RESEARCH COMMUNICATION

PiggyMac, a domesticated *piggyBac* transposase involved in programmed genome rearrangements in the ciliate *Paramecium tetraurelia*

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Programmed genome rearrangements drive functional gene assembly in ciliates during the development of the somatic macronucleus. The elimination of germline sequences is directed by noncoding RNAs and is initiated by DNA double-strand breaks, but the enzymes responsible for DNA cleavage have not been identified. We show here that PiggyMac (Pgm), a domesticated *piggyBac* transposase, is required for these rearrangements in *Paramecium tetraurelia*. A GFP-Pgm fusion localizes in developing macronuclei, where rearrangements take place, and RNAi-mediated silencing of *PGM* abolishes DNA cleavage. This is the first in vivo evidence suggesting an essential endonucleolytic function of a domesticated *piggyBac* transposase.

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Results and Discussion

A hallmark of ciliates is the separation of two versions of the genome—a germline and a rearranged somatic version—into two functionally distinct nuclei, a micronucleus (MIC) and a macronucleus (MAC), respectively, that coexist in the cytoplasm of these unicellular eukaryotes. The MAC is responsible for gene transcription but is lost at each sexual cycle, while the MIC undergoes meiosis

and gives rise to new MICs and MACs in sexual progeny. During new MAC development, the genome undergoes massive DNA amplification, together with extensive rearrangements (Jahn and Klobutcher 2002; Yao et al. 2002). In *Paramecium*, imprecise elimination of germline sequences, including essentially all transposable elements, leads to chromosome fragmentation or to internal deletions (Le Mouél et al. 2003). In addition, the precise excision of ~60,000 single-copy, noncoding internal eliminated sequences (IESs) is essential for the assembly of functional genes (for review, see Bétermier 2004). IESs are invariably bounded by two 5'-TA-3' dinucleotides, only one of which remains in the MAC sequence after excision, and a loosely conserved 8-base-pair (bp) consensus sequence (5'-TAYAGYNR-3') was defined from a statistical analysis of their ends (Klobutcher and Herrick 1995). This consensus shows some similarity to the ends of Tc/*mariner* class II transposons, which duplicate their TA target site upon insertion, and it was proposed that *Paramecium* IESs originate from an ancient invasion of the MIC genome by Tc/*mariner* elements that would have degenerated during evolution, while remaining under selective pressure for precise excision from the MAC genome. It was suggested that an ancestral Tc/*mariner* transposase could have been domesticated by the host to catalyze efficient excision of inactivated elements (Klobutcher and Herrick 1997). However, *Paramecium* IES excision starts with staggered double-strand breaks (DSBs) generating 4-base 5' overhangs at both IES ends, centered on the conserved TA (Gratias and Bétermier 2003). Subsequent pairing of the TA dinucleotides may drive the precise closure of IES excision junctions. This geometry differs significantly from the DNA cleavage that initiates Tc/*mariner* cut-and-paste transposition, which produces 3' overhangs and leaves the two flanking TAs at the donor site (Plasterk and van Luenen 2002).

In contrast, *piggyBac* class II transposons generate no footprint at their donor site, which is reconstituted precisely upon excision to restore the initial 5'-TTAA-3' target sequence (Elick et al. 1996). In vitro, the *piggyBac* transposase catalyzes double-strand cleavage at transposon ends, and the resulting DSBs exhibit 4-base 5' overhangs carrying the duplicated TTAA target site (Mitra et al. 2008). Our attention was drawn to a *Paramecium tetraurelia* gene, *GSPATG00016627001* or *PiggyMac* (*PGM*), which encodes a putative 1065-amino-acid protein with significant homology with a *piggyBac* transposase (Supplemental Fig. S1). Alignment of the predicted Pgm protein with *piggyBac* transposases (Fig. 1A; Supplemental Fig. S1) reveals the conservation of the DDD catalytic triad and of a downstream cysteine-rich putative plant homeodomain (PHD) finger (Sarkar et al. 2003; Mitra et al. 2008). Unlike transposable elements, which are eliminated from the somatic genome during programmed rearrangements, the *PGM* gene is found in one copy per haploid genome in the MAC (Aury et al. 2006; Arnaiz et al. 2007). Furthermore, it does not appear to be part of a transposable structure, since no terminal inverted repeats can be found in the sequences surrounding the conserved central region, in the very short upstream and downstream intergenic regions (91 and 32 bp, respectively), or up to 900 bp into the flanking genes (data not shown). Altogether, these observations suggest

