Sleeping Beauty Transposon Mutagenesis as a Tool for Gene Discovery in the NOD Mouse Model of Type 1 Diabetes

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ABSTRACT A number of different strategies have been used to identify genes for which genetic variation contributes to type 1 diabetes (T1D) pathogenesis. Genetic studies in humans have identified >40 loci that affect the risk for developing T1D, but the underlying causative alleles are often difficult to pinpoint or have subtle biological effects. A complementary strategy to identifying “natural” alleles in the human population is to engineer “artificial” alleles within inbred mouse strains and determine their effect on T1D incidence. We describe the use of the Sleeping Beauty (SB) transposon mutagenesis system in the nonobese diabetic (NOD) mouse strain, which harbors a genetic background predisposed to developing T1D. Mutagenesis in this system is random, but a green fluorescent protein (GFP)-polyA gene trap within the SB transposon enables early detection of mice harboring transposon-disrupted genes. The SB transposon also acts as a molecular tag to, without additional breeding, efficiently identify mutated genes and prioritize mutant mice for further characterization. We show here that the SB transposon is functional in NOD mice and can produce a null allele in a novel candidate gene that increases diabetes incidence. We propose that SB transposon mutagenesis could be used as a complementary strategy to traditional methods to help identify genes that, when disrupted, affect T1D pathogenesis.

Type 1 diabetes (T1D) is an autoimmune disease in which lymphocytes mediate the specific destruction of insulin-producing pancreatic β cells (Bluestone et al. 2010). Genetic studies in human populations have detected >40 genomic intervals that harbor T1D-associated alleles (Polychronakos and Li 2011; Pociot et al. 2010). However, identification of the underlying genes for these T1D loci and the biological effects of putative causative alleles is often difficult due to genetic heterogeneity and limited tissue availability (Polychronakos and Li 2011; Pociot et al. 2010). Instead, it has proven useful to complement human genetic studies with strategies that not only aim to discover “naturally” occurring alleles but also engineer “artificial” null alleles in putative and novel candidate genes to determine their effect on disease pathogenesis in inbred animal models (Ermann and Glimcher 2012).

The nonobese diabetic (NOD) mouse strain, in particular, has been widely used to investigate T1D pathogenesis (Driver et al. 2011; Jayasimhan et al. 2014). NOD mice spontaneously develop T1D, and genetic studies have identified >40 murine T1D susceptibility loci [termed insulin-dependent diabetes (idd) loci], several of which overlap human T1D susceptibility loci (Burren et al. 2011). Although congenic NOD mouse strains have confirmed the majority of these idd loci, relatively few of the underlying genes and their causative alleles have been definitively identified (Araki et al. 2009; Hamilton-Williams et al. 2001; Hung et al. 2006; Kissler et al. 2006; Laloraya et al. 2006; McGuire et al. 2009; Razavi et al. 2006; Tan et al. 2010; Yamanouchi et al. 2007). It has become apparent, however, that the NOD mouse strain has a combination of rare alleles (e.g., H2-Aku) and common alleles (e.g., H2-Enu and B2m) for different genes that interact and increase the risk for T1D (Driver et al. 2011; Ridgway et al. 2008). Intriguingly, some nonobese diabetic mouse strains harbor a more
diabetogenic allele than NOD mice for a given Idd locus (Brodnicki et al. 2003; Wang et al. 2014; Ghosh et al. 1993; McAleer et al. 1995). This complex genetic architecture for T1D susceptibility in the Mus species is similar to that described in humans and further complicates the identification of “natural” causative alleles within genes underlying Idd loci using traditional outcross and congenic mouse studies (Driver et al. 2011; Ridgway et al. 2008).

Here, we propose an alternative approach for disease gene discovery using the Sleeping Beauty (SB) transposon mutagenesis system to generate “artificial” alleles on the NOD genetic background. The SB transposon can insert within genes and disrupt transcript expression (Horie et al. 2003; Horie et al. 2004). Its ability to carry gene-trap elements and reporter genes, which increase gene disruption efficiency and accelerate identification of mice with a disrupted gene (Izsvak and Ivics 2004; Horie et al. 2004). Transposon transgenic mouse lines are NOD-TgTn(sb-Trans-SA-IRESLacZ-CAG-GFP-SD:Neo)1Tcb and NOD-TgTn(sb-Trans-SA-IRESLacZ-CAG-GFP-SD:Neo)2Tcb, but have been termed NOD-SBtson L1 and L2 in the text. Transposon transgenic mouse lines are NOD-Tg(Prm-sb10)1Tcb and NOD-Tg(Prm-sb10)2Tcb, but have been termed NOD-PrmSB L1 and L2 in the text. NOD-SBtson mice were mated to NOD-PrmSB mice and double-positive hemizygous male offspring (NOD-SBtson+/PrmSB+) were identified by PCR genotyping. NOD-SBtson+/PrmSB+ males were backcrossed to wild-type NOD females to produce G1 mice, carrying potential transposon insertions. Mice carrying transposon insertions, which activated the polyA trap, were noninvasively identified by fluorescent expression under UV light prior to weaning.

**MATERIALS AND METHODS**

**Constructs and production of transgenic and SB transposon mutant mice**

The transposase construct pRP1345 (Figure 1A) was obtained from Prof. J. Takeda (Osaka University, Japan) and has been previously described (Horie et al. 2004). The transposon construct, pTrans-SA-IRESLacZ-CAG-GFP_SD:Neo, has been described (Fischer et al. 2001). IR/DR: inverse repeat/direct repeat transposase recognition motifs. (B) Breeding scheme for SB transposon mutagenesis. NOD-SBtson mice (lines 1 and 2) were mated to NOD-PrmSB mice (lines 1 and 2). Double positive male offspring (seed mice) were backcrossed to wild-type NOD females to produce G1 mice carrying potential transposon insertions. (C) Mice carrying transposon insertions that activated the polyA trap were detected by fluorescence under UV light prior to weaning.

