaced with a linker containing restriction sites in the order PmeI, KpnI, PstI, EcoRI, SalI, NotI, SacI, PmeI), resulting in the pMiLR vector. The trap was subcloned as a SalI fragment in pMiLR, resulting in the transposon vector pMi-En2-SA-IRES-EGFP-pA (MESE). A double-trap construct was made by subcloning the IVS-IRES-enhanced yellow fluorescent protein (EYFP)-poly(A) BglII/XhoI fragment from pIRES-EYFP (Clontech) into BamHI/XhoI of the modified pUC18 vector (where the linker was cloned into a HindIII/ERI site introducing restriction sites in the order SalI, DraI, BglII, XhoI, BamHI, DraI, SalI) and subsequent cloning of the En2-SA-IRES-EGFP BamHI fragment into a BglII site. A SalI fragment containing the double-sided trap was cloned into the SalI site of pMiLR, resulting in a final double-transposon-trap (DTT) construct, pMiLR-DTT. A double trap with stop codons in all 3 frames was constructed by first inserting a linker containing a triple stop at the BglII/XbaI site of the En2-SA-SvpA vector and inserting another triple-stop-containing linker at ERI/BglII sites of pSCT vector, introducing 3 stops between the splice acceptor site of intron and poly(A). The pMiLR vector was modified by inserting a linker at PstI/NotI sites, containing restriction sites in the following order: PstI, BamHI, FseI, SwaI, AscI, AvrII, NcoI, Not. IVS-SA-3Xstop-p(A) from pSCT was cloned into this modified vector as an NcoI/AvrII fragment followed by cloning of the other IVS-SA-3Xstop-p(A) BamHI fragment from En2-SA-SvpA in the BamHI site, resulting in the final DTT-3Xstop vector.

Transposon-containing mice. Transposon-containing lines (STT A and B) were generated by injecting a 4.6-kb PmeI fragment from the transposon trap vector pMiEn2-SA-IRES-EGFP-p(A) into FVB/N fertilized oocytes. The DTT line was generated by injecting a 5.5-kb PmeI fragment from pMiLR-DT vector.

DTT-3Xstop lines were generated by injecting a 2.9-kb PmeI fragment from the DTT-3Xstop vector. Transgenic founders were identified by Southern blotting of tail DNA using a 700-bp EGFP fragment as a probe or a 485-bp NcoI/AvrII fragment in the case of the DTT-3Xstop lines.

Breeding scheme. H1t-MM knock-in or transgenic mice were crossed with a transposon trap containing transgenic mice. Double-positive transgenic males were bred with C57BL/6 females, and transposon-positive offspring were analyzed for the presence of transposition events. EcoRV-digested tail DNA was analyzed by Southern blotting using the trap-specific probes mentioned above.

Genotyping and detection of the truncated message in embryos containing the HirA insertion. Breeding was set between heterozygous mice for HirA gene insertion. Embryos were dissected at day 10.5. Yolk sacs were used for DNA isolation and genotyping, while embryos were scored for anomalies and used for RNA isolation. The primers used for genotyping were HirA fw (5′-CCTGGGC TCTAGTTATGGTGGCATACA-3′) and HirA rev (5′-CTTCCTTTATCTAC AGTGCTTCCTCCTC-3′) in combination with Int2 RB fw (5′-GAGGAGACA ATGGTTGTCAACAGAG-3′) or En2-Int (5′-GGCAGATGTAGCTAAAAG GCCTATC-3′), resulting in a product of 550 bp or 650 bp, respectively, in the case of transposon insertion and 464 bp in the case of a wt HirA allele. The primer set used for reverse transcription-PCR consisted of HirA exon I fw (5′-CTCTTGAAGCCAACCTGGGTCAACC-3′), HirA exon 3 rvB (5′-CGTC ATCCTCCTGGAGGACTGGAG-3′), and RB globin rev (5′-TGCCAAAATG ATGAGACAGCACA-3′), resulting in a 155-bp product in the wt situation and a 90-bp product in the transposon-mutated mouse.

Determination of transposition sites in the progeny of Trap/H1t transposase mice. Genomic DNA (0.2 μg) was used as a template in 3 steps of the thermal asymmetric interlaced PCR (TAIL PCR) procedure described by Matsuoka et al. (25). PCR products were gel purified, and direct sequencing of both strands was performed using Big Dye terminator chemistry version 3.1 (Applied Biosystems) as recommended by the manufacturer. Fragments were loaded on an ABI3100 genetic analyzer, and sequences were analyzed by DNA Sequencing Analysis (version 3.7) (Applied Biosystems).

All obtained transposon-flanking sequences were blasted against Ensembl mouse genome database and/or NCBI database to determine the chromosome positions of the insertion sites and to identify any insertion nearby or into the genes.

Quantitative RT-PCR for Cpvl gene transcript. The peritoneal cavity of the wt, DTT founder, and carboxypeptidase vitellogenic-like ($Cpvl^{+/-}$) mice was flushed with PBS. Cells were spun down, and total RNA was isolated using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc.) according to the manufacturer's instructions. cDNA was prepared using oligo(dT), and RT-PCR was performed using the Cpvl-specific primers CPVL10F (5′-TGCGAGAGGA CACAGTGAAG-3′) and CPVL11R (5′-CTGATGGAATTTGCCCACACG-3′) and murine hypoxanthine phosphoribosyltransferase (HRPT)-specific primers (6). The quantitative PCR was performed on a Bio-Rad C1000 Thermal cycler using the following program: 95°C for 4 min; 95°C for 30 s, 55°C for 25 s, 72°C for 30 min 30 times; and 60°C for 1 min and 65°C for 5 min.

