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Abstract: Modelling of human disease in genetically engineered pigs provides unique possibilities in biomedical research and in studies of disease intervention. Establishment of methodologies that allow efficient gene insertion by non-viral gene carriers is an important step towards development of new disease models. In this report, we present transgenic pigs created by Sleeping Beauty DNA transposition in primary porcine fibroblasts in combination with somatic cell nuclear transfer by handmade cloning. Göttingen minipigs expressing green fluorescent protein are produced by transgenesis with DNA transposon vectors carrying the transgene driven by the human ubiquitin C promoter. These animals carry multiple copies (from 8 to 13) of the transgene and show systemic transgene expression. Transgene-expressing pigs carry both transposase-catalyzed insertions and at least one copy of randomly inserted plasmid DNA. Our findings illustrate critical issues related to DNA transposon-directed transgenesis, including coincidental plasmid insertion and relatively low Sleeping Beauty transposition activity in porcine fibroblasts, but also provide a platform for future development of porcine disease models using the Sleeping Beauty gene insertion technology.

Pig transgenesis by Sleeping Beauty DNA transposition

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ABSTRACT

Modelling of human disease in genetically engineered pigs provides unique possibilities in biomedical research and in studies of disease intervention. Establishment of methodologies that allow efficient gene insertion by non-viral gene carriers is an important step towards development of new disease models. In this report, we present transgenic pigs created by Sleeping Beauty DNA transposition in primary porcine fibroblasts in combination with somatic cell nuclear transfer by handmade cloning. Göttingen minipigs expressing green fluorescent protein are produced by transgenesis with DNA transposon vectors carrying the transgene driven by the human ubiquitin C promoter. These animals carry multiple copies (from 8 to 13) of the transgene and show systemic transgene expression. Transgene-expressing pigs carry both transposase-catalyzed insertions and at least one copy of randomly inserted plasmid DNA. Our findings illustrate critical issues related to DNA transposon-directed transgenesis, including coincidental plasmid insertion and relatively low Sleeping Beauty transposition activity in porcine fibroblasts, but also provide a platform for future development of porcine disease models using the Sleeping Beauty gene insertion technology.

INTRODUCTION

Disease models in large animals play an increasing role in biomedical research and have important implications for the development of diagnostic tools and novel treatment of human diseases. Due to the anatomical and physiological similarities between humans and the pig, genetically engineered porcine disease models may be expected to recapitulate human pathophysiology more faithfully than do corresponding disease models in rodents. Also, a longer life-span may allow studies of chronic diseases, and a human-size body may enable studies of surgical intervention.

Pigs that are predisposed for a certain monogenic disease can be created by genetic engineering of the pig genome, in the least complicated approach by insertion of a proposed disease gene which may in a dominant fashion trigger development of the disease. Transgenesis in pigs has previously been achieved by transferring the transgene to oocytes or early embryos by pronuclear injection of foreign DNA (Hammer et al. 1985; Hirabayashi et al. 2001; Nottle et al. 2001), transduction with retro- and lentiviral vectors injected into the perivitelline space of the oocyte (Cabot et al. 2001; Hofmann et al. 2003; Whitelaw et al. 2004) or, alternatively, by sperm-mediated gene transfer (Naruse et al. 2005; Webster et al. 2005). With the successful cloning of animals by somatic cell nuclear transfer (SCNT), it is now possible to produce transgenic pigs from genetically engineered somatic donor cells. In SCNT, reported for a variety of animal species including the mouse (Wakayama et al. 2000; Wakayama and Yanagimachi 1999), the sheep (Wilmut et al. 1997), the cow (Cibelli et al. 1998; Kato et al. 1998; Kubota et al. 2000), and the pig (Betthausen et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000), the nucleus from a single differentiated somatic cell is transferred into an enucleated oocyte (unfertilized egg cell), and the reconstructed embryo is subsequently transferred to a surrogate mother. This procedure allows modification of the somatic donor cell in culture by transgene insertion or introduction of loss-of-function gene knockout mutations by homologous recombination. We have previously produced transgenic pig blastocysts by SCNT using a simplified and improved cloning procedure designated

handmade cloning (HMC) (Du et al. 2007; Kragh et al. 2004) and recently developed Göttingen minipigs expressing a randomly inserted human APP gene containing an Alzheimer's disease-causing dominant mutation (Kragh et al. 2009).

Permanent genetic modification of somatic donor cells for SCNT has been achieved by random genomic insertion of plasmid DNA (Hyun et al. 2003; Kragh et al. 2009; Kragh et al. 2004; Watanabe et al. 2005) or by genomic integration of transduced retroviral or lentiviral vectors (Lai et al. 2002; Park et al. 2001; Park et al. 2002). In both non-viral and viral approaches inclusion of a drug resistance gene in the transgene-encoding vectors allows selection for cells containing the integrated vector. Random insertion of plasmid DNA into the genome of somatic donor cells occurs as a result of breakage of the plasmid DNA. Breakage within the transgene cassette may result in drug-selected cell clones and eventually transgenic pigs that do not express the transgene. Moreover, randomly inserted plasmid DNA is thought to be more prone to formation of concatamers during the integration process which may affect gene expression (Garrick et al. 1998; Leahy et al. 1997). High gene transfer efficiency and genomic insertion of a defined genetic entity are major advantages of transgenesis by retro- and lentiviral vectors. However, both vector types preferentially target transcriptionally active regions of the genome (Mitchell et al. 2004; Schroder et al. 2002) and are, therefore, likely to insert near or into genes. Also, these technologies require classified laboratory facilities and extra precaution with respect to housing of the transgenic animals.

