Multimerization properties of PiggyMac, a domesticated piggyBac transposase involved in programmed genome rearrangements

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ABSTRACT

During sexual processes, the ciliate Paramecium eliminates 25–30% of germline DNA from its somatic genome. DNA elimination includes excision of ~45 000 short, single-copy internal eliminated sequences (IESs) and depends upon PiggyMac (Pgm), a domesticated piggyBac transposase that is essential for DNA cleavage at IES ends. Pgm carries a core transposase region with a putative catalytic domain containing three conserved aspartic acids, and a downstream cysteine-rich (CR) domain. A C-terminal extension of unknown function is predicted to adopt a coiled-coil (CC) structure. To address the role of the three domains, we designed an in vivo complementation assay by expressing wild-type or mutant Pgm-GFP fusions in cells depleted for their endogenous Pgm. The DDD triad and the CR domain are essential for Pgm activity and mutations in either domain have a dominant-negative effect in wild-type cells. A mutant lacking the CC domain is partially active in the presence of limiting Pgm amounts, but inactive when Pgm is completely absent, suggesting that presence of the mutant protein increases the overall number of active complexes. We conclude that IES excision involves multiple Pgm subunits, of which at least a fraction must contain the CC domain.

INTRODUCTION

Active eukaryotic DNA transposons are mobilized through the action of their self-encoded transposase, an enzyme that catalyzes DNA cleavage at its cognate transposon ends and promotes their integration into genomic target sites (1,2). Transposon dissemination drives host genome plasticity by introducing mutations, triggering ectopic recombination, modifying gene expression patterns or establishing new gene regulatory networks (3). As a consequence of mobility, transposase-encoding genes are largely over-represented in extant sequenced genomes and metagenomes relative to other genes (4). This observation led to the suggestion that transposases may have provided a selective advantage to their hosts, not only through their impact on genome diversification, but also through exaptation and emergence of novel cellular functions, a process referred to as transposase domestication (5–7). Only a few known domesticated transposases, however, have retained a catalytic function related to that of their ancestor. Rag1, exapted from a Transib transposon together with its cofactor Rag2, catalyzes V(D)J recombination during the assembly of vertebrate immunoglobulin genes (8,9). Mating type switching in the yeast Kluyveromyces lactis is initiated by two different domesticated transposases: α3 and Kat1, which derive from hAT and Mutator-like transposase elements, respectively (10–12). DNA cleavage activity was also reported for a human domesticated piggyBac transposase, Pgbd5, whose cellular function is still unclear (13,14), and for SETMAR/Metnase, a primate-specific domesticated mariner transposase possibly involved in DNA double-strand break repair (15,16). As exemplified by SETMAR, which is a fusion between a histone methyl-transferase and a partially active transposase, domesticated transposases may have acquired additional domains that contribute to their cellular function.

The study of developmentally programmed genome rearrangements in ciliates has provided a novel example of catalytically functional domesticated transposases (17,18). Ciliates are unicellular eukaryotes, in which two distinct types of nuclei coexist in the same cytoplasm (19). The highly polyploid somatic macronucleus (MAC, ~800 n in Paramecium), responsible for gene expression, is essential for cell growth and survival, while the diploid germline micronucleus is transcriptionally silent during vegetative growth. During sexual processes (conjugation between sexually complementary partners or autogamy, a self-fertilization...
process that can take place in some *Paramecium* species), the micronucleus undergoes meiosis and transmits the germline genome to the zygotic nucleus, while the old MAC is progressively destroyed and eventually lost. To ensure progeny survival, a functional new MAC has to differentiate from a copy of the zygotic nucleus, a process that involves large-scale genome rearrangements. In *Paramecium*, assembly of the functional new MAC genome includes elimination of 25–30 Mbp of DNA, representing 25 to 30% of the germline genome (20). DNA elimination is imprecise removal of repeated sequences (21) and precise excision of 45 000 short single-copy internal eliminated sequences (IES, a total of ∼3.5 Mbp), at least a fraction of which originate from ancestral *Tc/mariner* transposons (20,22). *Paramecium* IESs are flanked by two TA dinucleotides, one at each end, which are targets for programmed DNA double-strand breaks (DSB) (23). Even though IESs have derived from *Tc/mariner* elements, DNA cleavage at their boundaries requires the presence of PiggyMac (Pgm), a domesticated transposase from the distinct *piggyBac* family, which very likely plays a catalytic role in this step (17). The resulting DSBs (at chromosomal IES excision sites and on excised molecules) are repaired precisely by the classical non-homologous end-joining pathway (24), which involves a development-specific Ku70/Ku80 heterodimer (25). In addition to being essential for DSB repair, the Ku proteins interact with Pgm and are required to license DNA cleavage, indicating that IES excision depends upon pre-assembly of a multiprotein complex (25).

Pgm is a 1065-amino acid protein, in which several domains can be highlighted (Figure 1A). A core domain, characteristic of *piggyBac* transposases (Pfam domain PF13843, or DDE_Tnp1_7), harbors three conserved aspartic acids (D401D491D609) that were proposed to constitute a functional catalytic triad (17). This was further supported by studies on Tpb2p, a close homolog of Pgm that carries out IES elimination in a related ciliate, *Tetrahymena thermophila*: a triple mutation in the conserved Tpb2p DDD triad abolishes DNA cleavage in vitro (18) and blocks DNA elimination in vivo (26). Both Pgm and Tpb2p carry a cysteine-rich (CR) domain downstream of their core transposase domain, which was shown, for the *Tetrahymena* protein, to interact with heterochromatin-specific histone marks (26). Finally, a long C-terminal extension, predicted to adopt a coiled-coil structure, seems to have been acquired by ciliate-specific domesticated transposases, since it is not present in classical *piggyBac* transposases nor in their domesticated counterparts found in other species (Bouallégue, M., Rouault, J.D., Hua-Van, A., Makni, M. and Capy, P. (2016) Molecular evolution of piggyBac superfamily: from selfishness to domestication. Genome Biol. Evol., in press). The present study was initially designed to address the role of the different Pgm domains in *Paramecium tetraurelia*. For this purpose, we developed an in vivo complementation assay by expressing a functional Pgm-GFP fusion in cells, in which expression of endogenous *PGM* has been knocked-down, either through RNAi or somatic gene deletion. Using this assay, we established that the catalytic DDD triad and CR domain are essential for Pgm activity. Moreover, we present evidence that the C-terminal coiled-coil region is essential for productive MAC development, and that a mutant lacking this region can still form active complexes with full-length Pgm in vivo. Taken together, our data indicate that programmed IES excision involves assembly of multiple active Pgm subunits, among which at least some must carry an intact C-terminal domain.