RESULTS

Optimization of transposition. We optimized the Minos transposon system by taking into account the timing of transposition and the number of transposon targets, so as to provide maximum transposition activity before the genome is tightly condensed during late spermatogenesis. To this end, a Minos transposase gene with mammalian codon usage and Kozak sequence for optimal translation (21) was synthesized (Fig. 1). This transposase is 5-fold more active in a cell based transposition assay than the parent transposase (35) (Fig. 1). The reconstructed transposase gene was inserted into mice by recombination into the H1t gene locus in 129SV/C57BL/6J ES cells (Fig. 2A) or by oocyte injection of an H1t-driven transposase transgene (Fig. 2B), with indistinguishable outcomes. H1t is a histone variant unique to pachytene primary spermatocytes (13) and is already expressed at premeiotic stages (7) when the genome should be readily accessible to the transposase. H1t-targeted mice are fertile and undergo normal spermiogenesis (10, 22). Immunocytochemistry shows that the H1t-driven transposase is expressed exclusively in the testis (Fig. 2 [with all other tissues tested negative]) It is present at early stages of spermatogenesis (pachytene spermatocytes), but not in elongated spermatids found near the lumen of the seminiferous tubule comparable to the expression pattern of Tcfl5 found in primary spermatocyte and round spermatids (31) (Fig. 2).

The two transgenic C57BL/6J lines also express the transposase exclusively in pachytene spermatocytes (Fig. 2B [with other tissues negative]), implying that all regulatory elements necessary for sperm-specific expression were present in the ~100-kb transgenic construct. LoxP or FRT sites flanked the transposase cassette to remove the transposase gene when breeding the transposing males to females expressing either cis-acting replication element (CRE) or Flp recombinase, respectively, in the egg and thus preventing further transpositions postfertilization.

We generated a number of mouse lines carrying different transposons, either as a single transposon or as a single array of multiple transposons (Fig. 3). All of these heterozygous animals and those discussed below were normal. The transposon-bearing animals were bred with the transposase-expressing mice to obtain double-transgenic males that undergo transposition in the male germ line during early spermatogenesis.

No transposition occurred in offspring of the line carrying a single transposon in a limited breeding experiment (30 offspring) as opposed to mice carrying arrays, suggesting that transposition efficiency is transposon copy number dependent. This was confirmed by showing that new transpositions present in the first offspring of mice carrying an array could not be retransposed on further breeding with transposase-expressing animals.

Two single-transposon trap (STT) lines were used for further experiments: line STT-A contained ~5 copies of STT, and line STT-B contained around 40 copies (data not shown). Fluorescent in situ hybridization (FISH) analysis showed that the array integrated on chromosome 5 in line SST-A and on chromosome 4 in line SST-B (Fig. 3B).

Double-transgenic males (heterozygous for arrays and transposase) were bred, and offspring carrying transposons (as judged by PCR analysis of tail DNA) were analyzed for transposition events by Southern blots. The number of novel restriction fragments provides a measure of the number of transpositions. While the frequency of transposition events in line STT-A was low, each transposon-positive offspring of line STT-B has at least one but generally multiple bands, each representing a new transposition event (Fig. 3 and see the supplemental material).

FISH analysis revealed that inter- and intrachromosomal transpositions occur (see the example in Fig. 3C), but it lacks the resolution to score transpositions near or in the parental transposon array (see below). Importantly we did not observe any chromosomal deletions in our FISH analyses.

Transpositions in progeny from line B were therefore analyzed by DNA sequencing to determine the frequency of transposition into genes and hence suitability of the system for whole genome screening purposes. Thermal asymmetric interlaced (TAIL) PCR (25) and sequence analysis of insertion sites in the offspring showed on average 2 to 3 transpositions per animal. A total of 101 insertion sites from 46 progeny were