In this report, we describe the use of Sleeping Beauty (SB) DNA transposon-based vectors for creation of genetically engineered Göttingen minipigs expressing the green fluorescent protein (GFP) reporter gene. Transposable DNA elements are mobile genetic entities that integrate into genomic DNA in a transposition process catalyzed by the transposase enzyme encoded by the element itself. SB is a cut-and-paste DNA transposon belonging to the Tc1/*mariner* superfamily of transposon elements. SB was genetically reconstructed from fossil elements in the genome of salmoid fish (Ivics et al. 1997) and is

actively mobilized in cells derived from a variety of vertebrate species (Izsvak et al. 2000). SB-derived vectors efficiently jump from plasmid DNA introduced into cells to chromosomal DNA and are inserted without particular preference for actively transcribed genes (Liu et al. 2005; Yant et al. 2005). The element has been explored for the use in therapeutic gene transfer (Izsvak and Ivics 2004; Liu et al. 2006; Mates et al. 2009; Mikkelsen et al. 2003; Ohlfest et al. 2004; Singh et al. 2008; Yant et al. 2000) and is widely used as a tool in animal transgenesis. SB-directed transgenesis is well-established in zebrafish (Davidson et al. 2003), and germline mutagenesis by microinjection of transposon DNA and transposase-encoding nucleic acids into fertilized oocytes has been established in the mouse (Dupuy et al. 2002; Mates et al. 2009) and the frog (Sinzelle et al. 2006; Yergeau et al. 2009).

The properties of SB as an efficient and genetically simple non-viral transgene delivery system facilitating gene insertion with a random integration profile make SB-derived vectors relevant tools for transgenesis also of larger animals. A routine use of transposons may be expected to improve the efficiency of cellular transgenesis and to reduce concatamer formation and integration of CpG-rich plasmid sequence (Clark et al. 2007a). By transfecting skin-derived porcine fibroblasts with plasmid DNA carrying the GFP reporter gene, we previously reported production of blastocysts harboring randomly inserted plasmid DNA (Kragh et al. 2004). Active transposition of SB-derived vectors in porcine fibroblasts has recently been documented (Clark et al. 2007b). Using the GFP gene as a reporter, we here combine SB-directed engineering of neonatal porcine fibroblasts with HMC-based SCNT and production of transgenic cloned pigs. We describe events of SB transposition leading to the production of transgenic cloned pigs with uniform GFP expression in all analyzed organs, but also describe potential critical issues on the path towards creating porcine disease models using the SB technology.

RESULTS

Generation of transgenic pigs by SCNT using SB transposon-modified neonatal porcine fibroblasts

Production of transgenic pigs by SCNT is based on the genetic engineering of primary porcine cells prior to nuclear transfer. To investigate the potential use of SB-derived vectors in minipig transgenesis, we constructed a SB transposon vector, designated pSBT/cHS4.Ubi-GIN.cHS4, harboring a bicistronic gene cassette with a reporter gene encoding green fluorescent protein (GFP) linked to a neomycin resistance gene (neo^r) through an internal ribosomal entry site (IRES) (Fig. 1A). The human ubiquitin C promoter (Ubi) was utilized to drive expression, and the expression cassette was flanked on each side by 1.2-kb long 5'-HS4 chicken β -globin (cHS4) insulators, which have been shown to protect the genetic cargo of SB transposons from transcriptional silencing (Dalsgaard et al. 2009).

Transposition in neonatal porcine fibroblasts (NPFs) was carried out by co-transfecting pSBT/cHS4.Ubi-GIN.cHS4 with an expression construct, pCMV-HSB3, encoding the hyperactive HSB3 transposase variant. This transposase variant has previously been shown to increase levels of transposition more than 5-fold compared to the original SB10 transposase (Yant et al. 2004). By transfecting supercoiled transposon donor plasmid and HSB3-encoding plasmid in a 40:1 ratio (using 50 ng of pCMV-HSB3), the number of G418-resistant NPF colonies was increased 12-fold relative to an experiment in which HSB3-expressing plasmid was replaced by the pUC19 plasmid (Fig. 1B). This suggested that the SB transposon was actively mobilized by the HSB3 transposase from transfected plasmid DNA and inserted into genomic DNA of NPFs derived from a newborn Göttingen minipig.

We next created a pool of clones consisting of a total of 80 independent G418-resistant NPF clones. The pool was expanded, and transgenic blastocysts were created by HMC. A total of 102 blastocysts were transferred to the surrogate sow. Eight pigs were born alive and one pig was stillborn, and none of the pigs showed any sign of abnormalities. Five of the pigs survived past weaning (Fig. 1C).

Ear cuts from these five pigs (pigs #6-9, and #13) were analyzed for fluorescence under blue light, and four of the five pigs were found to express GFP (Fig. 1D; pigs #6-8 and #13). These findings were confirmed by fluorescence microscopy of cultured fibroblasts derived from the ear biopsies (data not shown). Furthermore, only fibroblasts derived from the four GFP-positive pigs survived 10 days of culturing in G418-containing medium, whereas fibroblasts from the GFP-negative pig proved not to be G418-resistant. These observations indicated that at least one intact GFP-IRES-neo^r cassette was present in four out of five pigs.

Multiple integrations of the transgene cassette in GFP-expressing pigs

To characterize the transgene cassette insertion in the cloned pigs, we first performed Southern blot analysis, using a neo^r probe, on genomic DNA isolated from blood samples. The genomic DNA was digested with *Xba*I, which cuts once in the transposon (in the GFP gene) and in the case of a random insertion in the plasmid region flanking the right IR of the transposon (Fig. 2A). Among the four GFP-positive pigs (#6-8 and #13), three different banding patterns were observed (Fig. 2B, lanes 2-4 and 6). The band patterns in lanes 2 and 3 were identical and suggested a total of 13 insertions. Hence, the two pigs (#6 and #7) originated from cells from the same NPF clone. Two of the pigs (#8 and #13), each containing eight insertions, had almost identical banding patterns except for two single bands (position marked with arrows in Fig. 2B) that varied between the two animals (Fig. 2B, lanes 4 and 6). These two pigs were most likely generated from the same NPF clone which happened to be polyclonal due to late SB insertions or, alternatively, due to re-mobilization of an inserted DNA transposon during clone development and selection. Interestingly, the GFP-negative pig (#9) was indeed transgenic but contained only randomly inserted plasmid DNA, as indicated by the detection of a 4.5-kb fragment (Fig. 2B, lane 5). Our data suggested that the five pigs were derived from a total of three NPF clones selected among the 80 clones of the original pool. This lends support to the notion that a strong selection pressure is evident

at one or more steps of the cloning procedure including expansion of pooled clones, generation of blastocysts, and/or embryo and fetal development.

The Southern blot analysis suggested that random plasmid insertion caused by plasmid breakage in the region outside the *Xba*I-flanked GIN cassette (resulting in a 4.5-kb fragment) occurred in all pigs but was prominent only in the GFP-negative pig. In this pig, the 4.5-kb band was representative of two independent random insertions, which was confirmed by the presence of two separate bands on a Southern blot, where enzymes cutting within the Ubi promoter and within the IRES sequence were used (data not shown).