**Transposon copy number analysis**

Southern blots were performed using standard protocols. An 898-bp probe specific for GFP labeled with α-32P-dCTP using the DECA prime II Random Priming DNA labeling kit (Ambion) was used to detect the presence of the SB transposon. Copy number was calculated by comparison with standards of known copy number.

**Transposon insertion site identification**

Ligation-mediated PCR (LM-PCR) was performed based on previously described protocols (Largaespada and Collier 2008; Takeda et al. 2008; Horie et al. 2003; Devon et al. 1995). Briefly, 1 μg genomic DNA was digested with HaeIII, Alul, BfI, or NruIII (New England Biolabs), and splinkerettes (Table 1) compatible with appropriate blunt or cohesive ends were ligated to the digested genomic DNA fragments using T4 DNA ligase (New England Biolabs). The two oligonucleotides to

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**Figure 1** Sleeping Beauty transposon mutagenesis strategy. (A) Constructs used for production of NOD-PrmSB and NOD-SBtson lines. The transposon construct, pTrans-SA-IRESLacZ-CAG-GFP_SD:Neo, has been described (Horie et al. 2003). The transposon construct pRP1345 comprises SB10 transposase driven by the mouse proximal protamine 1 promoter has also been described (Fischer et al. 2001). IR/DR: inverse repeat/direct repeat transposase recognition motifs. (B) Breeding scheme for SB transposon mutagenesis. NOD-SBtson mice (lines 1 and 2) were mated to NOD-PrmSB mice (lines 1 and 2). Double positive male offspring (seed mice) were backcrossed to wild-type NOD females to produce G1 mice carrying potential transposon insertions. (C) Mice carrying transposon insertions that activated the polyA trap were detected by fluorescence under UV light prior to weaning.
Table 1 Oligonucleotides used as splinkerettes and primers for LM-PCR

| Namea | Sequence | Purposea |
|-------|----------|----------|
| SplI-BLT | CGAATCGTAACCGCTTTGACGAGAATCGCTGCTCTCTCAAACAGACAGCAAGG | Splinkerette |
| Spl-top | CCTGGGCTGTTTGTGTTGCAA | Splinkerette |
| Nla linker+ | GAATAACGATCATATAGGGGCCTCCTAAGGGAGCACCAG | Splinkerette |
| Nla linker- | 5'-GCGCTCTTAAGGGCAAGG-3' | Splinkerette |
| Bfa linker+ | GAATAACGATCATATAGGGGCCTCCTAAGGGAGCACCAG | Splinkerette |
| Bfa linker- | 5'-GCGCTCTTAAGGGCAAGG-3' | Splinkerette |
| Spl-P1 | CGAATCGTAACCGCTTTGACGAGAATCGCTGCTCTCTCAAACAGACAGCAAGG | LM-PCR |
| Spl-P2 | TGGCGGAATTTTCCAAGCTGTTTAAAGGCACAGTC | Nested LM-PCR |
| T/BJA | TAACGACTCATTAAGGCAAGGAAATTTTAC | LM-PCR (left) |
| TJB1 | TTGACTCTGATTGATTGTTGTT | Nested LM-PCR (left) |
| T/BAL | TGGGCTGATGCAAAAGTTAGATGTC | LM-PCR (right) |
| LongIRDR(R) | TGGCGGAATTTTCCAAGCTGTTTAAAGGCACAGTC | Nested LM-PCR (right) |
| Nla linker | 5'-GCGCTCTTAAGGGCAAGG-3' | Nested LM-PCR |
| SplB-BLT | CGAATCGTAACCGCTTTGACGAGAATCGCTGCTCTCTCAAACAGACAGCAAGG | Nested LM-PCR |
| Spl-top | CCTGCTTGTGAAAGGCTACTCGAAAATGTTGGACCG | Nested LM-PCR |
| Spl-P2 | TGGCGGAATTTTCCAAGCTGTTTAAAGGCACAGTC | Nested LM-PCR |
| SplB-BLT | CGAATCGTAACCGCTTTGACGAGAATCGCTGCTCTCTCAAACAGACAGCAAGG | Nested LM-PCR |
| KJC1 | CCACTGGGAATGTGATGAAAGAAATAAAAGC | Nested LM-PCR |
| TDR2 | GCTTGTGGAAGGCTACTCGAAAATGTTGGACCG | Nested LM-PCR |
| Newl1 | GACCTTGGTGTGATGCAAAATGTTGGACCG | Nested LM-PCR |
| LongIRDR(L2) | GCTTGTGGAAGGCTACTCGAAAATGTTGGACCG | Nested LM-PCR |
| KJC1 | CCACTGGGAATGTGATGAAAGAAATAAAAGC | Nested LM-PCR |
| Linker primer | GAACTGCGATCATTAGTCAAAAGG | LM-PCR |
| Linker primer nested | AGGGTCCGCTTAAAGGGAC | Nested LM-PCR |

a Primer names and purpose are based on the previously described LM-PCR protocols (Keng et al. 2005; Largaespada and Collier 2008).