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Figure 1. Developmentally regulated expression and cellular localization of the PiggyMac domesticated transposase. (A) Structure of the predicted Pgm protein and comparison with the *piggyBac* transposase from *Trichoplusia ni*. The catalytic D residues are indicated by asterisks. (Black boxes) Conserved core. The conserved Cys-rich domain is shown in orange, and the C-terminal coiled-coil domain (predicted by the Lupas algorithm) is shown in gray. (B, top two panels) Northern blot analysis of *PGM* and *PtSPO11* mRNA levels during autogamy of 51new. (Bottom two panels) Ligation-mediated PCR (LMPCR) detection of DSBs at the left boundary of IESs 51A1835 and 51G4404 in the same autogamy time course (see Supplemental Fig. S2). (V) Vegetative cells. (C) Localization of the GFP-Pgm fusion in new developing MACs. (Panels a–f) A vegetative 51new cell was injected with linearized plasmid pPBL49-GFP-M, and autogamy was induced. MAC fragmentation was monitored by DAPI staining (panels a, c, e, g, i), and GFP fluorescence was examined with a Leica microscope (panels b, d, f, h, j). (White arrowheads) Developing new MACs. Note the punctuate nuclear GFP fluorescence pattern in panels d and f. Additional fluorescent speckles were sometimes observed with the GFP filter in noninjected cells (panels h, j) and in cells injected with GFP constructs (panel b), due to the presence of birefringent crystals in the cytoplasm. These do not colocalize with nuclear DAPI fluorescence.

that *PGM* has evolved through the domestication of a *piggyBac* transposase gene. In addition, the Pgm protein exhibits an ~300-amino-acid C-ter extension with a predicted coiled-coil structure not found in any *piggyBac* transposase (Fig. 1A).

We followed *PGM* expression during the sexual cycle, which starts with MIC meiosis and proceeds through the development of new MACs. Northern blot hybridization of total RNA with a *PGM* probe failed to detect any transcript in vegetative cells but showed the specific accumulation of a 3-kb mRNA during autogamy, a self-fertilization process (Fig. 1B). Expression of *PGM* takes place several hours later than expression of the *PtSPO11* gene, which encodes the *P. tetraurelia* homolog of the Spo11p endonuclease, essential for meiotic recombination (Keeney 2001). *PGM* mRNA can be detected when new MACs have started to develop, concomitantly with the detection of the first DSBs at IES ends (Fig. 1B). In addition, expression of a GFP-Pgm fusion showed that the protein localizes preferentially in the developing new MACs during autogamy (Fig. 1C), where programmed

DSBs take place. No detectable fluorescence was observed in vegetative cells (data not shown).

To test the function of the *PGM* gene, we knocked down its expression by RNAi during autogamy using the “feeding” technique, which consists of feeding *Paramecium* *Escherichia coli* bacteria that have been engineered to produce dsRNA from the target gene (Galvani and Sperling 2002). Following silencing of *PGM*, autogamy started normally, and developing new MACs could be detected by DAPI staining of cells (Supplemental Fig. S3). However, ~95% of post-autogamous progeny were unable to resume normal vegetative growth when returned to standard growth medium (Table 1). To control for possible off-target silencing artifacts, two nonoverlapping fragments of *PGM* were used independently to induce RNAi (Fig. 2A), and similar results were obtained with both constructs. For one construct, the efficiency of *PGM* silencing was checked by Northern blot hybridization of total RNA extracted throughout autogamy from control and *PGM* silenced cells (Supplemental Fig. S4): No accumulation of full-size mRNA was observed at early time points in cells submitted to RNAi.