**MATERIALS AND METHODS**

*Paramecium* strains and cell culture

*P. tetraurelia* 51 nd7-1 was obtained from a cross between d4-2 nd7-1 mt7 (27) and 51 new mt8 (23), followed by one round of autogamy: one viable homozygous nd7-1...
post-autogamous F2 clone was submitted to two additional backcross/autogamy cycles to obtain F6 stock 51 nd7-1 (25). Standard culture conditions were at 27°C in a wheat grass infusion (WGP; Pines International Inc.) inoculated with *Klebsiella pneumoniae* and supplemented with 0.8 μg/ml β-sitosterol. Autogamy and conjugation were carried out as described (28). During autogamy, total genomic DNA was extracted from ~200,000 cells for each time-point as described in (17) and quantified using Qubit dsDNA assay kits (Thermo Fisher Scientific).

**Molecular procedures**

Oligonucleotides were purchased from Eurofins Genomics (Supplementary Table S1). PCR amplifications were performed in a final volume of 25 μl, with 10 pmol of each primer, 5 nmol of each dNTP and 1 U of DyNAzyme II or DreamTaq DNA polymerases (Thermo Scientific) according to the enzyme supplier’s recommendations, and using an Eppendorf Mastercycler personal thermocycler. PCR products were analyzed on 0.8–1.5% UltraPure agarose gels (Invitrogen) or 3% NuSieve GTG agarose gels (Lanza). Sanger DNA sequencing was performed by GATC Biotech. Restriction enzymes and T4 DNA ligase were used as recommended by their supplier (New England Biolabs or Promega). Southern blot hybridization with 32P-labeled probes was carried out as described (see Supplementary Data and (17)).

Excision of nested IES 51A6649Δ29 was monitored by PCR on 20 ng total genomic DNA, using primers OMB099 and OMB100 (Supplementary Table S1) and 1.25 U DreamTaq DNA polymerase (Thermo Scientific). Unless otherwise stated, 25 cycles were performed as follows: 30 s at 95°C, 1 min at 52°C, 30 s at 72°C, with a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in a 3% NuSieve GTG agarose gel (Lanza) and visualized following ethidium bromide staining. Ethidium bromide-stained gels were washed in H2O for 10 min and acquisition of the fluorescence signal was carried out using a Typhoon scanner (GE Healthcare Life Sciences).

**Microinjection of GFP fusion transgenes**

Plasmid *PGM-GFP* and its derivatives (see Supplementary Data) were linearized with *AflIII* and microinjected into the MAC of vegetative 51 nd7-1 cells together with a *BsaAI*-restricted *ND7*-complementing plasmid, as described previously (25). To evaluate the copy number of each injected transgene, Southern blot hybridization with 32P-labeled PBL49-1/2 probe was performed on *HindIII*-restricted total genomic DNA of individual injected clones (17). All hybridization signals were collected using a Typhoon phosphorimager and quantified using the ImageQuant TL software (GE Healthcare Life Sciences). We used the *GSPATG0000091099001* gene (bp 1 to 522 relative to ATG start codon, see (29)) to normalize the injected copy number of the transgene relative to the haploid genome equivalent (cphg, see Supplementary Data).

**Localization of Pgm using GFP fusions or immunofluorescence**

During autogamy, cells were permeabilized and fixed as described (25), then washed twice in TBST (10 mM Tris pH 7.4, 0.15 M NaCl, 0.1% Tween20) + 3% BSA (bovine serum albumin, Sigma-Aldrich). For the localization of GFP fusion proteins, fixed cells were directly transferred into TBST + 3% BSA + 0.2 μg/ml DAPI (4’,6-diamidino-2-phenylindole, Sigma Aldrich) for 5 min before being mounted in Citifluor AF2 (Biovalley). For immunofluorescence analysis, cells were incubated for 2 h at room temperature with α-Pgm 2659 primary antibodies (1:300 in Signal + solution A for immunostaining, GenTex), and washed with TBST + 3% BSA prior to 40-min incubation with Alexa fluor 488 goat anti-rabbit IgG secondary antibody (1:300, ThermoFisher Scientific), followed by DAPI staining. Epifluorescence microscopy imaging was performed using a Zeiss Axioplan 2 Imaging epifluorescence microscope using a 63x/1.4 Plan-Apochromat oil objective. Unless otherwise indicated, fluorescence signals were acquired with the same exposure time for AF488 or GFP, and identical window settings were applied to the image display using both Photoshop and ImageJ softwares, in order to compare the fluorescence intensities. Confocal imaging was carried out using a Yokogawa CSU-X1-A1 Spinning Disk coupled with a Nikon Eclipse Ti E microscope. We used a Plan-Apochromat 100x/1.40 oil objective and images were collected by an Evolve 512 Delta EM-CCD camera mounted behind a 2x magnification lens. Z-series were done with a step of 0.2 μm. Quantifications were performed on the optical section that maximizes the surface of each new MAC, using the ImageJ public software (https://imagej.nih.gov/ij/). The fluorescence intensity of each new MAC was obtained after subtracting an estimate of the local background to its Integrated Density.