We have previously found evidence of random insertion of transposase-encoding plasmid sequences in a transgenic cloned Göttingen minipig (J. E. Jakobsen, A. L. Jørgensen, A. L. Nielsen, J. G. Mikkelsen; unpublished observations). A sample of genomic DNA from this animal was utilized as a positive control in a PCR-based analysis of the presence of such sequences in the five transgenic pigs studied here. We did not find any evidence of integration of transposase-encoding plasmid in any of the five transgenic pigs (Fig. 2C, lanes 1-5 and 8-12) or a negative control (Fig. 2C; pig #78, lanes 6 and 13), whereas PCR analysis of genomic DNA from the positive control pig showed evidence of the transposase gene (Fig. 2C; pig #58, lanes 7 and 14).

Evidence of transposase-directed gene insertion by analysis of integration sites in transgenic pigs

To confirm that SB transposase-directed gene integration was a prevalent mechanism of insertion during transgenesis, we performed long distance inverse PCR (LDI-PCR) on one of the pigs (pig #8) containing eight insertions to identify junction sites between transposon and genomic DNA. PCR amplification of genomic DNA digested with either *Bsr*GI or *Bsr*GI/*Nde*I resulted in a total of five amplicons, all of which were found by sequence analysis to carry the hallmarks of a SB transposon insertion. Four of these insertions were unique and could be mapped to different genomic loci, all situated in intergenic regions

of the pig genome (Fig. 3). In summary, our genetic analyses show that SB-directed transgenesis was achieved by gene insertion into intergenic regions.

Systemic expression of transgenic GFP in pigs harboring multiple SB-directed insertions

We sacrificed pig #8 to carry out careful fluorescence imaging of the animal in comparison with an age-matched animal as negative control. As shown in Fig. 4A through Q, GFP expression was detected in all seventeen analyzed tissues of the transgenic pig including brain, eye, skeletal muscle, heart, lung, liver, and skin. Some autofluorescence could be detected in the liver, heart, and rib bone of the control pig but fluorescence was still considerably stronger in the tissues from the transgenic pig (Fig. 4, panels B1/B2, G1/G2, and P1/P2). Heart and testis (Fig. 4, panels G2 and H2) did not show similar uniform expression of GFP compared to the remaining organs, and the fat layer covering the heart had increased fluorescence compared to the smooth heart muscle (Fig. 4, panels G1 and G2), whereas fluorescence in the epididymis was considerably higher than in the testis (Fig. 4, panels H1 and H2). This pig contained a minimum of four and possibly more SB transposase-directed transgene insertions flanked by cHS4 insulators. It was not possible at this stage to determine the contribution of each of the insertions to the overall expression of GFP. The animals are currently being bred and future transgenic offspring will be analyzed for persistency of and differences in transgene expression.

DISCUSSION

We describe here the production of transgenic cloned pigs by HMC-directed nuclear transfer using primary porcine fibroblasts modified by genomic insertion of a GFP-tagged Sleeping Beauty DNA transposon vector. By SB transposase-directed insertion of the GFP marker gene, we established Göttingen minipigs with systemic expression of the transgene. Hence, all of seventeen analyzed tissues, including brain, eye, muscle, heart, lung, liver, and skin, derived from pigs containing SB vector insertions were found to express GFP by fluorescence imaging.

SB facilitates efficient gene insertion in a wide panel of cell types (Izsvak et al. 2000) and represents an attractive non-viral approach for gene delivery and insertion in both gene therapy applications and animal transgenesis. The level of stable transfection mediated by SB in a particular cell type is under influence of the DNA transfection efficiency as well as the SB mobilization rate, both of which may vary considerably between cell types. SB transposition is supported by cellular factors like the DNA-bending protein HMGB1 (Zayed et al. 2003) and is influenced by the transposase concentration, which is likely to vary between cell types depending on efficacy of DNA uptake and expression vector design (Mikkelsen et al. 2003). In NPFs, the level of stable transfection obtained with the HSB3 transposase was significantly increased over background but was relatively low compared to levels that we routinely measure in murine or human cell lines. As the NPFs were quite efficiently transfected with at least 50 % of the cells transiently expressing the transgene (data not shown), our findings most likely reflect suboptimal transposition rates in NPFs. Still, the HSB3 transposase increased the level of gene insertion (as measured by the number of drug-resistant colonies) 12-fold relative to the negative control in which pUC19 plasmid was co-transfected with the transposon donor plasmid. In analogy, previous work by Clark and co-workers showed that the SB11 transposase variant increased colony formation in pig fetal fibroblasts 2.5-fold over fibroblasts transfected with plasmid encoding an inactive transposase variant (Clark et al. 2007b). To create a procedure for production of transposon-tagged transgenic pigs

with reduced risk of inserting the transposase gene in the animals, we utilized low amounts of SB transposase-encoding plasmid (50 ng plasmid) during *in vitro* manipulation of NPFs. Evidence of transposase-directed transgene insertion was obtained in the GFP-transgenic litter. Hence, in one of the pigs, for which the SB insertions were carefully mapped, at least four SB transposase-directed insertions out of a total of eight insertions could be detected.

Integrating viral vectors are directed towards transcriptionally active regions in the genome due to specific interactions between cellular and viral proteins (Ciuffi et al. 2005). One of the advantages of SB transposon vectors is the random integration profile (Liu et al. 2005; Staunstrup et al. 2009; Yant et al. 2005). Thus, SB vectors are less likely to target genes but may be more vulnerable to transcriptional silencing when inserted in heterochromatin. In cell lines the immediate silencing of SB vectors after insertion can be relieved by flanking the transgene with *chs4* insulators (Dalsgaard et al. 2009), and downstream analyses of the SB-transgenic pigs will assist in documenting the potential benefits of transgene insulation.

Although HSB3 mediated increased stable transfection in NPFs, at least one random insertion containing remnants of the transposon donor plasmid backbone was identified in each of the five pigs. Notably, one of the pigs was found to contain two random insertions only. This pig did not express the transgene, suggesting that the two transgene cassettes were transcriptionally silenced or, as cannot be formally excluded, that the plasmid breakage point in both cases affected both genes of the transgene expression cassette **at some point after initial selection**.