Autogamy includes meiosis of the MIC and karyogamy, followed by the development of new MACs: A defect in any of these steps would compromise the recovery of viable progeny. In contrast, conjugation—a sexual process involving reciprocal fertilization between two partners—allows us to distinguish the first set of events, which take place within *Paramecium* mating pairs, from the development of new MACs, which starts right after pair separation (Berger 1973). Therefore, we used conjugation to identify which steps of the sexual cycle are affected by the silencing of *PGM*. A parallel RNAi experiment was performed for the gene *PtSPO11*. Reactive cells of both mating types obtained in silencing medium were mixed, and stable mating pairs were transferred to the same medium to monitor exconjugant survival. In agreement with the data obtained during autogamy (Table 1), the silencing of either *PtSPO11* or *PGM* throughout meiosis and subsequent steps of conjugation strongly compromised exconjugant survival (Fig. 2B). In parallel, we crossed reactive cells obtained in standard *Klebsiella pneumoniae* medium, then transferred the mating pairs to *PtSPO11* or *PGM* silencing medium to allow MIC meiosis and karyogamy to take place normally, while ensuring that exconjugants would start to ingest dsRNA-producing bacteria as soon as they separate (Fig. 2C). Under these conditions, strong lethality was observed only in the *PGM* knockdown, while the silencing of *PtSPO11* had little or no effect, as expected for a meiosis-restricted function. Therefore, *PGM* gene expression is essential during MAC development for the production of viable sexual progeny.

To gain further insight into the role of Pgm in MAC development, we monitored DNA rearrangements in two independent autogamy time-course experiments, where the *PGM* gene was silenced by RNAi. In control RNAi experiments (Fig. 3A, left panel), IESs were amplified transiently together with their flanking sequences, before their excision from the new developing MACs: At later time points, only the rearranged forms, originating from both the old and new MACs, could be detected. In contrast, IES-retaining forms (IES⁺) accumulated throughout autogamy in the new MACs of *PGM* silenced cells, relative to the rearranged sequences present in the fragments of the parental MAC, as visualized by Southern

Table 1. Survival of post-autogamous progeny of *P. tetraurelia* cells silenced for genes *PGM* and *PtSPO11*

Medium	Silencing					Standard
Target gene	<i>PGM</i>		<i>PtSPO11</i>		<i>ND7</i>	None
Silencing plasmid	PGM-1	PGM-2	PtSPO11-1	PtSPO11-2	<i>ND7</i>	None
% Wild type	3	2	6	11	96	98
% sick	2	6	8	14	0	0
% dead	95	92	86	75	4	2
Total %	100	100	100	100	100	100
Cells	132	204	144	84	324	222
Number of experiments	(5)	(7)	(5)	(3)	(11)	(7)

The gene targeted in each silencing experiment is indicated in *italics*. For *PGM* and *PtSPO11*, the RNAi plasmids used for dsRNA production are displayed (see Fig. 2A). The *ND7* gene was used as a control RNAi target, since its silencing has no effect on sexual processes (Nowacki et al. 2005). Autogamy was also performed in standard *K. pneumoniae* medium (right column). Cells were starved in each medium to induce autogamy and, following 3–4 d of starvation, autogamous cells were transferred individually to *K. pneumoniae* medium to monitor growth of sexual progeny. The table summarizes the data from several independent experiments (see Supplemental Table S2 for details) and shows the total number of autogamous cells analyzed for each RNAi. The number of experiments is indicated in parentheses. (sick) Slow-growing progeny, often larger cells than wild-type and slow-swimming; (dead) death in progeny after less than three cell divisions.

blot hybridization (Fig. 3A; Supplemental Fig. S5A) or by PCR amplification (Supplemental Fig. S6A) of IES-containing regions. At the latest time points, IES⁺ forms accounted for up to ~30% of total genomic DNA for each locus, due to overamplification of the nonrearranged region in the developing new MAC and to the degradation of parental MAC fragments that occurs during prolonged starvation (Berger 1974). Because of the presence of the rearranged genome in the old MAC, it is generally difficult to detect new excision junctions in the developing MAC. The variant cell line 51Δ*A* carries a maternally inherited MAC deletion of the surface antigen A gene but retains the gene in its MIC. We therefore used this strain to monitor the transient appearance of IES excision junctions within this locus, but failed to detect any in cells depleted for Pgm (Supplemental Fig. S6B). Ligation-mediated PCR experiments established a clear correlation between the above defects in IES excision and a complete absence of DSBs at the ends of all IESs tested (Fig. 3B).

In the 51Δ*A* cell line, Southern blot hybridization with a probe specific for the *A* gene region (Fig. 4) clearly revealed the accumulation of a high-molecular-weight species (band 1) representing chromosomes carrying the full-length *A* gene. Consistent with our previous observations, these chromosomes retain their IESs, as demonstrated by the size difference relative to the normal MAC fragment (band 2) observed in the wild-type 51new strain (Fig. 4, middle panel) and by hybridization with an IES-specific probe (Fig. 4, bottom panel). Thus, like IES excision, maternally inherited elimination of the *A* gene is blocked in *PGM* silenced cells, and the nonrearranged germline locus is retained in the developing new MAC. Similar results were obtained for the germline region located downstream from the surface antigen G gene, which contains a transposon that is eliminated imprecisely during MAC development, leading to chromosome fragmentation (Supplemental Fig. S5B,C; data not shown). Again, the silencing of *PGM* led to retention of the MIC sequences located downstream from the normal telomere addition site.