**Gene silencing experiments**

Plasmid PGM-1, which carries the *HindIII*-NcoI fragment of the *PGM* gene, was used to trigger RNAi against endogenous *PGM* (17). Plasmids p0ND7c (30) and pICL7a (31) carry control RNAi inserts targeting non-essential *ND7* and *ICL7a* genes, respectively.

RNAi during autogamy was achieved as described (25), by transferring cells grown for 10 to 15 vegetative fissions in plasmid-free *Escherichia coli* HTT115 bacteria (32) to medium containing the same bacteria harboring each RNAi plasmid and induced for dsRNA production. Survival of post-autogamous progeny was tested by transferring 30 individual starved autogamous cells to standard growth medium. Cells with a functional new MAC were identified as normally-growing survivors that were unable to undergo a novel round of autogamy ifstarved after ~8 vegetative divisions.

**Recovery of somatic PGM deletions following conjugation**

The RNAi-resistant *PGM*-GFP transgene was microinjected into both 51 nd7-1 mt7 and 51 nd7-1 mt8 parents before mating. Conjugating pairs were transferred to *PGM* RNAi medium and, following their separation, individual
exconjugants were isolated and grown for two days in the same medium before transfer to standard growth medium. Efficient PGM silencing was confirmed by the failure to recover viable progeny from a control mating of non-injected parents. Among the viable F1 exconjugants obtained from injected parents, somatic PGM deletion was screened for by PCR and by monitoring lethality levels in their post-autogamous F2 progeny.

**Anti Pgm antibodies**

Peptide 2659 corresponding to Pgm amino acid sequence 2 to 288 and carrying a N-terminal His tag was expressed and purified by affinity chromatography, before rabbit immunization and purification of sera through protein A-affinity purification. The resulting α-Pgm 2659 antibodies (Proteogenix) were further purified by antigen affinity against peptide 2659 to yield α-Pgm 2659(AF) antibodies (Covalab). Polyclonal antibodies were also raised in guinea pig against peptide 2659 and purified by antigen affinity to obtain highly specific α-Pgm 2659-GP antibodies (Proteogenix).

**Protein extraction from Paramecium cells and Western blot analysis**

Between 2 and 7.5 × 10⁵ cells were collected by centrifugation at each autogamy time-point and washed in 10 mM Tris pH 7.4 before concentrated cells were frozen in liquid nitrogen. Prior to SDS-PAGE analysis, ~1 to 2 × 10⁶ cells were quickly transferred into 50 to 100 µl of boiling 10% sodium dodecyl sulphate containing 1X Protease inhibitor cocktail Set 1 (Calbiochem), and incubated at 100°C for 2 min for complete lysis. Cell lysates were kept at −20°C before gel electrophoresis. Unless otherwise indicated, electrophoresis was carried out in 4–15% pre-cast Tris-glycine polyacrylamide gels (Biorad). Protein expression in insect cells and co-precipitation assays

Plasmid pVL1392-MBP-PGM, harboring a synthetic Pgm coding sequence adapted to the universal genetic code (25), was used to construct pVL1392-MBP-PGMΔA, pVL1392-MBP-PGMΔCR and pVL1392-MBP-PGMΔCC, which encode N-terminal MBP fusions to the same Pgm derivatives as those expressed in microinjected Paramecium cells. Plasmid pVL1392-PGM-HA was constructed by introducing the synthetic Pgm coding sequence into the pVL-1392 vector (BD Biosciences) and adding an HA-tag to the C-terminus of Pgm. Each plasmid was transfected individually into High Five cells together with the BD BaculoGold Linearized Baculovirus DNA (BD Biosciences) to produce recombinant baculoviruses.

Recombinant baculovirus-infected High Five cells were lysed in buffer A (50 mM HEPES pH 7.5, 500 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 5% glycerol and 0.5% Tween-20), using a Dounce homogenizer. For the purification of MBP-tagged proteins, soluble protein extracts were incubated for 2 h with 25 µl of amylose resin (New England Biolabs). For the immunoprecipitation of HA-tagged proteins, 1.5 µg of monoclonal α-HA antibodies (HA-7 from Sigma Aldrich) were incubated overnight on a rotating wheel at 4°C with 10 µl of protein A sepharose beads (GE Healthcare), before incubating the coated beads with cell extracts for 2 h at 4°C. In both experiments, the beads were washed 3 times with 1 ml of lysis buffer A, and re-suspended in Laemmli buffer (33) before electrophoresis in polyacrylamide gels.

For the detection of Pgm-HA, western blots were incubated with HA-7 monoclonal antibodies (Proteogenix), whereas incubations with α-Pgm 2659-GP and α-GFP antibodies were performed in Signal+ solution for Western blot (GenTex), whereas incubations with α-Pgm 2659-GP and α-GFP antibodies were performed in TBST (50 mM Tris-HCl, pH8; 150 mM NaCl and 0.5% Tween) containing 4% low fat milk powder. Monoclonal Anti-αβtubulin TAP952 (α-Tub) antibody (1:300) or anti-αTubulin TEU435 (α-alpha Tub) antibodies (1:300 or 1:1000) were used as loading controls. Primary antibody was detected with the species-appropriate HRP-conjugated secondary antibody (anti-rabbit IgG, anti-mouse IgG, 1/2500, Promega or anti-guinea pig IgG, 1/5000, Thermo Scientific) using the ECL detection system (WesternBright, Advansta). Signal was visualized with the LAS-3000 Image Reader (Fujifilm) and densitometric analyses were performed with the ImageQuant TL software (GE Healthcare Life Science).