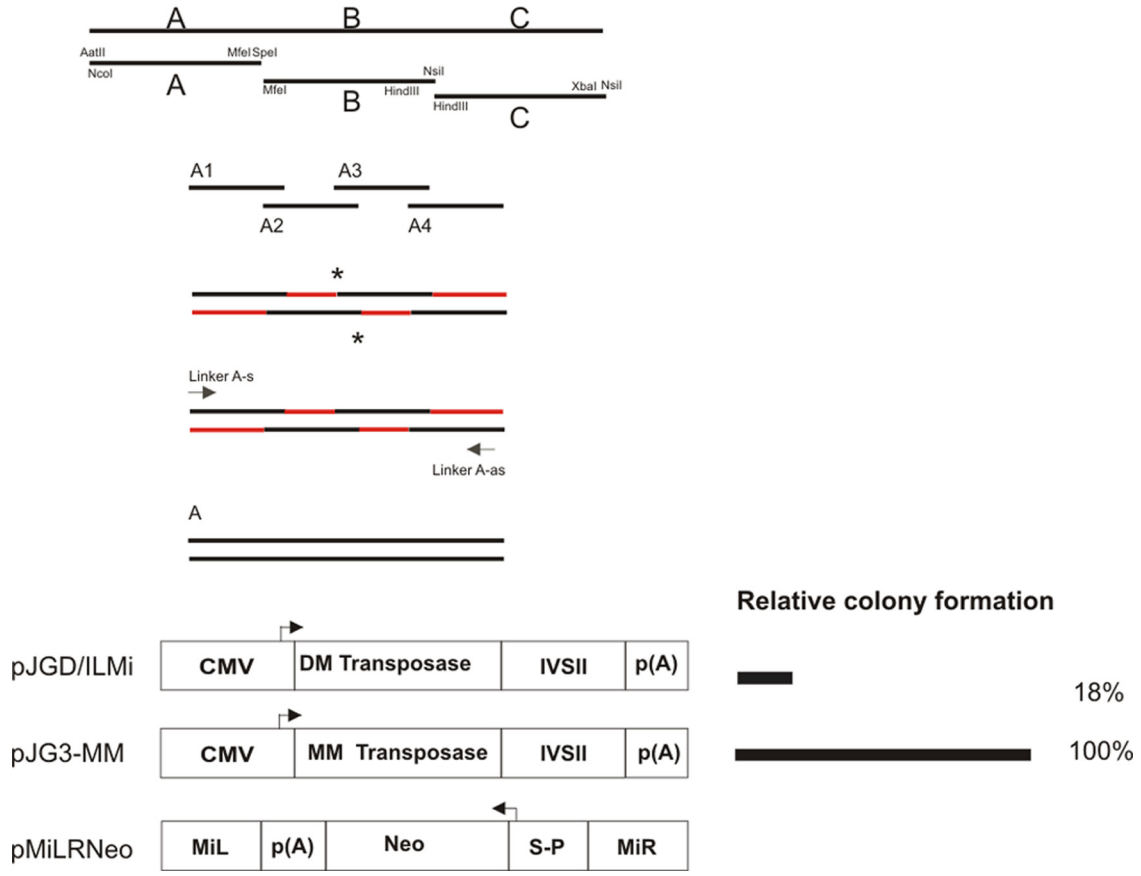

FIG. 1. (Top panel) Synthesis of the MM transposase gene. The complete synthesis of the gene results from the ligation of three DNA fragments (A, B, and C). Each linker is made of 4 to 6 overlapping oligonucleotides. After annealing, gaps are filled in using Klenow fragment (shown in red), nicks are ligated (depicted with asterisks), and the linker (here depicted as A) is subsequently amplified by the outer 5′ oligonucleotides of each DNA strand. (Bottom panel) Transposition assay in ES cells. Each transposase expression vector was cotransfected with the pMiLR Neo transposon vector, and G418-positive colonies were counted after 5 days of selection. Black bars represent an average frequency of colony formation for each pair of constructs from data collected in two independent experiments done in triplicates. CMV, cytomegalovirus.

characterized by sequence analysis (see the supplemental material), not including insertions back into the transposon array or into repetitive sequences that could not be assigned to any particular chromosomal location. New inter- and intrachromosomal transpositions were present in every transposon-positive offspring or embryo. Twenty-one transpositions were into genes. Forty-one transpositions were into unique sequences in one of 15 different chromosomes, of which 8 transpositions were into a gene (see the supplemental material). Sixty transpositions, including 13 into genes, were into unique sequences spread over 50 Mb on parental chromosome 4. We confirmed these numbers by a limited analysis of the lower-copy line A offspring. Out of 24 insertions (excluding transpositions back into the array) from 18 progeny, 11 were into unique sequences on 8 different chromosomes, including 5 into a gene. Thirteen were into unique sequences spread over 90 Mb on the parental chromosome, including two into a gene (supplemental material). Live-born litter sizes from the double-transgenic males were somewhat smaller (5 offspring/litter) when compared to halfway during gestation (6.6 embryos/mother) or the transposase-only-expressing parental line (7.3 offspring/litter). The latter was similar to the litter size obtained from the nontransposing single-copy transposon/transposase line (7.1 offspring/litter). This suggests that transpositions did not affect the number of fertilizations, but that the novel mutations are leading to some late developmental defects. Only 3 of 25 insertions were in a sense orientation relative to the gene, showing a clear bias toward the antisense orientation of the trap in introns for which we presently have no real explanation. We therefore generated a bidirectional gene trap (a double-transposon trap, or DTT) (Fig. 3A) where, regardless of the orientation, the gene will be trapped and one of the two marker genes (EGFP or EYFP) will be expressed, providing the possibility to follow the spatial and temporal expression of the mutated genes. We generated a new DTT mouse line with the bidirectional trap array containing over 10 copies of the transposon mapped to chromosome 2 by FISH analysis and confirmed to be at 80906987 (ENSEMBL version 43) by sequence analysis of the TAIL PCR product (data not shown). The results regarding transpositions were comparable to those described above. There are 1 to 6 insertions per animal/embryo, with an average of almost 3 per animal (75 insertions from 29 mice/embryos). Eighty-five percent of the jumps were on the same parental chromosome 2 (supplemental material). Thirty-one insertions were into a gene. Surprisingly, we found in one mouse two different hits in the carboxypeptidase vitellogenic-like (Cpvl)