produce the double-stranded splinkerette were annealed at a concentration of 50 μM in the presence of 100 mM NaCl by incubating at 95°C for 5 min and then allowing the mixture to cool to room temperature in a heat block before ligation. A second digest, with XhoI or KpnI, was used to remove ligated splinkerettes from transposon concatemer fragments. DNA was purified after each step using QiaQuick PCR Purification kit (Qiagen). Two rounds of PCR were performed using nested primers within the linker and transposon (Table 1) with the subsequent PCR product sequenced. Restriction enzymes, splinkerettes, and primers were used in the following six combinations (first digest: linker; second digest: primer set 1; primer set 2): (i) and (ii) Alul or HaeIII; SplB-BLT; XhoI; T/BJA × Spl-P1; TJB1 × Spl-P2; (iii) and (iv) Alul or HaeIII; SplB-BLT; KpnI; TDR2 × Spl-P1; T/BAL × Spl-P2; (v) Bfa linker; KpnI; LongIRDR(L2) × Linker primer; Newl1 × linker primer nested; and (vi) NlaIII; Nla linker; XhoI; LongIRDR(R) × Linker primer; KJC1 × Linker primer nested. The resulting sequence was aligned to mouse genome build GRCm38 to identify the transposon-flanking genomic sequence (i.e., insertion site) and determine which gene was disrupted based on current annotation for build GRCm38. The different combinations give a number of chances to identify genomic DNA adjacent to both the 3’ and 5’ ends of the transposon after transposition. Additional primers were designed for genotyping the transposon insertion site in established mutant mouse lines (Table 2).

Gene expression analysis
RNA was extracted from tissues using Trizol (Invitrogen) and cDNA was synthesized using Superscript III (Invitrogen), both according to manufacturer’s instructions. For SB4 mice, RT-PCR was performed with oligos specific for Scler1a10: Exon 1 forward: 5’-GACGGTTGCTGAGTGAAAC-3’; Exon 2 reverse: 5’-GACGGTTGCTGAGTGAAAC-3’; and Actb (forward: 5’-GGACTGCTCTTCCTGAGAG-3’; reverse: 5’-GGACTGCTCTTCCTGAGAG-3’). For SB7 mice, RT-PCR was performed with primers in GFP (5’-CCCTGAGCAAAGACCCCAAGAGG-3’ and Serinc1 exon 1 (5’-CCCTGAGCAAAGACCCCAAGAGG-3’ and Serinc1 exon 1 (5’-CCCTGAGCAAAGACCCCAAGAGG-3’) and Serinc1 exon 2 (5’-CCCTGAGCAAAGACCCCAAGAGG-3’) and Serinc1 exon 2 (5’-CCCTGAGCAAAGACCCCAAGAGG-3’), followed by Sanger sequencing of products. Transposase expression was determined using quantitative real-time PCR using LightCycler Probe Master Reagent (Roche Diagnostics) and the following primer/UPL probe combination for SB transposase (SB10): Fwd: 5’-ACCCAGCAAGCTGATAC; Rev: 5’-ACCCAGCAAGCTGATAC; and UPL probe #95: AGTCCCAG. Data were normalized to Hprt expression. For SB7, Serinc1 expression was determined by quantitative real-time PCR (LightCycler480, Roche) with the following primer/probe combination for Serinc1: (Fwd: 5’-ACCCAGCAAGCTGATAC; Rev: 5’-ACCCAGCAAGCTGATAC; and UPL probe #95: AGTCCCAG). Data were normalized to Hprt expression.

Diabetes monitoring
Mice were tested once per week for elevated urinary glucose using Diamon reagent strips (Bayer Diagnostics). Mice with a positive glycosuria reading (>110 mmol/L) and confirmed by a positive glucose reading (>15 mmol/L), using Advantia II Glucose Strips (Roche), were diagnosed as diabetic.

Data availability
Mouse lines are available upon request.

RESULTS

Generation of transposition events in NOD mice using SB transgenic NOD lines
To perform germline mutagenesis of the NOD mouse, we used two SB2 constructs previously used in mice (Figure 1A). The pP1345 construct contains the transposase gene under the control of the proximal promoter 1 (Prm1) promoter, which restricts expression to spermatogenesis and limits transposon mutations to the germline, thus preventing somatic mutations (Fischer et al. 2001). The SB transposon construct (sb-pTrans-SA-IRESlacZ-CAG-GFP)$^+$ contains splice acceptor and donor sequence motifs encompassing a promoter trap comprising the lacZ gene, and a polyA tract with the gene encoding enhanced green fluorescent protein (GFP) driven by the CAG promoter (Horie et al. 2003). This construct enables efficient identification of mutant mice in...
which the transposon has inserted in a gene, as activation of the polyA trap results in ubiquitous GFP expression, i.e., mutant mice fluoresce.

The transposase and transposon were introduced separately into NOD mice to establish independent transgenic NOD lines for each SB component. Briefly, SB transposon and SB transposase transgenic mice were produced by pronuclear injection of linearized constructs into fertilized NOD/Lt (NOD) oocytes. NOD-Tg(Prm-sb10) mice (called NOD-PrmSB hereafter) harbor the transposase; and NOD-TgTn(sb-pTrans-SA-IRES-LacZ-CAG-GFP-SD:Neo) mice (called NOD-SBtson hereafter) harbor the transposon. The breeding scheme to generate mutant NOD mice is outlined in Figure 1B: mice from the two transgenic lines are mated, bringing together the two components of the SB system, and transposition occurs within the sperm cells of the double-positive hemizygous “seed” males. These NOD seed males are mated to wild-type NOD females to generate G1 litters. G1 pups with potential transposon-disrupted genes are efficiently identified before weaning by visualization of bodily GFP expression under UV light (Figure 1C). This early detection allows cage space to be minimized; only those litters that contain fluorescent pups are weaned and kept for additional analysis.