Transposase-encoding plasmid could not be detected in any of the five transgenic pigs. This is an important finding since continued expression of the transposase could potentially facilitate transposon re-mobilization and re-insertion, resulting in reduced overall stability of the pig model. Although this aspect is rarely experimentally addressed in reports describing transposon-directed gene delivery, methods for co-delivery of transposon donor plasmid with *in vitro*-synthesized mRNA encoding the

transposase to somatic cells have been described (Wilber et al. 2006) and may be applicable in protocols of SB-directed pig transgenesis. Also, the use of *in vitro*-transcribed mRNA as a source of transposase has been successful in early embryos of frog (Sinzelle et al. 2006), zebrafish (Davidson et al. 2003), and mouse (Dupuy et al. 2002).

Cloning based on a pool consisting of 80 NPF clones resulted in birth of one set of genetically identical twins and a pair of pigs originating from a single NPF clone. This indicates that the genetic engineering process and cloning by HMC-directed SCNT is accompanied by a selection pressure. Whether this pressure is in effect during *in vitro* cell expansion or during blastocyst or embryo development is not clear. Also, it remains a matter of speculation whether these observations reflect any toxicity related to the actions of the SB transposase. Despite the fact that four of the five pigs contained at least 8 insertions, we find it unlikely that these clones had a selective advantage due to their high copy numbers. However, our data suggest that a cloning strategy based on expanded pools is more likely to succeed, less labor-intensive, and cheaper than cloning using individual clones. Moreover, by growing pools rather than individual drug-resistant clones we could minimize the number of cell cycles and limit the risk of chromosomal aberrations during *in vitro* culturing of the fibroblasts.

Retro- and lentiviral vectors are established potent tools for stable modification of porcine somatic donor cells for SCNT (Lai et al. 2002; Park et al. 2001; Park et al. 2002) and have been successfully used for transgene delivery to porcine embryos (Cabot et al. 2001; Hofmann et al. 2003; Whitelaw et al. 2004). SB-directed transgenesis combined with SCNT and HMC represents a nonviral alternative that offers insertion of a defined genetic unit, strong systemic transgene expression, and possibilities of multicopy transgene insertion. Still, the moderate efficiency of the cloning procedure requires that a significant amount of nuclear transfers is performed. In our hands, 102 blastocysts (resulting from more than 250 reconstructed embryos) were transferred to the surrogate sow to create a transgenic litter of eight piglets. Five of these pigs survived past weaning, whereas three pigs died

without signs of any macroscopic malformations, as suggested by the post-mortem examination. Reports of lentiviral transgenesis based on injection of viral particles into the perivitelline space of single-cell embryos have shown birth of as many as 34 transgenic pigs from 244 zygotes treated with human immunodeficiency virus-derived vectors (Hofmann et al. 2003) and 37 transgenic pigs from a total of 120 zygotes treated with vectors based on equine infectious anaemia virus (Whitelaw et al. 2004). Notably, a transposon-based approach does not require classified laboratory facilities for genetic engineering of somatic cells, production of early embryos, or housing of the animals. Our protocol may be further refined for example by use of the highly potent SB100X transposase (Mates et al. 2009) encoded by trace amounts of transfected plasmid DNA or *in vitro*-transcribed RNA. We believe that the SB system combined with SCNT has the potential of becoming a preferred tool for introduction of dominant mutations or shRNA genes in the porcine genome and that this approach will prove to be of high value for creation of disease models in the pig.

MATERIALS AND METHODS

Vector construction

The transposon donor vector, pSBT/cHS4.Ubi-GIN.cHS4, was generated by inserting PCR-amplified 1.2-kb cHS4 insulators into linkers flanking the gene expression cassette in the vector pSBT/Ubi-GIN. This vector contains a GFP-IRES-neo^r (GIN) cassette driven by the human ubiquitin C promoter (Ubi) and was derived from pSBT/RSV-FGIP (Moldt et al. 2008). The GFP sequence, originally derived from pEGFP.N1 (Clontech, Mountain View, CA, USA), contained a Flp recognition sequence (FRT) inserted between the start codon and the remaining part of the eGFP gene, as described in (Moldt et al. 2008). This chimeric variant of the gene is here simply designated 'GFP'. pSBT/Ubi-GIN was generated by replacing the SV40 promoter of pSBT/SV40-FGIP (similar, except for the promoter, to pSBT/RSV-FGIP) with the human ubiquitin C promoter PCR-amplified from pUbiC-Jun (Schorpp et al. 1996) using the following two primers: 5'-GTAAACGCGTAGATCTGGCCTCCGCGCC-3' and 5'-CGGTCCATGGAAGCTTCGTCTAACAAAAAAG-3'. The fragment was inserted into *MluI/NcoI*-digested pSBT/SV40-FGIP. The IRES-puro cassette of pSBT/Ubi-FGIP was replaced with an IRES-neo^r cassette PCR-amplified from pCMV-Bipep (Tolstrup et al. 2001) creating pSBT/Ubi-GIN. pCMV-HSB3 was previously described (Yant et al. 2004).

Generation of transgenic porcine fibroblasts by transfection of SB transposon vectors

Neonatal porcine fibroblasts (NPFs) were cultured from ear biopsies of newborn Göttingen minipig no. 74113 (Ellegaard Göttingen Minipigs A/S, Soro Landevej 302, DK-4261 Dalmore, Denmark) as previously described (Kragh et al. 2009). The cells were grown in DMEM to 50 % confluence, trypsinized, and resuspended for further expansion prior to freezing of aliquots. For production of a transgenic litter, 2×10^5 fibroblasts were transfected in a 60-cm² dish (in 10 ml medium) using 2 µg of the pSBT/cHS4.Ubi-GIN.cHS4 transposon plasmid and 50 ng pCMV-HSB3 or as control 50 ng pUC19 plasmid. 10 µl FuGENE 6

was used in the reaction mixture. On the following day, the cells were washed with PBS and subsequently cultured in G418-containing medium (0.75 µg/ml) for two weeks. A total of 80 colonies were pooled and grown for 9 days prior to SCNT by HMC.