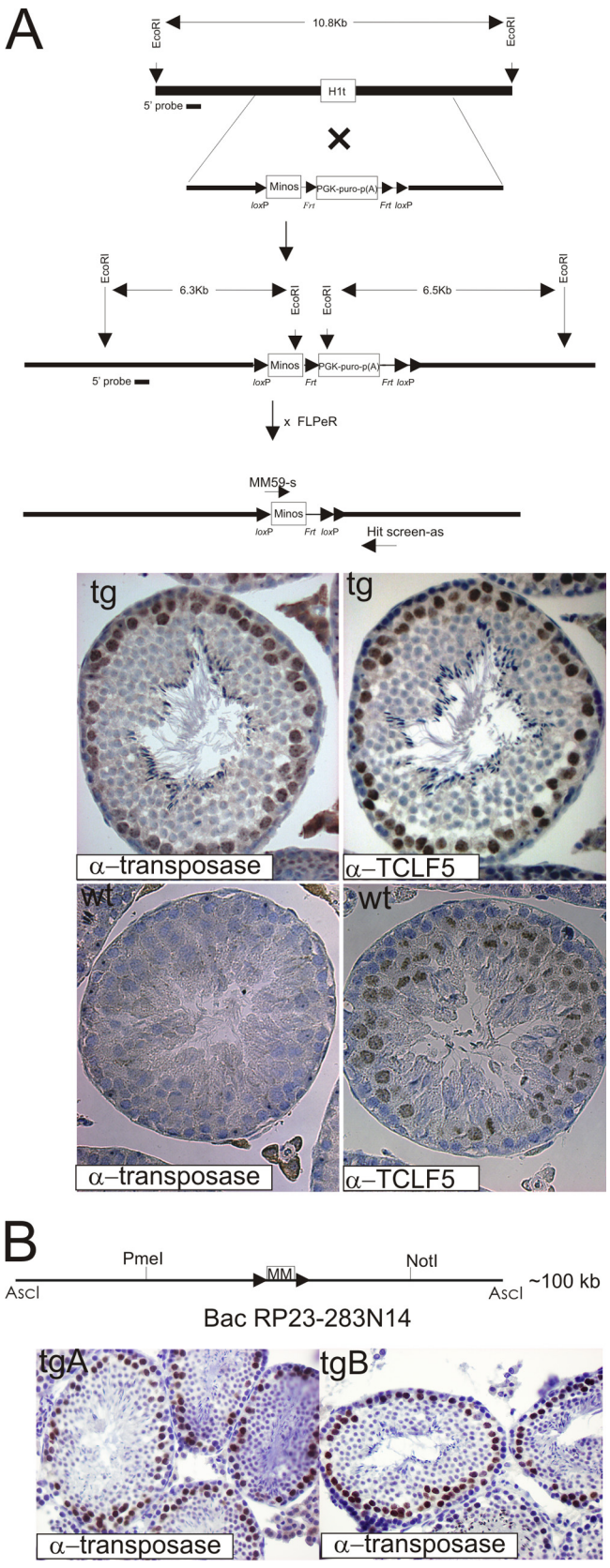

FIG. 2. Targeting and expression analysis of the Minos transposase. (A) Homologous recombination between the wt H1t locus and targeting vector H1t-MM below is shown. EcoRI-digested DNA, probed with a 5′ probe, gives a wt band of 10.8 kb and a targeted band of 6.3 kb. The puromycin cassette is removed after crossing with FLPeR mouse line (11). Immunohistochemical analyses of seminiferous tubule cross-sections from adult H1t-MM knock-in and wt mice. Consecutive sections from the genetically modified mouse (tg) and wild-type mouse are stained with antitransposase (α-transposase) and anti-TCFL5 (α-TCFL5) antibody as a marker of primary spermatocytes at the pachytene stage. (B) Transgenic construct containing Minos transposase and analysis of two transgenic lines. Arrows surrounding Minos transposase are FRT sites.

gene on chromosome 6 (supplemental material). However, we did not detect any EYFP expression in macrophages where Cpvl is specifically expressed (24). Out of 95 progeny generated by breeding heterozygous Cpvl-trapped mice, no Cpvl$^{-/-}$ mice were born, suggesting that Cpvl knockout might be embryonic lethal. The quantitative RT-PCR on cDNA obtained from intraperitoneal cells (material reached in macrophages) with the Cpvl-specific primers and HPRT primers for standardization, shows that Cpvl heterozygous mice have half of the amount of transcript that is detectable in the wt mouse or DTT founder mouse (Fig. 4). We also did not detect marker expression in late spermatocyte development in mice with the trapped Fsip2 gene, because the trap was inserted into 11th exon of the transcript that is not translated. We next constructed a new generation of transposons in which the splice acceptor side is followed by stops in each frame in both orientations (see the supplemental material), but leaving out the sequences coding for the fluorescent marker proteins. Two of these DTT-3Xstop parental lines with a transposon array on chromosome 7 were analyzed in detail (see the supplemental material and Fig. 3). Line DTT-3Xstop A (containing ~15 copies) has ~3 insertions per mouse. Seven out of 19 were in a gene, and half of these on a different chromosome. Line DTT-3Xstop L (containing ~50 copies) had over 2.5 insertions per mouse: 12 out of 33 insertions were in a gene, and 30 out of 33 were on a different chromosome (Table 1). One of the insertions was in HirA (supplementary material), a gene reported to be essential for embryogenesis. Homozygous mutants die by embryonic day 10.5 (E10.5), showing growth retardation and abnormal patterning in various degrees in most tissues (30). We observed the identical phenotype when breeding the HirA trap in the first intron to homozygosity (Fig. 5), and based on the detection of truncated message and phenotypes we conclude that the trap is functional.

Screen for cardiovascular defects. We concluded that the frequency of gene insertion using the optimized Minos transposon system is sufficient to carry out a small functional screen as a proof of principle experiment. Since a substantial proportion of diseases in the human population are heterozygous in origin or are caused by quantitative changes in levels of particular proteins, we based our screening strategy on immediate screening of the offspring for a phenotype in a heterozygous mouse. Complex breeding strategies are not required. A small DTT transposon screen was set up to score for cardiovascular defects by nuclear magnetic resonance (NMR) on day 15.5 of gestation; 120 embryos (33 wt and 87 transposon positive) were collected, coded, and phenotypically scored, with the observer blinded to the genotype. DNA was prepared from yolk sacs to