To assess the feasibility of the SB mutagenesis system in the NOD mouse strain, two NOD-SBtson lines were established for which the number of copies of the transposon within the transgene concatemer was determined by Southern Blot analysis (Figure 2A) and the site of transgene integration was determined by ligation-mediated PCR (LM-PCR) (Figure 2B). These lines were bred with two established NOD-PrmSB lines, in which expression of SB transposase in testes had been confirmed by quantitative RT-PCR (data not shown), to produce seed males. NOD seed males (i.e., double-positive, hemizygous for both SB constructs) were backcrossed to NOD females to produce G1 mice. Three different breeding combinations of transposon/transposase transgenic strains gave rise to GFP-positive offspring at the rate of 2.0%, 2.3%, and 2.7% respectively (Figure 2B). A fourth combination transgenic strains gave rise to GFP-positive offspring at the rate of 3.0% in mice sired by NOD seed males (called NOD-SBtson hereafter) harbor the transposon. The breeding scheme to generate mutant NOD mice is outlined in Figure 1B: mice from the two transgenic lines are mated, bringing together the two components of the SB system, and transposition occurs within the sperm cells of the double-positive hemizygous “seed” males. These NOD seed males are mated to wild-type NOD females to generate G1 litters. G1 pups with potential transposon-disrupted genes are efficiently identified before weaning by visualization of bodily GFP expression under UV light (Figure 1C). This early detection allows cage space to be minimized; only those litters that contain fluorescent pups are weaned and kept for additional analysis.

Identification and prioritization of transposition sites in GFP-positive G1 NOD mice

LM-PCR followed by sequencing and genome alignment was used to identify the transposition sites for nine of the 11 GFP-positive mice (Table 3). Of the two GFP-positive mice that were not determined, one died before characterization. It was not clear why the second GFP-positive mouse was refractory to site identification by LM-PCR despite using six different restriction digest/PCR combinations. Consistent with the published >30% rate of local chromosomal hopping (Keng et al. 2005), seven of the nine identified transposition sites fell on the same chromosome as the transposon donor concatemer. Of the seven insertion sites derived from the NOD-SBtsonL1 concatemer on chromosome (Chr) 10, five were on Chr10, with the other two identified on Chr1 and Chr4. The two insertion sites arising from the NOD-SBtsonL2 concatemer on Chr1 were both mapped to Chr1. LM-PCR also indicated that each GFP-positive mouse contained a single, rather than multiple, transposon insertion site.

Once GFP-positive NOD mice and their transposon mutations are identified, there are two options. One option is to generate and monitor diabetes onset in cohorts of mice from every GFP-positive G1 mouse. Although such a full-scale phenotype-driven approach is aimed at identifying novel genes not suspected to play a role in diabetes, as well as known and putative candidate genes, this option requires substantial animal housing capacity and monitoring numerous cohorts for diabetes over a >200-d time course. Like many investigators, we have limited resources and this option was not feasible. However, the SB transposon mutagenesis strategy allows for a second option: prioritizing mutant mice based on the genes that are disrupted and establishing homozygous mutant lines for expression and diabetes monitoring. We therefore prioritized transposon-disrupted genes based on the following criteria:

1) The transposon insertion site is predicted to disrupt the gene in some way. Has the transposon inserted within an exon, an intron, or in a regulatory region? Is the mutation predicted to disrupt gene expression? An insertion that is predicted to completely abrogate expression of the normal gene product would be of high priority; however, in some instances a predicted hypomorphic allele may also be of value.

2) The gene is a known or putative candidate for a described mouse and/or human T1D susceptibility locus. T1D loci and candidate genes are curated in a searchable form at T1Dbase (https://t1dbase.org/) (Burren et al. 2011).

3) The gene, not previously considered as a putative T1D susceptibility gene, encodes a known protein involved in an immune-related molecular pathway that could affect T1D pathogenesis (e.g., cytokine or chemokine signaling/regulation, pattern recognition pathways, costimulatory molecules, apoptosis, regulation of immune tolerance), but is not likely required for general immune cell development.

4) The gene, not previously considered as a putative T1D susceptibility gene, is expressed in relevant cells (e.g., immune cell subsets, β cells) as determined in the first instance using gene expression databases [e.g., Immunological Genome Project (https://www.immgen.org/)] (Heng et al. 2008) followed by additional expression analyses if needed.

| Line | Fwd | Rev | Size | Allele |
|------|-----|-----|------|--------|
| SB4  | AGCGCGAAGACAACTCCTGGT | AAAGGGGCGTGCGCTAAACA | 123 bp | WT     |
| SB4  | CTGTTGTCATGCACAAAGTAGATGTCC | AAAGGGGCGTGCGCTAAACA | 224 bp | SB     |
| SB7  | ATTCGCCAGTGTGCTGTGGTAC | CTTGGAAATCATCCCGTGAGAGA | 647 bp | WT     |
| SB7  | ATTCGCCAGTGTGCTGTGGTAC | CTTGGTCTATGCACAAAGTAGATGTCC | 632 bp | SB     |
Characterization of transposon effects in two prioritized GFP-positive G1 NOD mice