Handmade cloning (HMC) using SB-treated NPFs

Handmade cloning was performed as previously described (Du et al. 2007). Briefly, oocytes with partially digested zonae pellucidae were enucleated by oriented bisection according to the polar body position. For each oocyte, the part without chromatin, i.e. the cytoplasm, was collected and electrofused with one transgenic fibroblast modified by SB to carry the cHS4.Ubi-GIN.cHS4 cassette. Another cytoplasm was then electrofused with each cytoplasm-fibroblast pair during a second fusion round which also activated the reconstructed embryo. After 6 days the *in vitro* cultured embryos were developed into transgenic blastocysts. Day 5 and 6 blastocysts with clearly visible inner cell mass were surgically transferred to a Danish landrace sow on day 4 or 5 after weaning. Pregnancy in the surrogate sow was diagnosed by ultrasonography on day 21 and confirmed during later stages of the pregnancy. Pigs were delivered by natural birth on day 114 and raised by their surrogate mother. Pregnancy was established by transfer of 102 cHS4.Ubi-GIN.cHS4-transgenic blastocysts to the surrogate sow. Among nine delivered pigs, one was stillborn and five pigs (referred to as pigs #6-9 and #13) survived past weaning. The non-transgenic wildtype pig included as a negative control pig was designated #78.

Analysis of transgene expression

5-mm ear cuts and fibroblasts derived from ear biopsies were analysed by fluorescence microscopy. Green fluorescent protein (GFP) was monitored with a 492 nm excitation filter and a 508 nm emission filter, exposing for 0.5 sec. Organs and tissues from selected transgenic pigs and control pigs were imaged using a Kodak Multispectral In-Vivo FX machine (Carestream Inc, New Haven, CT, USA). For each

organ or tissue two images were acquired, one at white light (excitation: 0 nm; emission: 600 nm; exposure time: 0.175 sec; F-stop:22.14; no binning) and one by which GFP fluorescence was monitored by using the multi-wavelength function with a 480 nm excitation filter and a 535 nm emission filter, exposing for 1 sec. with f-stop: 2.51 and 2 x 2 binning. Every two images were acquired using the same field of view (FOV). After acquisition, pictures taken in full light were added a scale bar. The fluorescence emission picture was artificially coloured green in the Kodak IM Software v1.0 according to the signal detected using the specified filter set.

Copy number analysis by Southern blotting

Six µg of genomic DNA was digested with restriction enzymes, loaded on a 0.7 % agarose gel and electrophoresed overnight at 1 V/cm. The DNA was transferred to a nylon membrane (Roche, Basel, Switzerland) by vacuum suction. During transfer, the DNA was nicked and denatured by 0.25 M HCl and 1.5 M NaCl plus 0.5 M NaOH, respectively. The membrane was prehybridized in 5xSSPE (0.75 M NaCl, 0.05 M NaH₂PO₄, 0.005 M EDTA), 5x Denhart (0.1 % Ficoll400, 0.1 % polyvinylpyrrolidone, 0.1 % BSA), 1 % SDS, 50 % formamide, 5 % dextranulphate and single-stranded DNA carrier (0.5 mg/ml) overnight at 42 °C. The DNA probe, a 670-bp fragment of the neomycin resistance gene, was labelled with P³²-labelled CTP by random priming, heat-denatured, added to prehybridization solution, and incubated with the membrane overnight at 42 °C. The membrane was washed in 2x SSPE for 2x5 min at 26 °C, in 2x SSPE, 0.5 % SDS for 2x15 min at 63 °C, and in 0.2x SSPE, 0.5 % SDS for 15 min at 63 °C. The membrane was air-dried prior to X-ray film exposure.

Detection of genomically integrated transposase gene by PCR

To investigate whether or not the SB transposase-encoding helper plasmid had been incorporated into the genome of the SB-transgenic fibroblasts, we used PCR with two set of primers. The first primer pair

(5'-GCTTAGGGTTAGGCGTTTTG-3' and 5'-GGTTATGTCGATATAGGACTCG-3') spanned part of the CMV promoter and the 5' ORF in the transposase. The second primer pair (5'-GCCAGACTACGGTTTGCAAC-3' and 5'-CACCTTCCAGGGTCAAGGAAG-3') amplified the transposase ORF.

Identification of SB vector insertion sites by long distance inverse PCR (LDI-PCR)

Templates for amplification of junction sites between transposon and genomic DNA were generated as follows: 0.5 µg genomic DNA was digested with either *BsrGI* alone or together with *NdeI*, purified on a GFX column and eluted in 40 µl ddH₂O. After adjustment with ddH₂O and 10x T4 ligase buffer, T4 ligase was added, and the 500 µl ligation mix was incubated at 16 °C overnight. Ligated DNA was purified on a GFX column and eluted in 40 µl ddH₂O, and 2 µl was used as template in the first PCR reaction (33 cycles, 20 pmol primers and annealing temperature of 59 °C) using primers with recognition sequences within the right inverted repeat (5'-GTCCTGCTGGAGTTCGTGAC-3') and within the *neo^r* gene (5'-GGCACAGTCAACTTAGTGTATG-3'). Subsequently, 1 µl of this PCR reaction was used as template in a nested PCR (30 cycles, 10 pmol primers and annealing temperature of 58.5 °C) using the primers 5'-ATCACTCTCGGCATGGAC-3' and 5'-GATGTCCTAACTGACTTGCC-3'. The resulting PCR products were gel-purified and sequenced.

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FIGURE LEGENDS

Figure 1. Generation of GFP-transgenic pigs by insertion of insulated GFP-encoding SB vectors in porcine fibroblasts. (A) Schematic map of the SB transposon vector, pSBT/cHS4.Ubi-GIN.cHS4, used to create transgenic pigs. The borders of the transposon are indicated by grey arrows representing the left and right inverted repeats (LIR and RIR). The vector contains a transgene GFP-IRES-neo^r cassette flanked by 1.2-kb long 5'-HS4 chicken β -globin (cHS4) insulators. (B) Stable transfection efficiency in NPFs. The diagram depicts the number of G418-resistant colonies measured by co-transfection of NPFs with pSBT/cHS4.Ubi-GIN.cHS4 with either pUC19 or the HSB3-encoding plasmid pCMV-HSB3 using a 40:1 plasmid ratio. The experiment was performed in triplicates and the data are presented as mean \pm SD. A mean of 7 and 90 colonies was observed in cultures transfected with pUC19 and pCMV-HSB3, respectively. (C) Five young Göttingen minipigs (pigs #6-9 and #13) established by SCNT. (D) GFP expression in ear cuts from the five Göttingen pigs. Ear cuts were analyzed in normal light (a, c, e, g, and i) and under blue light (b, d, f, h, and j). Samples were derived from the pigs (#6-9 and #13) indicated above each set up of pictures.