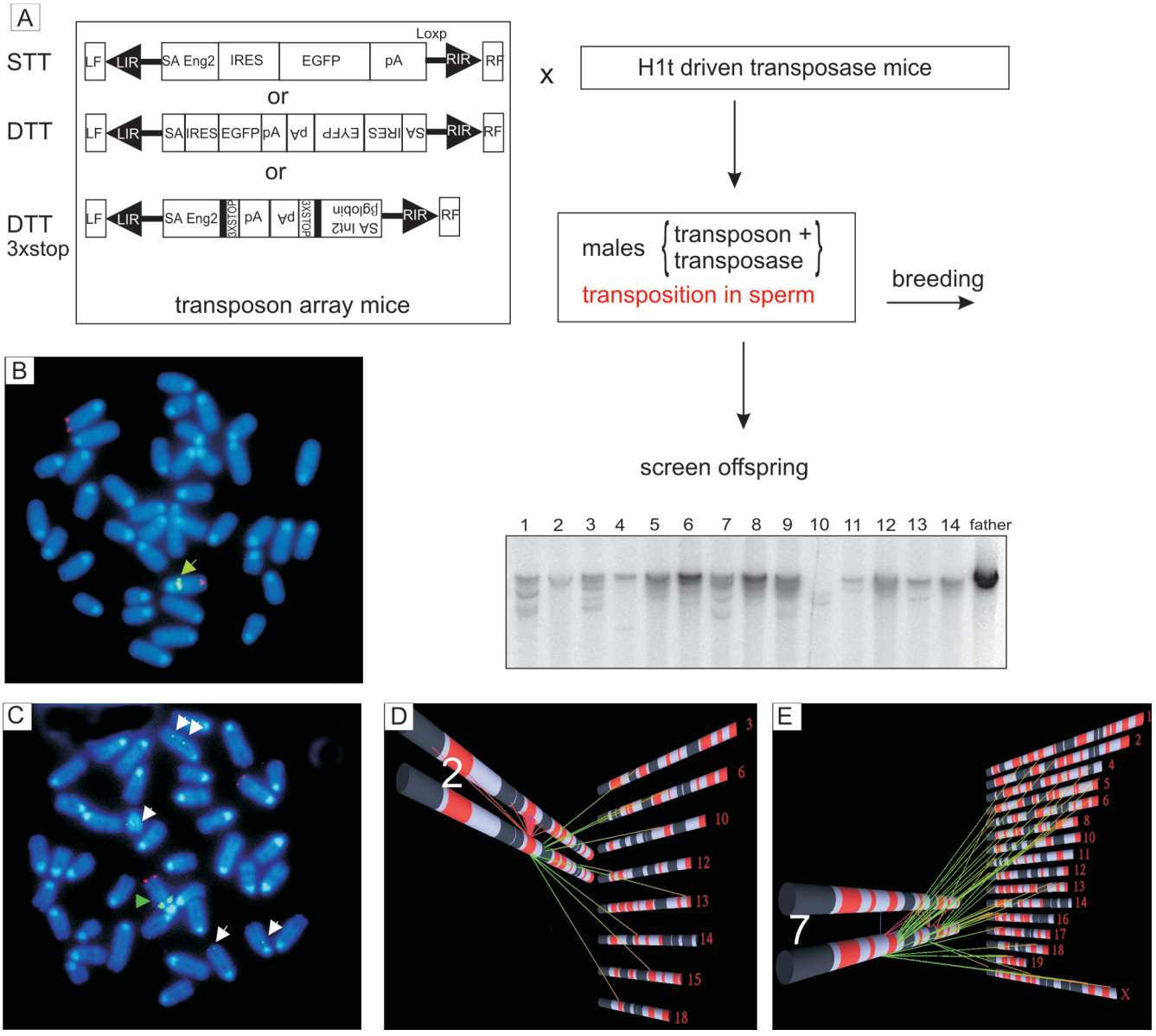

FIG. 3. (A) Breeding scheme to generate transpositions. Three different transposon constructs were used: the single-transposon trap (STT), double-transposon trap (DTT), and double-transposon trap with 3 stops (DTT-3Xstop). SA, splice acceptor site of the engrailed 2 gene or rabbit beta-globin gene; IRES internal ribosome start site; EGFP, humanized enhanced green fluorescent protein coding region; EYFP, humanized enhanced yellow fluorescent protein; pA, polyadenylation site, LIR and RIR, left and right inverted repeats of Minos transposon; RF and LF, right and left flanks—unique *Drosophila* sequences of <100 bp which are not part of the transposon. At the end of the chain of events shown in panel A is a blot showing transpositions. Each band represents a novel transposition event. (B) FISH of the starter line STT-B; (C) FISH analyses of one of its offspring. The transposon probe is in green, and the chromosome 4-specific probe is in red. Green arrows, parental array of transposons; white arrows, novel transpositions. (D) Three-dimensional (3D) viewer illustrating transpositions from the starter line DTT. Intrachromosomal jumps are shown by red lines, and interchromosomal jumps are shown by green lines. Blue line, integration site of the transposon array on chromosome 2. (E) 3D viewer illustrating transpositions from starter line DTT-3Xstop L. Line L shows preferentially interchromosomal jumps (green lines).

determine the sequence of the insertion sites. From each of the selected embryos, 1 to 6 different integration site sequences were obtained. Twenty out of 87 transposon-positive embryos showed some abnormalities such as edema, narrow aortic arch, ventricular septal defect (VSD), bilateral cysts in jugular lymphatic sacs, dextrocardia, and other abnormalities not directly related to the cardiovascular system, such as double renal pelvis, small eyes, enlarged thymus, etc. None of those anomalies was seen in 33 wt embryos or DTT founder animals. Three different embryos with 3 insertions each had a strikingly similar cardiovascular phenotype (E15.5 to 26, 97, and 99) (supplemental material). Each had insertions in *Nckap1*. The other insertions were unrelated. Severe edema was a common factor for all 3; 2 also had enlarged jugular lymphatic sacs, while one