The essential role of the *PGM* piggyBac transposase in *P. tetraurelia* genome rearrangements may be an addi-

tional example of how capture of transposon-coding sequences by a host genome can allow for the emergence of a new cellular function. A well-known example of such domestication is the V(D)J recombination system of

Figure 2. Post-transcriptional silencing of the *PGM* gene during MAC development. (A) Maps of genes *PGM* and *PtSPO11* showing fragments used as Northern blot hybridization probes (gray line for *PGM*; the whole gene was used for *PtSPO11*), and for the construction of RNAi plasmids PGM-1 and PGM-2 (1 and 2 on *PGM* map) and PtSPO11-1 and PtSPO11-2 (1 and 2 on *PtSPO11* map). The two *PGM* introns (thin lines) were confirmed by fine restriction analysis and sequencing of RT-PCR products. Restriction sites are indicated. (M) MscI; (H) HindIII; (N) NcoI; (Bs) BsmBI. (B) *PGM* silencing during conjugation. 51new mt7 and mt8 reactive cells were obtained in induced silencing bacteria harboring RNAi plasmids PGM-2 (P) or PtSPO11-1 (S). The silencing of *ND7* (N) was used as a control RNAi. Following conjugation, mating pairs were transferred to each corresponding silencing medium. A conjugation in standard *K. pneumoniae* medium (K) was also performed. The phenotype of exconjugants is displayed as histograms (see footnote for Table 1). (C) *PGM* silencing following mating pair separation. Reactive cells were obtained in standard *K. pneumoniae* medium, and mating pairs were transferred to silencing medium.

Figure 3. Inhibition of IES 51G4404 excision in *PGM* silenced 51ΔA autogamous cells. (A) Quantification of IES-retaining chromosomes (IES⁺) on Southern blots of *Pst*I-restricted DNA during autogamy. (Gray box) Hybridization probe Gmac; (Vk) vegetative cells grown in *K. pneumoniae* before transfer to each silencing medium. All time points are in hours (see Supplemental Fig. S4). (B) LMPCR analysis of DSBs at the left arm of each IES excision site in the same time-course experiment.

immunoglobulin genes in vertebrates, which originates from *Transib* class II transposons (Kapitonov and Jurka 2005). Although this will need to be definitively established by future biochemical studies, we propose that the *Pgm* domesticated transposase is the endonuclease responsible for IES excision in *P. tetraurelia* because it is essential for IES end cleavage in vivo and it has retained the conserved catalytic site of *piggyBac* transposases. Interestingly, depletion of this protein also blocks the imprecise elimination of germline DNA that is associated with chromosome fragmentation. This suggests that *Pgm*-induced DNA DSBs are involved in the two types of programmed DNA elimination described in *Paramecium*, as has been proposed to explain the common presence of TA dinucleotides in both types of MAC junctions (Le Mouél et al. 2003).

Paramecium IESs are thought to be degenerated copies of ancestral *Tc/mariner* transposons that would have evolved under selective pressure for precise excision during MAC development. This is somewhat reminiscent of the evolution of nonautonomous miniature transposable elements (MITEs), which have derived from class II transposons through the loss of their coding capacity but still carry conserved terminal inverted repeats (TIRs) at their ends (Wessler et al. 1995; Casacuberta and Santiago 2003). However, IESs and MITEs differ by several aspects. While MITEs can be found in thousands of homogeneous copies in a given genome, each IES is a single-copy element in the haploid genome. In addition, the recognizable TIRs found at MITE ends can be targets for the transposase of related full-length transposons (Feschotte et al. 2005; Loot et al. 2006), whereas IESs do not have canonical TIRs, but they are similar to those of their putative *Tc/mariner* ancestors or to the ends of known *piggyBac* transposons (Penton et al. 2002). This raises the question of how the *Pgm* domesticated transposase is targeted to its cleavage sites. It was shown previously that a specialized RNAi pathway mediates *trans*-nuclear