The transposon insertion site in the SB7 mouse (NOD. Serinc1Tn(ub-Trans-SA-JRESLauZ-CAG-GFP-SD-NooI1.7Cub)) was localized 4.6 kb upstream of Serinc1 (Table 3, Figure 3A). SERINC1 facilitates the synthesis of serine-derived lipids, including the essential membrane lipids phosphatidylserine and sphingolipid (Inuzuka et al. 2005). These are important components of membrane structures known as “ordered membrane domains” or “lipid rafts,” which are required for appropriate signaling in immune cells (Szoor et al. 2010; Yabas et al. 2011). Due to the position of the transposon insertion, we postulated that, rather than completely disrupting expression of Serinc1, the mutation may affect gene regulation. RT-PCR analysis of homozygous SB7 splenic RNA using primers within GFP and Serinc1 identified two fusion transcripts in addition to the normal transcript. The first contains GFP spliced to sequence upstream of exon 1 with normal splicing of the entire gene. The second contains GFP spliced directly to exon 2 of Serinc1 (Figure 3A). The skipped exon 1 encodes the first 13 amino acids of the Serinc1 coding sequence. The production of any SERINC1 protein from these fusion transcripts is unlikely because there is a stop codon following the GFP sequence and no obvious internal ribosomal entry site prior to the Serinc1 sequence, but this still remains to be tested. In either case, the presence of the transposon was reported to develop spontaneous disease. The strains either have not been analyzed for immunological phenotypes (Slc16a10) or have only been analyzed in small cohorts (n < 4) with no differences observed (Serinc1). Despite this, Serinc1 and Slc16a10, although not directly attributed in the literature with immune-related roles, have functions that could be postulated to affect immune cell responses and/or β-cell activity, which could be revealed in the context of the “sensitized” NOD genetic background (i.e., the NOD mouse strain enables detection of mutated genes that increase or decrease diabetes incidence). Notably, both genes according to expression databases (The Immunological Genome Project and BioGPS) (Heng et al. 2008; Wu et al. 2009) were highly expressed in macrophages, an immune cell population with a key role in T1D pathogenesis (Driver et al. 2011; Jayasingham et al. 2014). SB4 and SB7, which were both male G1 mice, did not carry the transposase construct; consequently, secondary jumping of the transposon was not possible in their offspring. SB4 and SB7 G1 mice were thus prioritized for establishment of new lines, bred to homozygosity, and investigated for the effect of their transposon insertions.

Table 3 Sleeping Beauty transposon insertion sites in GFP-positive G1 NOD mice

| Mutant Mouse | NOD-SBtson Line | NOD-PrmSBL Line | Site of Transposition Insertion (STI)a | Closest Gene in Correct Orientation | Gene Coordinatesa | Position of STI with Respect to Gene |
|--------------|-----------------|-----------------|---------------------------------------|-----------------------------------|-------------------|-------------------------------------|
| SB1          | L1              | L1              | chr:19,247,537                         | Cnbd1                             | chr:18,860,454-19,122,526 | 125 kb 5’ of gene                  |
| SB2          | L1              | L1              | chr:10:30,040,682                       | Rspos3                            | chr:10,29,453,107-29,535,867 | 505 kb 5’ of gene                  |
| SB3          | L1              | L1              | chr:10,54,845,255                      | Ms3I2                             | chr:10,56,106-116,516,880 | 1.2 Mb 5’ of gene                  |
| SB4          | L1              | L1              | chr:10,122,645                         | Slc16a10                          | chr:10,40,033,535-40,142,254 | Intron 1                           |
| SB5[c]       | L1              | L2              | n.d.                                  | n.d.                              | n.d.                           | n.d.                                |
| SB6          | L1              | L2              | n.d.                                  | n.d.                              | n.d.                           | n.d.                                |
| SB7          | L1              | L2              | chr:10,57,537,126                      | Serinc1                           | chr:10,57,515,775-57,532,529 | 4.6 kb 5’ of gene                  |
| SB8[c]       | L1              | L2              | chr:10,56,499,234                      | AK018981                          | chr:10,56,504,301-56,505,287 | 5 kb 5’ of gene                    |
| SB9[c]       | L1              | L2              | chr:1,61,954,348                       | Pard3bos1                         | chr:1,61,767,415-61,851,462 | 102 kb 5’ of gene                  |
| SB10         | L2              | L2              | chr:1,49,094,718                       | C230029F2Rik                      | chr:1,49,244,616-49,340,431 | 150 kb 5’                         |
| SB11         | L2              | L2              | chr:1,48,800,150                       | Slc39a10                          | chr:1,46,807,544-46,853,509 | 1.9 Mb 5’ of gene                  |

a Coordinates are based on genome build GRCm38.

b The SB9 transposon site of integration falls within intron 2 of Pard3b in the opposite orientation.

c SB5, SB8, and SB9 mice died of unknown causes before homozygous lines could be established.

n.d., not determined.
insertion results in a relatively small, but significant, decrease in expression of Serinc1 as measured by quantitative RT-PCR using a Taqman probe spanning the exon 1/2 splice junction (Figure 3B). This splice junction is used in both the “normal” Serinc1 and the fusion transcript that includes exon 1; therefore, the reduction in “normal” Serinc1 expression is greater than measured by this assay. These analyses suggest that, rather than completely disrupting expression of Serinc1, this mutation modulates expression and represents a hypomorphic allele. Nonetheless, homozygous mutant SB7 mice exhibited a similar diabetes incidence compared to wild-type littermate females (Figure 3C), indicating that a minor reduction in Serinc1 expression does not affect T1D pathogenesis.

The transposon insertion identified in the SB4 mouse (NOD. Slc16a10^IgTn(ab-Trans-SA-RESLacZ-CAG-GFP-SD:Neo)1.4Tcb) localizes to intron 1 of Slc16a10 (Table 3, Figure 4A), which encodes the aromatic amino acid transporter SLC16A10 (also known as TAT1) (Mariotta et al. 2012; Ramadan et al. 2006). It is becoming increasingly evident that regulation of amino acid transport is crucial for the proper regulation of immune cell activation and function (Nakaya et al. 2014; Sinclair et al. 2013; Thompson et al. 2008), and also impacts glycemic control (Jiang et al. 2015). We predicted that the strong splice acceptor encoded by the transposon would result in splicing from exon 1 of Slc16a10 into the transposon sequence, thus truncating the Slc16a10 transcript. Expression analysis of homozygous mutant SB4 mice showed that the Slc16a10 transcript was not detected (Figure 4B), which was associated with a significant increase in diabetes incidence compared to wild-type littermate females (Figure 4C). This result demonstrates that SB transposon mutagenesis can be used to identify novel genes that affect T1D pathogenesis. Moreover, SB4 is a promising mutant mouse line for investigating the role of Slc16a10 and aromatic amino acid transport in macrophage function and the development of T1D in NOD mice.