**RESULTS**

**A functional PGM*-GFP transgene resistant to RNAi against endogenous PGM**

To set up a functional complementation assay, we used a tagged version of PGM that allowed us to monitor production of Pgm from the complementing transgene, follow its localization and distinguish it from endogenous Pgm. We constructed a PGM*-GFP transgene, in which the GFP coding sequence was fused to the 3’ end of the PGM coding sequence, just upstream of the stop codon (Supplementary Figure S1). To obtain an RNAi-resistant transgene, the original HindIII-BsrXI fragment of PGM was replaced by an extensively mutagenized synthetic DNA fragment, designed to maximize nucleotide sequence divergence with the endogenous genomic locus without modifying the amino acid sequence of the encoded Pgm protein (Supplementary Figure S2). The resulting transgene was named PGM*-GFP (Figure 1B) and encodes a wild-type Pgm protein fused to GFP at its C-terminal end.

Following microinjection of PGM*-GFP into the MAC of vegetative cells, GFP fluorescence accumulated preferentially in the developing new MACs and distinct foci were...
observed over a granular nucleoplasmic signal in fixed autogamous cells (Figure 2A, Supplementary Figure S3), as previously described for other GFP-tagged Pgm derivatives (17). A similar pattern was detected in live cells (Supplementary Figure S5A), indicating that foci formation is not an artefact of the fixation procedure used for sample preparation. We confirmed that GFP foci are not attributable to overexpression from the microinjected transgene, because a punctuated pattern was also observed for endogenous Pgm in fixed non-injected cells, as revealed by immunofluorescence staining using specific α-Pgm rabbit antibodies (Supplementary Figure S5B). Confocal microscopy analysis of the same slides showed a nucleoplasmic granular pattern with variably-sized foci (Supplementary Figure S5C). A dynamic localization pattern was observed for Pgm relative to large DAPI-poor regions inside the developing MAC: prominent Pgm foci were detected inside these regions at early developmental stages, before splitting into smaller foci and eventually becoming dispersed at late stages, until a diffuse Pgm signal filled the whole compartment (Supplementary Figure S5C). These nuclear DAPI-poor compartments are reminiscent of prominent spherical DNA-poor structures that were described previously in the developing and mature MAC of ciliates (34–36) and exhibit similar features to those of putative nucleoli (37). The biological significance of Pgm localization in these compartments will have to be explored in future studies. At the cell population level (Supplementary Figure S6), Western blot analysis of whole proteins extracted during an autogamy time-course of the same non-injected clone confirmed the progressive accumulation of endogenous Pgm, with a T5-T10 peak corresponding to the time when DNA DSBs are introduced at IES ends (17), and a decrease at later time-points.

In injected cells, the expression patterns of Pgm and Pgm-GFP were shown to be quite similar, as judged by Western blot analysis (Figure 2B). Fluorescence microscopy of fixed cells confirmed that Pgm-GFP is detected preferentially in early developing MACs and disappears at later stages (Supplementary Figure S3). For the particular clone analyzed in Figure 2B, the ratio between Pgm-GFP relative to endogenous Pgm (between 0.3 and 0.5) was consistent with the measured copy-number of injected PGM*-GFP (copy-number per haploid genome or cphg = 0.44). Within a wide-range of cphg (0.44 to 79), no toxicity was observed during vegetative growth nor following autogamy of injected cells grown under control conditions (Figure 2C, black bars), indicating that the Pgm-GFP fusion has no dominant-negative effect. To check whether Pgm-GFP is functional, injected cells were submitted to RNAi targeting the endogenous PGM sequence (Figure 2C, grey bars). In non-injected cells (cphg = 0), no viable progeny was obtained, confirming that the endogenous PGM gene is efficiently silenced. At low cphg (0.44), partial recovery of viable sexual progeny was observed (60% progeny with functional new MACs), while full rescue was obtained for higher cphg levels. To confirm that the rescue is actually due to RNAi complementation by the PGM*-GFP transgene rather than simple release of endogenous PGM silencing, we checked for the presence of Pgm and Pgm-GFP using specific α-Pgm antibodies. Endogenous Pgm was found to disappear following PGM RNAi (Figure 2D), while the Pgm-GFP fusion was still detectable both on western blots (Figure 2D) and by epifluorescence microscopy analysis of fixed cells (Figure 2A bottom panels). Taken together, these data show that the synthetic PGM*-GFP transgene is resistant to RNAi against endogenous PGM and encodes a functional protein.

Complementation of a PGM knockdown by mutant PGM transgenes reveals strict requirement for the DDD catalytic triad and the CR domain

Using the above functional complementation assay, we tested the activity of three different mutant derivatives of Pgm-GFP (Figure 1A). The D401A D491A D609A triple mutant transgene, designated as PGM*-3A-GFP or 3A, harbored an alanine residue in place of each of the three conserved aspartic acids of the putative catalytic triad. We also constructed two deleted transgenes: PGM*-ΔCR-GFP (or ΔCR) encodes a PgmΔCR variant carrying a deletion of the CR domain, from residues 701 to 758, while PGM*-ΔCC-GFP (or ΔCC) encodes a deletion of the C-terminal coiled-coil (CC) extension, from residues 759 to 1065.