cross-talk between the *Paramecium* germline and somatic genomes, through a sequence homology-dependent comparison of two noncoding RNA populations produced from the meiotic MIC and the old MAC, respectively (Lepère et al. 2008; 2009). As a result, MIC-specific small RNAs are thought to trigger epigenetic modifications on homologous sequences in the developing new MAC, which could provide a signal for recruitment of the IES excisase. The putative PHD domain of *Pgm* may participate in the recognition of differentially methylated histones at epigenetically marked sites, as was demonstrated previously for the RAG2 PHD domain, which directs the V(D)J RAG1/2 recombinase to chromatin regions carrying trimethylated H3K4 residues (Matthews et al. 2007). The long C-ter extension found in *Pgm* may also allow protein–protein interactions with other components of the excision machinery.

Close survey of the MAC genome of another ciliate, *Tetrahymena thermophila* (Eisen et al. 2006), revealed the presence of two genes encoding proteins homologous to *Pgm* and carrying the conserved DDD catalytic triad (Supplemental Fig. S1). The closest one (TTHERM_01107220) also carries a coiled-coil C-ter extension and is specifically expressed at late stages of conjugation, when germline DNA elimination takes place (Miao et al. 2009). In addition, the developmental DSBs detected at *Tetrahymena* IES boundaries also exhibit 4-base 5' overhangs, like those observed at *Paramecium* IES ends, although no conserved TA dinucleotide was reported at DNA cleavage sites in *Tetrahymena* (Saveliev and Cox 1996). The participation of *piggyBac*-derived proteins in programmed DNA rearrangements needs to be investigated in *Tetrahymena*, but their existence suggests that domestication of a *piggyBac* transposase gene occurred

Figure 4. Inhibition of the imprecise deletion of the surface antigen A gene. A Southern blot of *Bgl*II–*Xho*I-restricted genomic DNA was hybridized with probe A (middle panels and histograms on the top), then with IES 51A2591 probe (bottom panels). Species 1 and 2 are absent from the vegetative old MAC (lanes Vk and V) and carry the full-length A gene (1: with IES; 2: without IES, as in 51new). Species 3 is present in the old MAC of the particular clone used here and carries an internal deletion of the A gene. Species 4 is also found in the old MAC and carries a telomere branched upstream of A, as in the MAC of the d48 control (a macronuclear variant harboring a telomeric deletion of the A gene) (Forney and Blackburn 1988).

early during the evolution of oligohymenophorean ciliates. As already suggested by others (Klobutcher and Herrick 1997), *Paramecium* may have taken advantage of this early domestication to cope with the putative invasion of its germline genome by the Tc/*mariner* elements that led to present-day TA-IESs. More distant ciliates, such as *Oxytricha trifallax*, appear to have evolved a different strategy to rearrange their somatic genome and rely on the use of the DDE transposase encoded by their own germline transposons, which do not belong to the *piggyBac* family (Nowacki et al. 2009). Our work provides the first demonstration of an essential role for a domesticated *piggyBac* transposase in developmentally programmed DNA rearrangements. Cellular genes encoding proteins related to *piggyBac* transposases have been reported in many other eukaryotic genomes, and one human gene (PGBD4) carries a potentially active catalytic site (Sarkar et al. 2003). Exploring the function of these genes will certainly be a fascinating avenue for future research.

Materials and methods

Paramecium strains, cultivation, and autogamy

All experiments were carried out with strain 51new (Gratias and Bétermier 2003). The 51ΔA cell line was obtained as described (Gratias et al. 2008), and the absence of the rearranged version of the *A* gene in the MAC was checked by a PCR assay on total genomic DNA, using primers flanking IESs 51A1835 and 51A4404. Cultivation and autogamy were carried out at 27°C as described previously (Gratias and Bétermier 2001). In each time-course experiment, the *t* = 0 time point was defined as the time when 50% of cells exhibited a fragmented old MAC.

Injection of GFP fusion transgenes

Plasmids carrying GFP fusion transgenes (see the Supplemental Material) were linearized by *Afl*III and microinjected into the MAC of vegetative 51new cells, as described (Nowacki et al. 2005). Both C-ter and N-ter fusions gave similar results, and no lethality was observed in the post-autogamous progeny of injected cells (data not shown), indicating that the GFP-Pgm fusions did not interfere with normal progression of autogamy (Fig. 1C; data not shown).