**DISCUSSION**

We demonstrate here that SB transposon mutagenesis can successfully generate both null and hypomorphic mutations in the NOD mouse. A significant advantage of this strategy is the use of the GFP reporter to screen out, before weaning, those mice unlikely to be carrying a functional mutation, thereby significantly reducing mouse handling and housing. Mutation sites can then be determined in individual GFP-positive mice and prioritized for further analysis based on the requirements of the investigator. We used our prioritization criteria to select two mutant NOD mice (SB4 and SB7) for further characterization and subsequently found that disruption of Slc16a10 expression in NOD mice resulted in an increased T1D incidence. Although further studies are required to determine how Slc16a10 and amino acid transport contributes to diabetes pathogenesis, this result indicates that SB transposon mutagenesis can be used as a complementary approach to other T1D gene discovery strategies.
We observed 2–3% fluorescent G1 offspring, which is lower than that reported for a similar mutagenesis scheme using the same transposon construct (~7%) (Horie et al. 2003). If one aims for a single transposition event per G1 offspring, it would be expected that ~20% of the G1 offspring should fluoresce [i.e., ~40% of the mouse genome contains exons/introns (Sakharaki et al. 2005), with a 50% chance of the transposon landing in the correct orientation to trap the polyA sequence motif]. There are a number of possibilities that could explain a lower rate of fluorescent G1 offspring, pointing to improvements that can be made to our screen. The NOD-SBtson mutator transgenic lines we generated harbored few copies of the transposon. A higher copy number in the donor transposon concatemer may increase mutation efficiency due to more “jumping” transposons in the sperm of NOD seed males (Geurts et al. 2006). Use of a more efficient transposid could also increase transposition efficiency. As the first described transposon for use in vertebrates, the SB system has been the most widely used and developed. Improvements from the first-generation SB transposases, such as that used in our study, have seen 100-fold increases in transposition efficiency (Mates et al. 2009).

Trying to obtain too high of a mutation rate, however, is not necessarily favorable. For example, increasing transposon copy number by using transgenic mice with large transposon concatemers (>30 copies) may lead to local chromosomal rearrangements in subsequent offspring and complicate characterization of causative transposon mutations (Geurts et al. 2006). Increasing transposon number and/or transposidase efficiency will also lead to GFP-positive offspring with multiple gene mutations. It would then require additional work to identify and confirm all the gene mutations in a given mouse, as well as additional breeding to segregate mutations and test mutant mice with only one gene mutation, all of which increases breeding times and cage costs. Thus, an advantage of a lower efficiency is that it leads to mice with only one gene mutation, eliminating the need for extensive segregation analysis and facilitating more efficient gene identification and prioritization. Although we did not establish and characterize all of our mutant mouse lines due to limited resources, cryopreservation of sperm from mutant male mice could be used to allow archiving of mutants for future investigation. Empirically, it will be up to an individual laboratory to determine how many offspring they can efficiently screen and how they will prioritize mutant mice for subsequent analysis based on the mutation rate of their transgenic lines and available resources.

Interestingly, several of our transposon insertion sites mapped to regions at some distance from annotated genes. Although it is possible that the GFP could be activated by splicing into a cryptic polyA site, polyA gene trap strategies have been successfully used to identify novel unannotated genes (Zambrowicz et al. 1998). However, it may be difficult to predict a priori if an unannotated gene will be of interest, especially if it has little to no sequence homology with known genes. In this regard, the SB mutagenesis approach enables a phenotype-driven (i.e., forward genetics) approach to test novel genes that might not otherwise be targeted using a candidate gene approach (i.e., reverse genetics). SB mutagenesis may also benefit characterization of regions (e.g., Idd loci) that are known to contain putative susceptibility genes, but for which the “natural” causative alleles have not yet been identified. Saturation mutagenesis could effectively be performed in these regions by generating a transgenic NOD mouse line containing a transposon concatemer near the region of interest and taking advantage of the propensity of SB transposons to reintegrate close to the donor transposon concatemer (Keng et al. 2005). Nonetheless, investigating unannotated genes is riskier (i.e., the disrupted gene may not affect T1D) or more costly in the case of saturation mutagenesis (i.e., more mutant
lines need to be generated and characterized). Hence, prioritizing genes based on function postulated to contribute to T1D pathogenesis may be more favorable to most labs.

Transposon mutagenesis is one of a range of techniques that can be used to identify gene variants that affect development of T1D. Conventionally, “artificial” null alleles for candidate genes have been generated in other strains and bred onto the NOD background by serial backcrossing. This approach, however, results in the null allele being encompassed by a “hitchhiking” congenic interval from the other strain, which may also affect T1D susceptibility (Simpfendorfer et al. 2015; Armstrong et al. 2006; Leiter 2002; Kanagawa et al. 2000). Although NOD ES cell lines are available (Hanna et al. 2009; Nichols et al. 2009; Ohta et al. 2009), there are relatively few reports of their use in targeting genes (Kamakaka et al. 2009; Morgan et al. 2013). Alternatively, random mutagenesis using N-ethyl-N-nitrosourea (ENU) does not require ES cells or prior knowledge about candidate genes. ENU mutagenesis, however, creates tens to hundreds of mutations per mouse. Substantial breeding and sequencing, more than for SB transposon mutagenesis, would be required to segregate and test individual ENU mutations for their effect on T1D susceptibility (Hoyne and Goodnow 2006). Finally, emerging gene-editing techniques using the CRISPR-Cas9 system (Ran et al. 2013) can facilitate hypothesis-driven investigation of known and putative candidate genes in NOD mice (Ran et al. 2013; Li et al. 2014). Modifications of the CRISPR-Cas9 system also allow a range of strategies to be used, including the production of conditional alleles, insertion of reporters, activation or repression of alleles, and specific gene editing allowing the recreation of particular variants affecting immune function (Pelletier et al. 2015). Nonetheless, this approach requires genes to be specifically targeted, whereas SB transposon mutagenesis is random and may identify genes not otherwise considered. Thus, a combination of different approaches for gene discovery and characterization of allelic effects is available and will likely prove useful for understanding the genetic architecture of T1D.