Our strategy was to complement an endogenous PGM knockdown (KD) using each mutant transgene, with PGM*-GFP as a positive control. Gene KD in P. tetraurelia is routinely achieved through two different methods: somatic gene deletions (30) or feeding-induced RNA interference (38). To ensure that all complementing transgenes would be tested under similar PGM KD conditions, we opted for a somatic deletion of the endogenous PGM gene. In Paramecium, somatic deletions can be induced experimentally during sexual processes—either conjugation or autogamy—following RNAi-mediated silencing of a target gene (30 and Saudemont et al., in preparation). Because Pgm is essential for the recovery of viable sexual progeny, we applied RNAi against endogenous PGM to conjugating cells harboring the RNAi-resistant PGM*-GFP transgene in their parental MAC. As described in Materials and Methods, we obtained viable exconjugants, which harbored different deletion levels of endogenous PGM in their new MAC and had completely lost the parental PGM*-GFP transgene. We selected one deleted clone (ΔPGM), which gave the lowest level of viable progeny after autogamy (Supplementary Figure S7B), and confirmed the somatic deletion of PGM by PCR, with only few residual copies of endogenous PGM (Supplementary Figure S7C). A significantly reduced level of endogenous Pgm in this clone (~25% relative to a wild-type non-deleted control) was confirmed on Western blots (Figure 3A).

The 3A, ΔCC and ΔCR transgenes, and a wild-type PGM*-GFP (WT) control were microinjected separately into the vegetative MAC of ΔPGM cells. We selected transformed clones harboring medium-range copy numbers of each transgene (cphg = 21–68), and followed their behavior during an autogamy time-course in standard growth medium. At given time-points, the localization of each GFP fusion was monitored by fluorescence microscopy, the total amount of fluorescent protein was checked on Western blots using α-GFP antibodies, and total genomic DNA was extracted to follow IES excision at the molecular level. After autogamy, the fraction of viable progeny bearing a
Figure 2. The Pgm-GFP fusion expressed from the PGM*-GFP transgene is functional. (A) Localization of Pgm-GFP expressed in wild-type cells following microinjection of the RNAi-resistant PGM*-GFP transgene (cphg = 0.44). Autogamy was monitored following RNAi against a control gene (ICL7) or endogenous PGM (Supplementary Figure S4A). The GFP fusion is expressed under the control of PGM endogenous transcription signals. Scale bar = 5 µm. (B) Expression pattern of Pgm-GFP during autogamy, relative to endogenous Pgm. PGM*-GFP was injected into wild-type cells (cphg = 0.44). Expected sizes: 129 kD for Pgm, 156 kD for Pgm-GFP. Top panel: Western blot of total proteins extracted during an autogamy time-course of cells grown in a control RNAi medium (ICL7, Supplementary Figure S4A). Pgm was revealed using purified α-Pgm 2659(AF) rabbit antibodies. The α-tubulin signal obtained with anti-αTub TEU345 antibodies (α-αTub, middle panel) was used to normalize the quantification (middle panel). (C) Complementation of PGM RNAi following microinjection of the PGM*-GFP transgene into wild-type cells. Following microinjection, copy-numbers of the PGM*-GFP transgene (expressed in cphg, for copies per haploid genome) were measured for each injected clone as indicated in Materials and Methods. For each RNAi, the percentage of post-autogamous progeny with a functional new MAC is plotted as a function of transgene copy number (cphg). (D) Expression of the PGM*-GFP transgene is resistant to RNAi against endogenous PGM. Top panel: Western blot of whole proteins extracted from autogamous non-injected cells (n.i.), or from autogamous cells harboring PGM*-GFP (cphg = 0.44), following RNAi against control ICL7 gene or PGM (see Supplementary Figure S4A for details about autogamy time-points). α-Pgm 2659-GFP antibodies were used for Pgm detection. Bottom panel: Western blot of the same membrane, following stripping and incubation with α-αTub antibodies.

Functional new MAC was measured by transferring individual autogamous cells to fresh growth medium and letting them resume vegetative growth. Non-injected cells gave rise to 17% viable sexual progeny (Figure 3B), consistent with the presence of residual Pgm in the ΔPGM clone (see above). Injection of the WT transgene restored 67% viable progeny, confirming that expression of Pgm-GFP complements a PGM KD. Intermediate levels of viable sexual progeny (33%) were obtained in the presence of PgmACC and PgmACR-GFP, which suggests that deletion of the C-terminus coiled-coil region still allows partial activity. In contrast, Pgm3A-GFP and PgmΔCR-GFP were unable to complement a PGM KD, as indicated by the complete absence of viable sexual progeny following autogamy of microinjected cells. Strikingly, the 3A and ΔCR transgenes completely prevented ΔPGM cells from producing viable sexual progeny, suggesting that Pgm3A-GFP and PgmΔCR-GFP even have dominant-negative properties. To confirm this observation, we repeated the microinjection of 3A, ΔCC and ΔCR into wild type (WT) cells harboring normal levels of endogenous Pgm (Figure 3C). Strong lethality was observed in the post-autogamous progeny of cells harboring 3A or ΔCR, for copy-numbers above cphg = 19. In contrast, little or no lethality was observed in the progeny of cells that had been transformed with ΔCC or the WT PGM*-GFP construct.

An important control was to check whether Pgm3A-GFP, PgmACC-GFP and PgmACR-GFP are produced in the micro-injected ΔPGM clones used for the complementation assay, and whether they localize in the new MACs during sexual processes. Western blot analysis of total protein extracts using α-GFP antibodies showed that all three mutant Pgm-GFP derivatives are indeed produced during au-
Figure 3. Complementation assay in ΔPGM cells using the PGM*-GFP transgene or its mutant PGM*ΔCC-GFP, PGM*ΔA-GFP or PGM*ΔCR-GFP derivatives. (A) Western blot detection of residual endogenous Pgm in ΔPGM cells. Total protein extracts were prepared at comparable autogamy stages (T5, see Supplementary Figure S4B) from non-injected wild-type (WT) and ΔPGM cells submitted to ICL7 RNAi. Endogenous Pgm was detected using α-H9251-Pgm 2659-GP antibodies. Bottom panel: Western blot of the same membrane, following stripping and incubation with α-Tub antibodies. (B) Complementation of a PGM knock-down in ΔPGM cells using PGM*-GFP (WT), PGM*ΔCC-GFP (ΔCC), PGM*ΔA-GFP (3A) or PGM*ΔCR-GFP (ΔCR) transgenes. Each bar represents the percentage of post-autogamous progeny with a functional new MAC, for control non-injected ΔPGM cells (n.i.), or cells harboring each construct in their parental MAC. Transgene copy-numbers are indicated at the bottom. (C) Dominant-negative effect of PGMΔA-GFP and PGMΔCR-GFP in wild-type cells. PGM*ΔA-GFP (3A), PGM*ΔCR-GFP (ΔCR) and PGM*ΔCC-GFP (ΔCC) transgenes were microinjected into wild-type cells. PGM*-GFP (WT) was used as a control. Injected cells were allowed to undergo autogamy in standard culture medium. Each symbol represents, for each individual injected clone, the percentage of post-autogamous progeny harboring a functional new MAC, as a function of transgene copy-number (cphg).