Figure 2. Genetic analysis of transgenic pigs harboring the SBT/cHS4.Ubi-GIN.cHS4 cassette. (A) Schematic map of the pSBT/cHS4.Ubi-GIN.cHS4 plasmid with relevant restriction sites, used in the Southern blot analysis, indicated by dashed lines. Restriction sites used for LDI-PCR are shown in bold and marked with arrows. The small arrowheads indicate location of primers used in the LDI-PCR. (B) Southern blot analysis of genomic DNA isolated from SBT/cHS4.Ubi-GIN.cHS4-transgenic pigs and digested with *Xba*I. A 670-bp neo^r fragment was used as probe. Lanes 2-6 contain *Xba*I-digested genomic DNA from the five SB-transgenic pigs shown in Figure 1C. Negative controls are provided in lanes 7 and 8 which contain *Xba*I-digested DNA from a control pig (#78) and a porcine cell line (PFV) which did not

contain the *neo^r* gene, respectively. Pig identification numbers are provided above each lane. Lane 1 contains a marker. (C) The transposase gene cassette could not be detected in genomic DNA. Lanes 1-5 and 8-12 represent PCR reactions performed on genomic DNA from each of the five GFP-transgenic pigs using two primer sets (one amplifying part of the CMV promoter plus upstream part of the HSB3 gene and one amplifying the HSB3 ORF). PCR reactions performed on DNA from a wildtype pig were loaded in lanes 6 and 13, whereas PCR reactions performed on genomic DNA from a control pig containing a genomic insertion of the transposase gene were included as positive controls (lanes 7 and 14).

Figure 3. Identification of SB transposon insertion sites in intergenic regions of the porcine genome. Junction sites between transposon DNA and flanking DNA were identified by LDI-PCR in transgenic pig #8 harboring eight copies of the transgene cassette. Genomic DNA was digested with *BsrGI* or *BsrGI/NdeI* prior to PCR amplification with the primers shown in Figure 2A. Four SB insertion sites, all located in intergenic regions, were identified in pig #8 by a Blast searching the porcine genome using sequence tags identified by LDI-PCR. For one of the insertions (indicated with *), only a 25-bp sequence tag could be identified due to the short length of the LDI-PCR product. The vector insertion into chromosome 3 was flanked by a short stretch of TA-repeats which represents a potential consensus SB insertion site (Vigdal et al. 2002).

Figure 4. Systemic GFP expression in the SBT/cHS4.Ubi-GIN.cHS4-transgenic pig #8. Organs and tissues isolated from pig #8 and one wild type pig were analyzed by normal and blue light excitation. The left and right side of each picture shows organ/tissue from the wild type pig and the pSBT/cHS4.Ubi-GIN.cHS4 pig #8, respectively. The following organs or tissues were analyzed: A, Brain; B, Rib bone; C, Skeletal muscle; D, Salivary gland; E, Tongue; F, Eye; G, Heart; H, Testis; I, Subcutaneous fat; J, Pancreas; K, Spleen; L, Bladder; M, Colon; N, Kidney; O, Lung; P, Liver, and Q, Skin. Diagrams A1 through Q1 show

samples under normal light displayed in black and white. A2 through Q2 show samples under blue light excitation (480 nm) allowing visualization of GFP.

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ABSTRACT

Modelling of human disease in genetically engineered pigs provides unique possibilities in biomedical research and in studies of disease intervention. Establishment of methodologies that allow efficient gene insertion by non-viral gene carriers is an important step towards development of new disease models. **In this report, we present transgenic pigs** created by Sleeping Beauty DNA transposition in primary porcine fibroblasts in combination with somatic cell nuclear transfer by handmade cloning. Göttingen minipigs expressing green fluorescent protein are produced by transgenesis with DNA transposon vectors carrying the transgene driven by the human ubiquitin C promoter. These animals carry multiple copies (from 8 to 13) of the transgene and show systemic transgene expression. Transgene-expressing pigs carry both transposase-catalyzed insertions and at least one copy of randomly inserted plasmid DNA. Our findings illustrate critical issues related to DNA transposon-directed transgenesis, including coincidental plasmid insertion and **relatively low Sleeping Beauty** transposition activity in porcine fibroblasts, but also provide a platform for future development of porcine disease models using the Sleeping Beauty gene insertion technology.