The increasing number of causative “natural” alleles identified in human populations and inbred mouse strains will undoubtedly aid our understanding and prediction of genetic risk for T1D, as well as aid future clinical trials in selecting appropriate patient treatment groups based on their genetic profile (Bluestone et al. 2010; Polychronakos and Li 2011). Nonetheless, many of the identified T1D loci and underlying causative alleles have subtle biological effects that are not therapeutically amenable or are difficult to investigate due to tissue availability. Generating random “artificial” null alleles in the NOD mouse provides an alternative strategy to test and identify both putative and novel genes that: (i) have larger diabetes effects when more grossly disrupted; (ii) represent potential drug targets; and (iii) are less likely to be identified in population-based studies of natural variation. The NOD mouse exhibits a number of immunological abnormalities that are associated with T1D pathogenesis and provides a “sensitized” background to investigate the effect of artificial mutations upon the development of T1D (Driver et al. 2011; Ridgway et al. 2008; Jayasimhan et al. 2014). Our study indicates that SB transposon mutagenesis in NOD mice is feasible and provides a new strategy that combines the advantage of both forward genetics (random mutagenesis) and reverse genetics (gene prioritization) for the potential discovery of new genes that affect T1D pathogenesis.

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LITERATURE CITED

Araki, M., D. Chung, S. Liu, D. B. Rainbow, G. Chamberlain et al., 2009 Genetic evidence that the differential expression of the ligand-independent isoform of CTLA-4 is the molecular basis of the Idd5.1 type 1 diabetes region in nonobese diabetic mice. J. Immunol. 183: 5146–5157.

Armstrong, N. J., T. C. Brodnicki, and T. P. Speed, 2006 Mind the gap: analysis of marker-assisted breeding strategies for inbred mouse strains. Mamm. Genome 17: 273–287.

Bluestone, J. A., K. Herold, and G. Eisenbarth, 2010 Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 464: 1293–1300.

Brodnicki, T. C., F. Quirk, and G. Morahan, 2003 A susceptibility allele from a non-diabetes-prone mouse strain accelerates diabetes in NOD congenic mice. Diabetes 52: 218–222.

Burren, O. S., E. C. Adlem, P. Achuthan, M. Christensen, R. M. Coulson et al., 2011 T1DBase: update 2011, organization and presentation of large-scale data sets for type 1 diabetes research. Nucleic Acids Res. 39 (Database issue): D997–D1001.

Carlson, C. M., A. J. Dupuy, S. Fritz, K. J. Roberg-Perez, C. F. Fletcher et al., 2003 Transposon mutagenesis of the mouse germline. Genetics 165: 243–256.

Devon, R. S., D. J. Porteous, and A. J. Brookes, 1995 Splinkerettes–improved vectorettes for greater efficiency in PCR walking. Nucleic Acids Res. 23: 1644–1645.

Driver, J. P., D. V. Serreze, and Y. G. Chen, 2011 Mouse models for the study of autoimmunity type 1 diabetes: a NOD to similarities and differences to human disease. Semin. Immunopathol. 33: 67–87.

Dupuy, A. L. L. M. Rogers, J. Kim, K. Nannapaneni, T. K. Starr et al., 2009 A modified Sleeping Beauty transposon system that can be used to model a wide variety of human cancers in mice. Cancer Res. 69: 8150–8156.

Ermann, J., and L. H. Gimlicher, 2012 After GWAS: mice to the rescue? Curr. Opin. Immunol. 24: 564–570.

Fischer, S. E., E. Wienholds, and R. H. Plasterk, 2001 Regulated transposition of a fish transposon in the mouse germ line. Proc. Natl. Acad. Sci. USA 98: 6759–6764.

Geurts, A. M., L. S. Collier, J. L. Geurts, L. L. Osset, M. L. Bell et al., 2006 Gene mutations and genomic rearrangements in the mouse as a result of transposon mobilization from chromosomal concatamers. PLoS Genet. 2: e156.

Ghosh, S., M. Palm, N. R. Rodrigues, H. J. Cordell, C. M. Hearne et al., 1993 Polygenic control of autoimmune diabetes in nonobese diabetic mice. Nat. Genet. 4: 404–409.

Hamilton-Williams, E. E., D. V. Serreze, B. Charlton, E. A. Johnson, M. P. Marron et al., 2001 Transgenic rescue implicates β2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice. Proc. Natl. Acad. Sci. USA 98: 11533–11538.

Hanna, J., S. Markoulaei, M. Mitalipova, A. W. Cheng, J. P. Cassidy et al., 2009 Metastable pluripotent states in NOD-mouse-derived ESCs. Cell Stem Cell 4: 513–524.

Heng, T.S., and M.W. Painter, and Immunological Genome Project Consortium, 2008 The Immunological Genome Project: networks of gene expression in immune cells. Nat. Immunol. 9: 1091–1094.