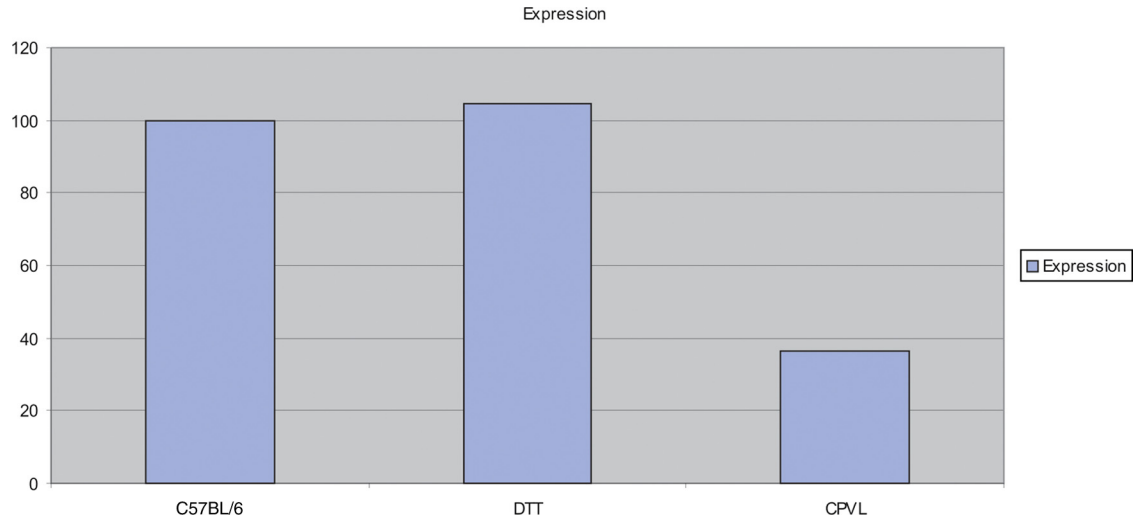

FIG. 4. Quantitative RT-PCR, performed with CPVL-specific primers on cDNA obtained from intraperitoneal cells of a wt mouse, DTT founder mouse, and the mouse heterozygous for "jump" in the CPVL gene, which originated from the DTT founder. HPRT-specific primers were used for standardization.

had a VSD (Fig. 6). *Nckap1* was previously reported to cause embryonic/perinatal lethality by affecting the cardiovascular, digestive, and nervous system in homozygous knockout mice (29), but in contrast to our results, not in heterozygous mice. However, that analysis was in C3HeB/FeJ mice while our analysis was in C57BL/6J mice, known to be prone to metabolic and cardiovascular disease.

The *Pde1a* (phosphoesterase 1A, calmodulin dependent) gene was targeted in two different embryos (E15.5 to 89 and 118) (supplemental material) with a very similar phenotype (growth retardation, edema, and VSD). The Pde1a protein is also known to be important for cardiac muscle contraction and vascular relaxation (18).

Since the NMR was performed at gestation day 15.5 on fixed animals, the interupted transcripts could not be analyzed nor could the phenotype be transmitted for further analysis.

DISCUSSION

Early sperm-specific expression of modified "mammalianized" Minos transposase in combination with high-copy-number transposon starter lines leads to high-frequency transposition. It can potentially be applied to any animal system (e.g., the rat—using transgenesis with an H1t-transposase), irrespective of genetic background. Insertions are easily identified by sequence analysis, and stable lines for a given mutation are easily generated due to transposase gene excision postfertilization. The process of generating progeny is fast, with essentially every one in two animals carrying a new intragenic mutation (Table 1). Although we do not see large chromosomal defects, we cannot exclude that there may be some background mutations. However, we do not consider this to be a problem as such mutations would automatically fall out of screen when positive animals are bred further for a detailed phenotypic analysis.

Different transposon systems show different levels of efficiency in the male germ line. The Sleeping Beauty (SB) system as used, with continuous ubiquitous expression of the SB transposase and SB arrays (9, 12, 15, 16), is less efficient with respect to the number of transpositions, ranging from transposition in 20% (12) of offspring to two transpositions per offspring (9). SB also transposes most often back into the parental chromosome. Recent work (3, 8) using an improved SB transposase and large transposon arrays also shows high efficiency of transposition in somatic tissues. The results with the piggyBac transposon (5) and a similar use of double-transgenic males (transposase plus transposon) are difficult to interpret. A 67% efficiency of transposition is reported, but only 3 out of 19 germ line transposi-

TABLE 1. Summary of the progeny characteristics

Transposon	Copy no.	Donor side	% Transposon-positive progeny	% Transposition-positive progeny among transposon-positive progeny	No. of progeny analyzed	No. of new insertions	Avg no. of new insertions/ progeny	% Interchromosomal insertions	No. of insertions into gene	Avg no. of gene hits/ animal
STT-A	5	Chromosome 5	50	25	18[a]	24	1.3	33	7	0.39
STT-B	40	Chromosome 4	>50	100	46	101	2.2	40.5	21	0.45
DTT	10	Chromosome 2	>50	100	29	75	2.6	15	31	1.06
DTT-3Xstop A	15	Chromosome 7	50	100	7	19	2.7	47	7	1
DTT-3Xstop L	50	Chromosome 7	50	100	13	33	2.5	90	12	0.92

[a] In the case of the STT-A line, the 18 progeny analyzed were those transposition positive among the transposon-positive progeny (25%). For all other lines, all transposon-positive progeny contain new insertions.