To
Dr. Bruce Whitelaw
Editor in Chief
Transgenic Research

Dear Dr. Whitelaw

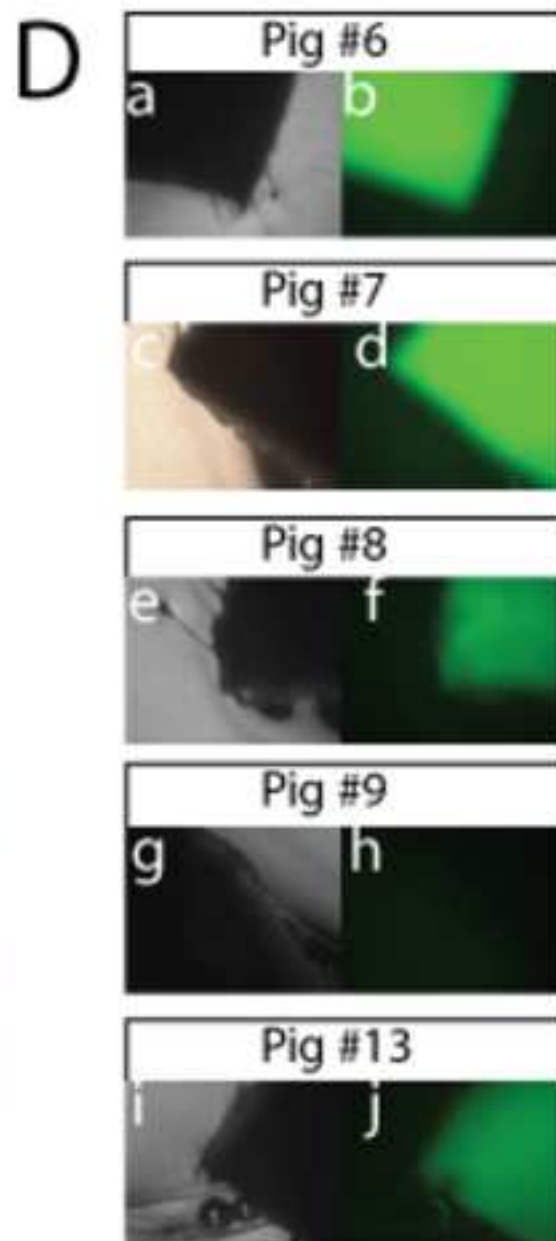
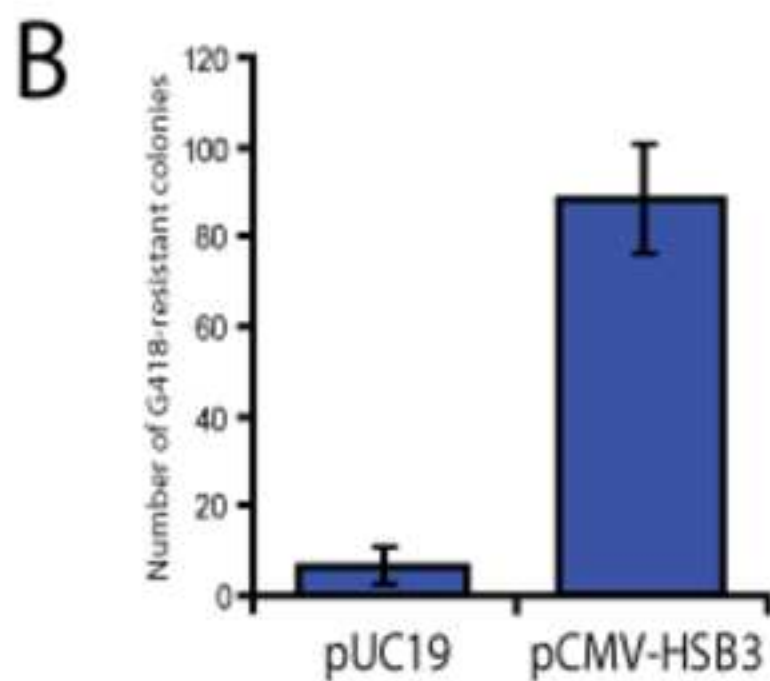
We appreciate the nice words and are grateful that the manuscript has now been finally accepted for publication in Transgenic Research.

We have considered the two specific comments mentioned and have revised the manuscript accordingly. However, regarding the first comment, we chose not to include 'immortalised'. In the work by Clark and co-workers transposition is carried out in both pig fetal fibroblasts (called PFFs in the paper) and in the immortalized pig cell line PEGE. The results that we refer to (2.5-fold increase in the presence of SB11) were created by transposition in PFFs whereas transposition in PEGE cells gave a 10-fold increase. We think that the comparison with the data from PFF is most relevant for our study and therefore did not introduce further changes in the manuscript.

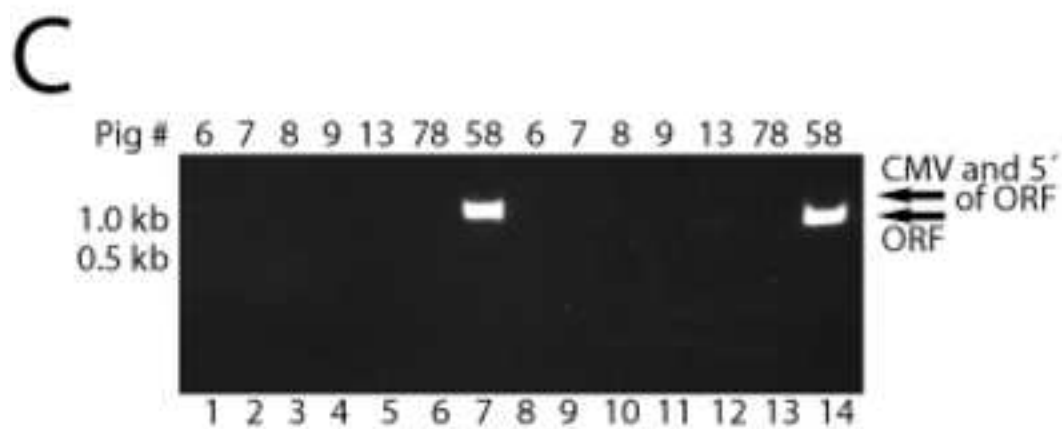
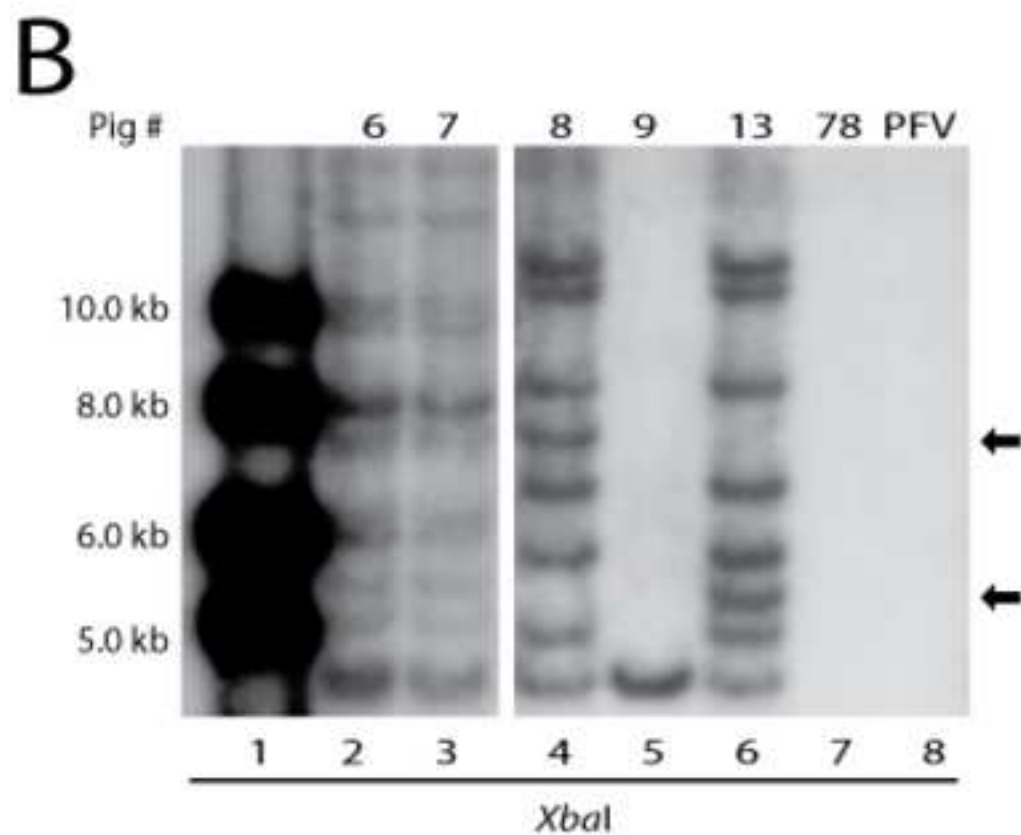
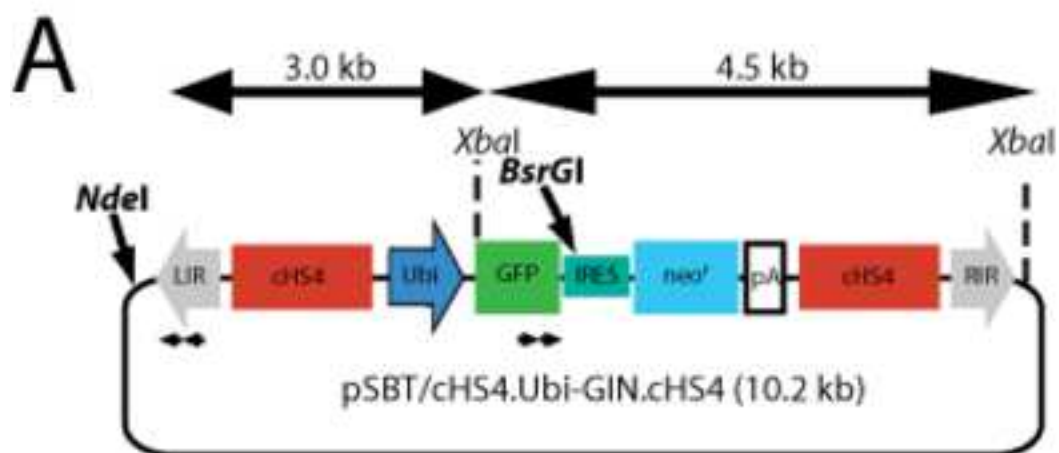
Sincerely,