Figure 4. Expression and localization of Pgm-GFP and its mutant derivatives in ΔPGM cells. (A) Western blots showing expression of WT and mutant Pgm-GFP fusions, and the aberrant accumulation of Pgm3A-GFP and PgmΔCR-GFP at late autogamy stages. For each microinjected clone, total proteins were extracted at different time-points during autogamy (see Supplementary Figure S4B) and loaded on 0.1% sodium dodecyl sulphate, 8% polyacrylamide gels. Western blots were first incubated with α-GFP antibodies for the detection of variant Pgm-GFP fusions (top panel) and stripped before using α-Tub monoclonal antibody. Black arrowheads indicate the position of each GFP fusion. (B) Localization of GFP fusion proteins expressed following microinjection of PGM*-GFP (WT), PGM*ΔA-GFP (3A), PGM*ΔCR-GFP (ΔCR) or PGM*ΔCC-GFP (ΔCC) into ΔPGM cells (same injected cells as in Figure 3B). GFP signals were acquired with the same exposure time (1000 ms) using an epifluorescence microscope. Scale bar = 5 μm. The size of each developing new MAC was measured at its maximal area section using ImageJ: 18 μm² for WT, 60 μm² for 3A, 55 μm² for ΔCR and 38 μm² for ΔCC. (C) Accumulation of PgmΔA-GFP and PgmΔCR-GFP in late developing MACs of injected ΔPGM cells. GFP fluorescence signals were acquired with the same exposure time for Pgm-GFP and PgmΔCC-GFP (1000 ms), shorter acquisition times (500 ms) were used for PgmΔA-GFP and PgmΔCR-GFP to avoid saturation (scale bar = 5 μm). Developing new MAC size was measured at the maximal area section: 122 μm² for WT, 181 μm² for 3A, 172 μm² for ΔCR and 167 μm² for ΔCC.

togamy (Figure 4A and Supplementary Figure S8A). Moreover, epifluorescence microscopy confirmed the localization of GFP fluorescence in developing MACs for each fusion construct (Figure 4B). Therefore, the failure of PgmΔA-GFP and PgmΔCR-GFP to complement a ΔPGM somatic deletion cannot be attributed to defective protein expression or nuclear import. Taken together, our data indicate...
that PgmΔA-GFP and PgmΔCR-GFP are inactive and have a dominant-negative effect in the presence of endogenous Pgm. We conclude that the putative catalytic DDD triad and the CR domain are essential during MAC development.

Different localization patterns could be noted, however, for the mutant proteins relative to wild-type Pgm-GFP (Figure 4B). The WT fusion formed nuclear foci, while PgmΔA-GFP displayed a more homogeneous pattern without conspicuous foci, suggesting that Pgm foci are associated with DNA cleavage. A variable localization pattern was observed for PgmΔCR-GFP in young developing MACs, with a homogeneous GFP nuclear signal in some cells and accumulation of GFP fluorescence in DAPI-poor regions in others. Similarly, PgmΔCC-GFP tended to accumulate in DAPI-poor regions. At later stages, the global intensity of PgmΔA-GFP and PgmΔCR-GFP fluorescence was consistently higher in late developing MACs than for WT Pgm-GFP, despite a twofold shorter acquisition time (Figure 4C). Strong accumulation of PgmΔA-GFP and PgmΔCR-GFP at late autogamy stages was confirmed by western blot analysis (Figure 4A and Supplementary Figure S8B). The accumulation of inactive mutant Pgm derivatives at late time-points is reminiscent of our previous observation that Pgm is overexpressed at late autogamy stages in cells depleted for Ku, an essential factor involved in IES excision (25), and confirms the correlation between aberrant late Pgm accumulation and the failure to produce a functional new MAC.

The ΔCC mutant is partially active in IES excision, but only in the presence of endogenous Pgm

Expression of PgmΔCC-GFP in ΔPGM cells resulted in a twofold increase in the yield of viable sexual progeny relative to non-injected cells (Figure 3B). To gain molecular insight into the ability of PgmΔCC-GFP to carry out IES excision, we monitored excision of a 29-bp IES nested inside the 370-bp IES 51A6649 from the AΔ1 surface antigen gene (Figure 5A). We previously showed that quick and efficient excision of the nested IES precedes that of IES 51A6649 in WT cells, resulting in the release of 341-bp circular molecules when the large encompassing IES eventually gets excised (39). During autogamy, excision of the nested IES is detected by PCR using primers hybridizing to the 51A6649 confirmed that PgmΔCC-GFP restores excision of the nested IES in partial PGM KDs, while no rescue was observed in complete PGM KDs (Figure 5D). Restoration of IES excision was only partial, however, since no obvious increase in the survival of sexual progeny was observed in partial PGM KDs for cells harboring ΔCC relative to the non-injected control (Supplementary Figure S9B) and the larger IES 51A6649 was clearly retained in the developing MAC of injected cells in all PGM KDs (Supplementary Figure S9C). We conclude from these experiments that the C-terminus of the ΔCC-expressing transgene constitutes a functional domain which, together with the truncated PgmΔCC protein, exhibits partial activity in the presence of endogenous Pgm.