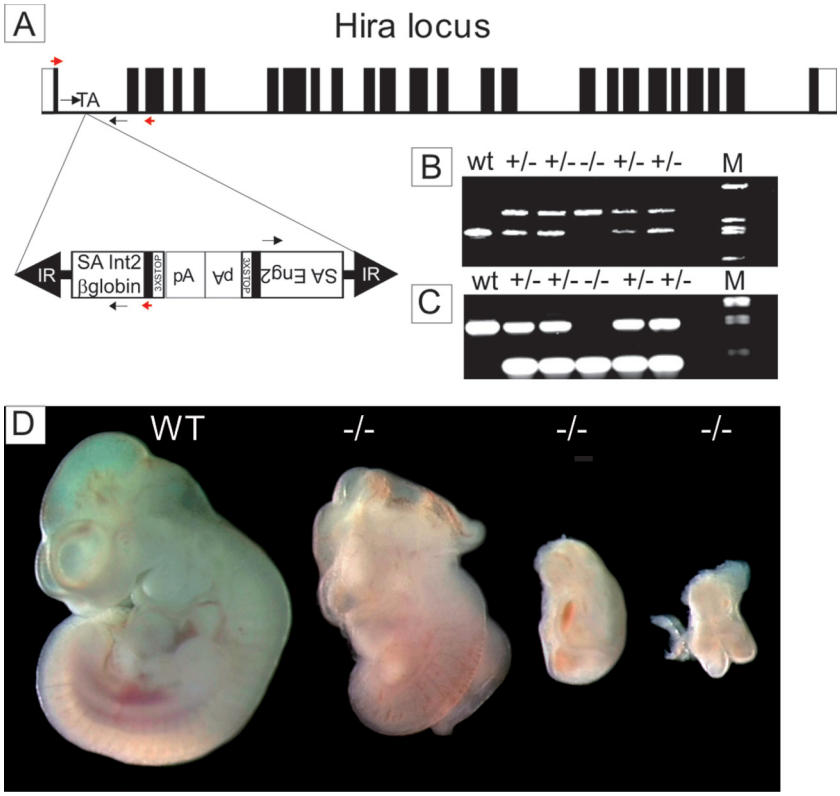

FIG. 5. (A) Schematic transposon-based HirA gene disruption. Exons are in black. Black arrows, genotyping primers; red arrows, RT-PCR primers. Between the left and right Minos inverted repeats (IR) is a double trap consisting of splice acceptors from the second intron of the rabbit β-globin gene followed by the 3rd exon, 3 stop codons, and pA sequences and in the opposite orientation the SA of mouse engrailed-2, followed by an exon, 3 stop codons, and pA. (B) Example of 6 HirA genotyped embryos (two litters) at day E10.5 showing an ~550-bp product in HirA-mutated embryos and an ~464-bp product in wt embryos. (C) Detection of a fusion transcript: i.e., truncated message in the same 6 embryos. Truncated message generated as a result of splicing of the endogenous splice donor with the trap-provided acceptor gives a product of ~90 bp, while wt message gives a product of 155 bp. Marker (M), λPst. (D) Example of wt and mild to severely affected $HirA^{-/-}$ embryos at day 10.5 (same scale).

tions were analyzed. The other 101 transpositions are not informative with respect to genomewide mutagenesis as they were obtained using a selection protocol in ES cells (5). By using the Prm1 promoter to drive the expression of piggyBac (34), it was possible to obtain on average 1 new transposition event per offspring. In their case, the transposase is active only after meiosis, and hence only offspring containing both transgenes (transposon and transposase) can contain new transpositions. In our system, the transposase is expressed earlier and transposition events are also seen in transposase-negative progeny. Our transposition efficiency is obtained from all transposon-positive offspring (Table 1) as opposed to transposon/transposase-positive offspring, and hence the numbers in the two studies are not quite comparable. Like Minos, piggyBac shows a marked preference for introns, a phenomenon also observed in *Drosophila melanogaster* (26). This is advantageous since the trap ensures a productive insertional event by truncating a gene product. Recognition of a simple TA motif (26) is also advantageous for genomewide analysis as it should lead to nearly random integration.

The data usually show a focus of insertions around the parental array, suggesting that distance from the array in the nucleus is an important parameter for transposon insertion. The data suggest this is also the case for transpositions to other chromosomes. For example, in STT-B with the chromosome 4 array, 13 out of 41 insertions observed to other chromosomes were to chromosome 6, 6 to chromosome 5, and 4 each to chromosome 14 and chromosome 8 (see Table S1 in the supplemental material), suggesting that chromosome 4 is spaced close to chromosomes 6, 5, 14, and 8 in the male germ cell nucleus. Alternatively, the large tandem repeats of the transposon may pair with nonhomologous chromosome in a nonrandom manner, depending on sequence content and degree of repetition. However, among a limited number of transpositions analyzed by Horie et al. (16), the converse was seen—a transposition from chromosome 14 to 4—suggesting the spacing is more likely explanation. Similarly a parental chromosome 3 transposon array showed 4 out of 20 transpositions to a different chromosome had occurred to chromosome 5, suggesting chromosomes 3 and 5 are also spaced closely together. Interestingly, while the DTT starter line shows extreme preference for intrachromosomal jumps on chromosome 2, two different founder lines DTT-3Xstop A and DTT-3Xstop L, with transposon arrays on chromosome 7, showed high degrees (50% and 94%, respectively) of interchromosomal events. The latter may be explained by the initial presence of the transposon array in the Ruvbl2 gene, which is highly expressed in mouse testis and thus in an active chromatin conformation at