Jacob Giehm Mikkelsen, PhD
Corresponding author

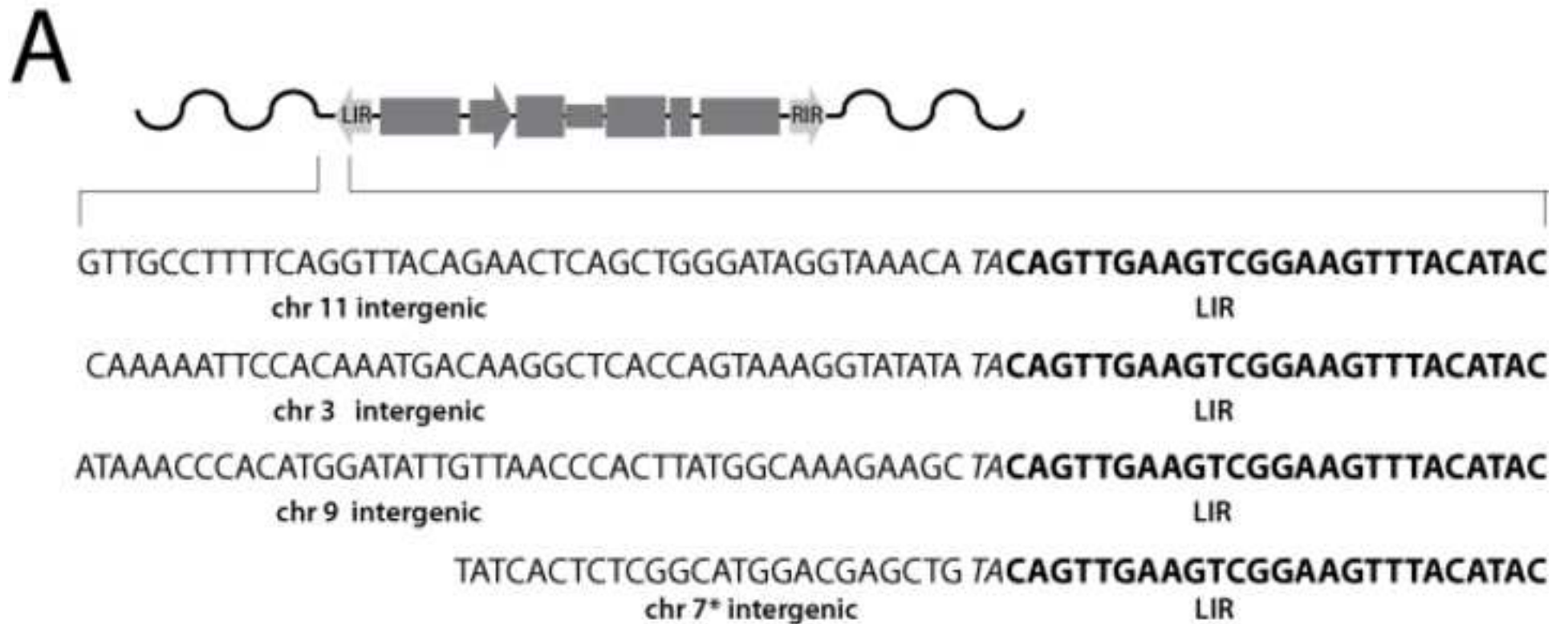
Jakobsen et al. Fig. 1



Jakobsen et al. Fig. 2



Jakobsen et al. Fig. 3



Jakobsen et al. Fig. 4