Horie, K., K. Yusa, K. Yae, J. Odajima, S. E. Fischer et al., 2003 Characterization of Sleeping Beauty transposon and its application to genetic screening in mice. Mol. Cell. Biol. 23: 9189–9207.

Hoyne, G. F., and C. C. Goodnow, 2006 The use of genomewide ENU mutagenesis screens to unravel complex mammalian traits: identifying genes that regulate organ-specific and systemic autoimmunity. Immunol. Rev. 210: 27–39.
Jiang, Y., A. J. Rose, T. P. Sijmonsma, A. Broer, A. Pfenninger et al., 2014 Advances in our understanding of the pathophysiology of type 1 diabetes: lessons from the NOD mouse. Clin. Sci. (Lond.) 126: 126–133.

Jayasimhan, A., K. P. Mansour, and R. M. Slattery, 2014 Molecular reconstruction of Sleeping Beauty, a Tcl1-like transposon from fish, and its transposition in human cells. Cell 91: 501–510.

Izsvak, Z., and Z. Ivics, 2004 Sleeping Beauty transposition: biology and applications for molecular therapy. Mol. Ther. 9: 147–156.

Jayasimhan, A., K. P. Mansour, and R. M. Slattery, 2014 Advances in our understanding of the pathophysiology of type 1 diabetes: lessons from the NOD mouse. Clin. Sci. (Lond.) 126: 1–18.

Jiang, Y., A. J. Rose, T. P. Sijmonsma, A. Broer, A. Pfenninger et al., 2015 Mice lacking neutral amino acid transporter B0AT1 (Slc6a19) have elevated levels of PGP21 and GLP-1 and improved glycemic control. Mol. Metab. 4: 406–417.

Kamanaka, M., D. Rainbow, C. Schuster-Gossler, E. E. Eynon, A. V. Chervonsky et al., 2009 Amino acid polymorphisms altering the glycosylation of IL-2 do not protect from type 1 diabetes in the NOD mouse. Proc. Natl. Acad. Sci. USA 106: 11236–11240.

Kanagawa, O., G. Xu, A. Tevaarwerk, and B. A. Vaupel, 2000 Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN-γ receptor loci. J. Immunol. 164: 3919–3923.

Keng, V. W., K. Yae, T. Hayakawa, S. Mizuno, Y. Uno et al., 2005 Region-specific saturation germline mutagenesis in mice using the Sleeping Beauty transposon system. Nat. Methods 2: 763–769.

Kissler, S., P. Stern, K. Takahashi, K. Hunter, L. B. Peterson et al., 2006 In vivo RNA interference demonstrates a role for Nrampl1 in modifying susceptibility to type 1 diabetes. Nat. Genet. 38: 479–483.

Kohjima, M., Y. Noda, R. Takeya, N. Saito, K. Takeuchi et al., 2002 PAR3beta, a novel homologue of the cell polarity protein PAR3, localizes to tight junctions. Biochem. Biophys. Res. Commun. 299: 641–646.

Laloraya, M., A. Davoodi-Semiromi, G. P. Kumar, M. McDuffie, and J. X. She, 2006 Improved Crkl expression contributes to the defective DNA repair capacity of the tranposon from Sleeping Beauty. J. Cell. Physiol. 206: 771–779.

Larosa, D. A., and L. S. Collier, 2008 Transposon-mediated mutagenesis in somatic cells: identification of transposon-genomic DNA junctions. Methods Mol. Biol. 435: 95–108.

Leiter, E. H., 2002 Mice with targeted gene disruptions or gene insertions for diabetes research: problems, pitfalls, and potential solutions. Diabetologia 45: 296–308.

Li, F., D. O. Cowley, D. Banner, E. Holle, L. Zhang et al., 2014 Efficient genetic manipulation of the NOD-Rag2–/–2LZgammac-null mouse by combining in vitro fertilization and CRISPR/Cas9 technology. Sci. Rep. 4: 5290.

Mariotta, L., T. Ramadan, D. Singer, A. Guetg, B. Herzog et al., 2012 T-type amino acid transporter TAT1 (Slc16a10) is essential for extracellular amino-acid transport by antigen receptors coordinates the metabolic reprogramming of the tranposon system. Nat. Methods 2: 763–769.

Matsuda, D. A., and L. S. Collier, 2008 Transposon-mediated mutagenesis in somatic cells: identification of transposon-genomic DNA junctions. Methods Mol. Biol. 435: 95–108.

Leiter, E. H., 2002 Mice with targeted gene disruptions or gene insertions for diabetes research: problems, pitfalls, and potential solutions. Diabetologia 45: 296–308.

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Mariotta, L., T. Ramadan, D. Singer, A. Guetg, B. Herzog et al., 2012 T-type amino acid transporter TAT1 (Slc16a10) is essential for extracellular amino-acid transport by antigen receptors coordinates the metabolic reprogramming of the tranposon system. Nat. Methods 2: 763–769.

Matsuda, D. A., and L. S. Collier, 2008 Transposon-mediated mutagenesis in somatic cells: identification of transposon-genomic DNA junctions. Methods Mol. Biol. 435: 95–108.

Leiter, E. H., 2002 Mice with targeted gene disruptions or gene insertions for diabetes research: problems, pitfalls, and potential solutions. Diabetologia 45: 296–308.

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Mariotta, L., T. Ramadan, D. Singer, A. Guetg, B. Herzog et al., 2012 T-type amino acid transporter TAT1 (Slc16a10) is essential for extracellular amino-acid transport by antigen receptors coordinates the metabolic reprogramming of the tranposon system. Nat. Methods 2: 763–769.