Multimerization properties of Pgm

The above experiments suggest that PgmΔCC interacts with Pgm to form an active complex, consistent with the known ability of many cut-and-paste transposases to assemble into oligomers during transposition (1). To check whether Pgm
Indeed forms oligomers, we co-expressed Pgm-HA with an MBP-Pgm fusion in a heterologous insect cell expression system. We found that both proteins consistently co-purify on amylose beads, while only background levels of Pgm-HA are recovered with the MBP tag alone or in the absence of an MBP-tagged partner (Figure 6A). Reciprocally, MBP-Pgm co-immunoprecipitates with Pgm-HA following incubation with anti-HA antibodies (Figure 6B). This shows that Pgm can oligomerize in insect cell extracts, as expected for a transposase-related protein. We obtained the same results when a MBP-PgmΔCC mutant fusion (ΔCC) was co-expressed with Pgm-HA (Figure 6A and B). In the antigen immunoprecipitation experiments, similar amounts of MBP-tagged protein and Pgm-HA were recovered in the co-precipitate, even though exceeding amounts of MBP fusions were present in input cell extracts (Figure 6B). We conclude from these experiments that PgmΔCC has retained the ability to interact with Pgm and that the resulting complexes are close to equimolar. The nearly 2-fold increase in sexual progeny survival observed upon injection of moderately high copy-numbers (cphg = 21) of the ΔCC transgene into ΔPGM cells expressing low but detectable levels of endogenous Pgm (Figure 3), suggests that active equimolar Pgm/PgmΔCC complexes form instead of endogenous Pgm/Pgm multimers, therefore increasing the number of available DNA elimination complexes. Using the same experimental strategy, we observed that N-terminal MBP fusions with Pgm3A and PgmΔCR were also each able to co-precipitate with Pgm-HA in heterologous cell extracts (Figure 6A and B). The ability of the two inactive mutants, Pgm3A and PgmΔCR, to associate with Pgm provides a possible explanation for their dominant-negative properties (Figure 3), because this association would give rise to inactive complexes and compete with assembly and/or function of the WT DNA elimination complex (see Discussion).

**DISCUSSION**

Transgene-mediated complementation of gene knockdowns in *P. tetraurelia*: a tool for functional domain analysis

Gene functional analyses are often carried out through siRNA-mediated silencing of endogenous genes and complementation with RNAi-resistant transgenes (see 40–42). In *P. tetraurelia*, RNAi is generally induced by feeding cells with dsRNA-producing bacteria (38). Complementation of
suggests that no overproduction inhibition, or OPI (45, 46), transgenic do not strongly inhibit MAC development, which over, we found that higher copy numbers of the PGM\(^*-\)GFP
1 to 10 copies of the transgene per haploid genome. More-
ential. Maximal complementation levels were observed for
clude that the Pgm-GFP fusion used in this study is func-
NAs have little or no impact on RNAi (44). We also con-
icient complementation, confirming that secondary siR-
outside of the mutagenized region. However, we observed
siRNAs might have been able to anneal to
we show that a
nous
by bacterial dsRNAs is resistant to RNAi against endoge-
pressing the human ortholog of a silenced gene (43). Here,
dsRNAs produced from the feeding insert (44). Secondary
the target mRNA outside of the region homologous to the
previous observation that feeding-induced RNAi triggers
RNAi was achieved for the first time in Paramecium by ex-
pressing the human ortholog of a silenced gene (43). Here,
we show that a Paramecium transgene (PGM\(^*\)) carrying
multiple silent base substitutions within the region targeted
by bacterial dsRNAs is resistant to RNAi against endoge-
nous PGM and can be used for functional complementation
assays. Our strategy might have been complicated by the previous observation that feeding-induced RNAi triggers
the production of secondary siRNAs complementary to the
target mRNA outside of the region homologous to the
dsRNAs produced from the feeding insert (44). Secondary
siRNAs might have been able to anneal to PGM\(^*\) mRNA
outside of the mutagenized region. However, we observed
efficient complementation, confirming that secondary siR-
NAs have little or no impact on RNAi (44). We also con-
clude that the Pgm-GFP fusion used in this study is func-
tional. Maximal complementation levels were observed for
1 to 10 copies of the transgene per haploid genome. More-
over, we found that higher copy-numbers of the PGM\(^*-\)GFP
transgene do not strongly inhibit MAC development, which
suggests that no overproduction inhibition, or OPI (45, 46),
takes place when Pgm is present in excess. Our observation
is consistent with the previously reported absence of OPI
for the T. ni PiggyBac transposase in human cells (47).

Complementation was also observed in the absence of
dsRNA-producing bacteria, following microinjection of
PGM\(^*\)-GFP into cells harboring a PGM somatic deletion.
Use of the same ΔPGM clone for all microinjections al-
lowed accurate comparison of the relative activities of com-
plementing mutant transgenes. Moreover, the presence of
residual levels of endogenous Pgm in ΔPGM cells allowed
us to detect the dominant-negative properties of the Pgm\(_{3A}\)
and Pgm\(_{ΔCR}\) complementing constructs. We found that
Pgm\(_{3A}\) is inactive, which shows that the conserved DDD
triad is essential for IES excision and that Pgm plays an es-
sential catalytic role in DNA cleavage at IES ends. A similar
finding was reported previously for the Tetrahymena Pgm
homolog, Tbp2p (18, 26), highlighting the functional im-
portance of catalytically active domesticated piggyBac trans-
posases in ciliates. The inability of Pgm\(_{ΔCR}\) to support IES
excision indicates that the CR domain is also essential. Fur-
ther investigations will clarify its exact function. It may in-
teract with the H3K27me3 heterochromatin mark involved
in the control of programmed DNA elimination in Parame-
cium (35), as reported for Tbp2p (26). The CR domain may
also interact with other targets, such as nucleic acids, as
demonstrated for some CR domains (48). Moreover, the
dominant-negative properties of Pgm\(_{3A}\) and Pgm\(_{ΔCR}\)
indicate that either mutant inhibits the normal action of WT
Pgm, similarly to what was proposed in other systems for
inactive transposase variants that act as natural repressors
(46, 49). Alternatively, Pgm\(_{3A}\) and Pgm\(_{ΔCR}\) may associate
with and titrate out essential IES excision factors, such as
the Ku proteins (25), or compete with Pgm for binding to
its natural chromatin targets.