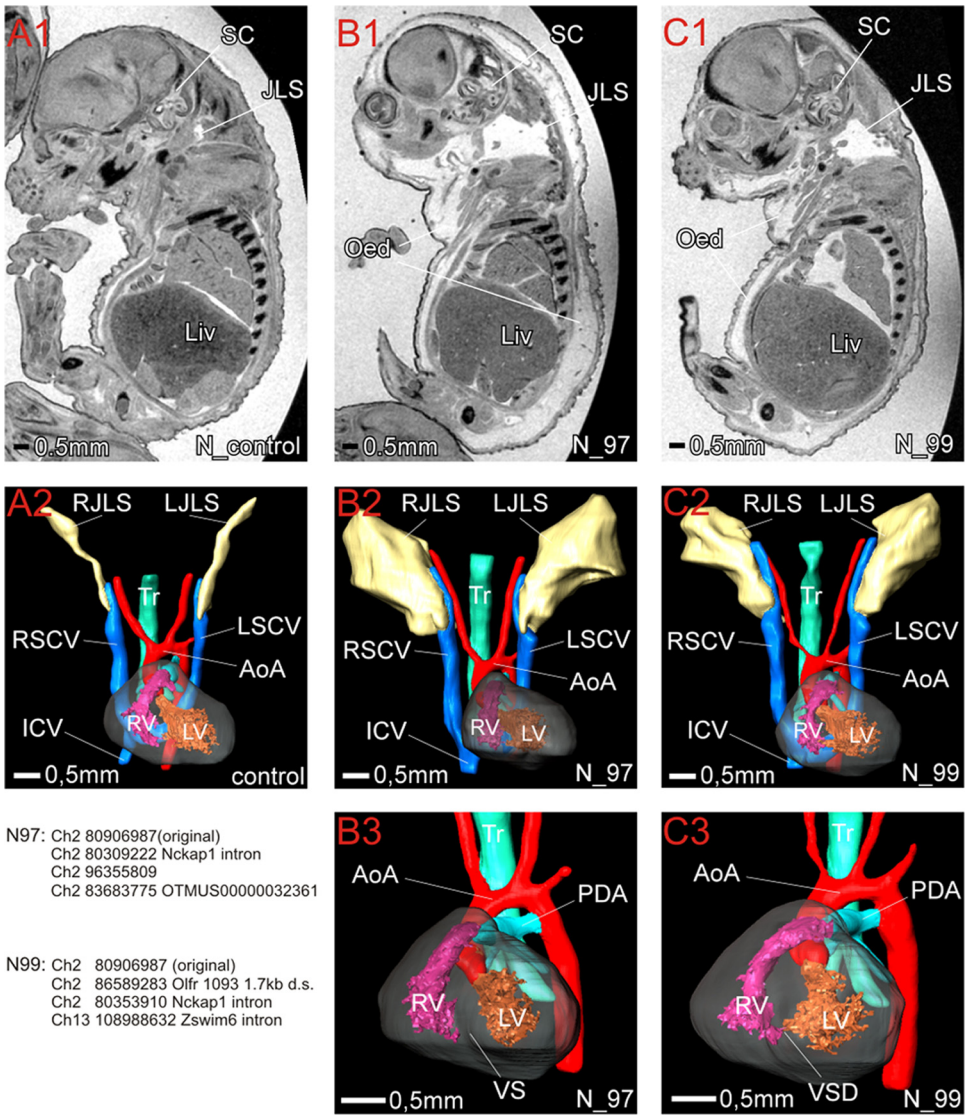

FIG. 6. Magnetic resonance imaging (MRI) pictures of a 15.5-day-old wt embryo (A1) and 2 transposon-positive embryos (N97 and N99) with an *Nckap1* gene insertion among others (B1 and C1) showing edema (Oed), small eyes, and enlarged jugular lymphatic sacs (JLS). A 3D reconstruction of wild-type embryo (A2) and 2 transposon-positive embryos (B2 and C2) shows enlarged left and right lymphatic sacs (LJLS and RJLS). (B3 and C3) Enlargement of the heart area, showing a ventricular septal defect (VSD) in embryo N99. Ao, aorta; AoA, aortal arch; PDA, pulmonary ductus arteriosus; RV and LV, right and left atrium; VS, ventricular septum; RSCV and LSCV, right and left superior caval vein; ICV, inferior caval vein; Tr, trachea; SC, semicircular canal; Liv, liver.

the time of transposase activity. It should be noted that genes can share transcription factories including genes on separate chromosomes (1). Data from translocations suggest that preferentially coassociated genes recombine more easily in a cell-type-specific manner. Thus, a genomewide transposon mutagenesis screen should be carried out with multiple parental lines ideally containing arrays on each chromosome to minimize a possible spatial bias in transpositions.

The Minos transposition system shows sufficient efficiency to allow a mammalian functional screen in the absence of multiple intragenic insertions per progeny. To keep a real genomewide screen workable, the top 10% of a phenotype analyzed by a simple procedure would be selected for further breeding and careful phenotypic analysis. This would separate the different transpositions in a single mouse to single transpositions. Other germline gene transposition systems (4, 5, 9, 15, 16) would probably show similar properties to the Minos system, but they have as yet not been optimized so as to be workable for functional analysis by phenotypic screening. If the transposition of single copies of transposon would be efficient, piggyBac would be best as it does not leave a footprint after excision. However, in practice single transposition is not efficient and arrays would be needed.

Unlike ENU mutagenesis (28), transpositions using any of the systems can be rapidly identified and linked directly with a measurable phenotypic change. Most importantly, transposon mutagenesis could be carried out rapidly in existing mutants to aggravate or ameliorate the phenotype, leading to the rapid

identification of genetic modifiers and interactors, which are likely to be important in complex genetic disease.

The Minos system is robust and can be easily transferred into potentially any mammal by transgenesis independent of the genetic background. Our preferred background is C57BL/6J, a strain prone to metabolic and cardiovascular abnormalities. In humans, these and other diseases have complex genetics, with the time of onset and severity presumably dependent on the nature of mutations in as yet to be defined disease pathways.

We believe that the application of gene transposition now has much to offer relative to alternative approaches for the study and understanding of normal and disease-related genes and pathways in mammals. Success now depends on the measurement of meaningful phenotypic end points, not the identification of the causative gene.

ACKNOWLEDGMENTS

We thank Yasunori Sasakura for the TAIL PCR protocol, John Kong-a-San for blastocyst injections, Angela Franklyn for NMR embryo preparations, Maria Kapetanaki and Willy Baarends for antibodies, Martine Jaegle and Charalambos Savakis for plasmids, Michael Lesnussa for three-dimensional viewer pictures, and Dies Meijer for fruitful discussions.

This work was supported by CGC (NGI, NL) and the British Heart Foundation.

T.D.W., S.B., R.C., F.G., and D.D. designed research; T.D.W., S.D., A.M., A.L., D.S., and D.D. performed research; T.D.W., S.D., A.M., D.S., and D.D. analyzed data; G.B. and T.A.K. contributed new reagents/analytic tools; and R.C., S.B., F.G., and D.D. wrote the paper.

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