Gene silencing experiments

Preparation of silencing medium. All dsRNA-producing plasmids (see the Supplemental Material) were transformed into *E. coli* HT115 (Timmons et al. 2001). Silencing media were prepared basically as described in Galvani and Sperling (2002), by inoculating precultures of the appropriate bacterial strains into WGP medium containing 0.1 mg/mL ampicillin to a starting OD₆₀₀ = 0.35–0.45. Following 6–8 h of shaking at 37°C, bacterial cultures were diluted sixfold into the same medium containing 0.4 mM IPTG to induce the synthesis of dsRNA. Following overnight induction at 37°C, silencing media were supplemented with 0.8 µg/mL β-sitosterol (Merck) before use.

RNAi during autogamy. *P. tetraurelia* cells were grown in standard *K. pneumoniae* medium for 20–30 vegetative fissions then washed twice in silencing medium. Cells were grown for eight to 10 additional vegetative fissions (2 d) in silencing medium (freshly induced medium was added the second day) before starvation-induced autogamy. Progression of autogamy was monitored by DAPI staining, and cells were generally 100% autogamous at day 1 of starvation. At day 3 or 4, 24–30 autogamous cells were picked and transferred individually to 200 µL of *K. pneumoniae* medium to monitor growth of sexual progeny.

RNAi during conjugation. *P. tetraurelia* reactive cells of both mating types were obtained in either *K. pneumoniae* or silencing medium. Following mixing, they were incubated for 1 h 45 min at room temper-

ature to allow the first conjugating pairs to form. Freshly induced silencing medium (2 vol) was added, and cells were incubated for 45 min at 27°C to stop further conjugation. In each experiment, 12 mating pairs were picked and transferred individually into silencing medium to monitor growth of exconjugants.

Northern and Southern blot hybridization

Total RNA or genomic DNA was extracted from 200–400 mL of *Paramecium* cultures during vegetative growth (500–1000 cells per milliliter) or at different time points during an autogamy time course. For RNA extraction, we used the Trizol procedure (Invitrogen) modified by the addition of glass beads to lyse cells. Twenty micrograms of denatured total RNA were separated on a 1% agarose gel in Tris Borate EDTA, transferred to Hybond N⁺ membrane in 20× SSC buffer, and UV-cross-linked. Total genomic DNA extraction was performed as described (Gratias and Bétermier 2003). Following restriction enzyme digestion, 2 µg of total genomic DNA (quantified using the Invitrogen QuBit assay kit) were electrophoresed on 0.8% (in Tris Acetate EDTA) or 1% (in Tris Borate EDTA) agarose gels and transferred to Hybond N⁺ membranes in 0.4 N NaOH.

Double-stranded probes were labeled by random priming with [α -³²P]dATP (3000 Ci/mmol; NEN Radiochemicals, Perkin Elmer), using the Prime-a-gene kit (Promega). Oligonucleotide probes were labeled with [γ -³²P]ATP (3000 Ci/mmol; NEN Radiochemicals, Perkin Elmer) and T4 polynucleotide kinase (Fermentas). Northern blots were hybridized at 42°C in Ultrahyb buffer (Ambion) and washed as recommended by the supplier. Southern blots were hybridized at 60°C as described (Bétermier et al. 2000) and washed in 0.2× SSC and 0.1% SDS at 60°C prior to image plate exposure. Plates were scanned with a Typhoon scanner (GE Healthcare Life Sciences). All details about PCR probes can be found in Supplemental Table S1. Probe *A* (referred to as probe *d* in Garnier et al. 2004) is an ~3-kb *Eco*RI MAC fragment located ~5 kb upstream of the *A* gene.

Detection of DSBs by ligation-mediated PCR (LMPCR)

For the detection of broken chromosome ends, LMPCR was performed as described in Gratias et al. (2008). Briefly, linkers I'/(ATAA)J', I'/(GTAT)J', and I'/(ATAC)J' were ligated to broken chromosome ends at the left boundary of IESs 51A1835 (from the surface antigen *A* gene), 51G4404 (from the surface antigen *G* gene), and sm19-576 (from the η tubulin *SM19* gene), respectively. Primer "PCR haut" was used in combination with a specific primer for the amplification of each ligation product. Following primer extension from a ³²P-labeled nested specific primer, LMPCR products were visualized on 6% polyacrylamide and 7 M urea sequencing gels. Primer and linker sequences can be found in Gratias et al. (2008).

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