**The ciliate-specific C-ter coiled-coil extension is essential for activity of the Pgm-associated IES excision complex**

DNA ‘cut-and-paste’ transposition mediated by DD(E/D)
transposases involves assembly of a highly ordered protein–
DNA complex, in which both transposon ends, each bound
by one or more transposase subunits, are synapsed together
through protein–protein interactions to assemble an active
transpososome (1). Whatever the number of transposase
subunits present in the synaptic complex, a single active cat-
alytic site carries out all DNA cleavage and strand transfer
reactions at each end. PiggyBac-related transposons are ex-
tensively used as tools for genome engineering (50). How-
ever, still little is known about the structural and func-
tional mechanisms that underlie transpososome assembly
for transposons of the piggyBac family, and information is
lacking on the stoichiometry of the transposition reaction.
The present work shows that the Paramecium domesticated
transposase Pgm acts as a multi-subunit complex in vivo,
as described for canonical transposases. Indeed, the abil-
ity of Pgm\(_{ΔCC}\) to rescue IES excision only when residual
amounts of Pgm are present suggests a mechanistic model,
in which productive genome rearrangements rely on assem-
bly of an active excision complex including several Pgm sub-
units. Co-precipitation experiments (Figure 6) show that
Pgm oligomerizes in solution and that Pgm and Pgm\(_{ΔCC}\)

![Figure 6. Multimerization properties of Pgm and its mutant derivatives.](image-url)
can also interact, indicating that one oligomerization domain lies within the transposase-related portion of the protein. We propose, therefore, that a complex including both Pgm and Pgm\textsubscript{ACC} is formed \textit{in vivo} and that a Pgm/Pgm\textsubscript{ACC} multimer is active for IES excision, at least partially. Alternatively, individual subunits within the complex may each carry out one step of the excision reaction without directly interacting with each other, and Pgm\textsubscript{ACC} may only be active for one step. Further studies will establish the exact number of Pgm subunits present in the IES excision complex and whether direct Pgm multimerization is actually required for catalytic activity. Similar activation of a wild-type transposase by inactive truncated mutants was reported for other transposons, such as the plant \textit{Ac} element (51), suggesting that truncated transposases may sometimes retain the ability to form an active transposition complex with their cognate full-length counterpart. Interestingly, Pgm\textsubscript{ACC} is defective if full-length Pgm is absent: therefore, an active IES excision complex requires the presence of the C-terminal domain in at least some Pgm subunits.

The C-terminal domain of Pgm was proposed to adopt a coiled-coil structure (17). Coiled coils are generally involved in protein–protein interactions, either protein oligomerization or interaction with other partners (52,53). According to the Multicoil sequence prediction software (54), the Pgm coiled-coil domain may even preferentially form trimeric structures. Interestingly, the coiled coil has been acquired by ciliate-specific domesticated \textit{piggyBac} transposases (Bouallegue et al., in press). IES excision in \textit{Paramecium} is a transposition-related mechanism, during which both ends of a given IES establish a long-range interaction, as reported for cut-and-paste transposons (55). However, because of their short size (30% are 26–30 bp in length) (20), \textit{Paramecium} IESs are probably not flexible enough to bend easily and form a DNA loop. Instead, higher-order interaction between the coiled coils of Pgm subunits bound at each IES boundary may contribute to assemble a catalytically active ‘excisome’. We also note that programmed DNA elimination in ciliates differs from standard cut-and-paste transposition in two other aspects. First of all, no recognizable conserved DNA motif was identified that may constitute a specific Pgm binding site on germline eliminated sequences (56). This may be linked to general properties of transposases from the \textit{piggyBac} family or their domesticated derivatives. Indeed, previously published work demonstrated that binding of the purified \textit{piggyBac} transposase from \textit{Trichoplusia ni} (PB) to DNA fragments carrying the transposon left or right terminal inverted repeats induces multiple shifted bands, but sequence-specific binding motifs were not determined experimentally (57). Recently, the vertebrate domesticated \textit{piggyBac} transposase, Pgbd5 (58), which is very distant from PB and Pgm (Bouallegue et al., in press), was shown to mobilize the \textit{piggyBac} transposon from \textit{T. ni} (14) and to trigger genomic rearrangements when overexpressed in human cells (59). Pgbd5-specific signal sequences were identified at the rearranged sites, but have little to do with the inverted repeats of the original \textit{piggyBac} transposon, suggesting that relaxed sequence specificity may be a common property of domesticated \textit{piggyBac} transposases. A second common property between IES excision and cut-and-paste transposition is the strong coupling that exists between DNA cleavage and DSB repair at IES excision sites (25). Taking these differences into account, an alternative function may be proposed for the Pgm coiled-coil domain, i.e. interacting with other components of the DNA elimination machinery, perhaps involved in IES recognition or DSB repair. Future studies will provide more insight into the composition of the DNA elimination complex and how the Pgm C-ter coiled coil is involved in interacting with other components of the complex.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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