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DNA Binding Specificity and Cleavage Activity of *Pacmmar* Transposase[†]

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ABSTRACT: *Mariner*-like elements (MLEs) are members of the Tc1/*mariner* superfamily of transposable elements which transpose by a "cut and paste" mechanism. Most of the MLEs characterized to date are transpositionally inactive due to the accumulation of mutations in their transposase gene. Here, we report the biochemical study of two copies of the *Pacmmar* element (*Pacmmar1.1* and *Pacmmar1.2*), isolated from the coastal crab *Pachygrapsus marmoratus*. These two copies present an open reading frame encoding a putative active transposase. Using an in vitro transposition assay, we show that *Pacmmar* transposases are unable to perform by themselves the transposition reaction. However, we demonstrate by an electrophoretic mobility shift assay that both transposases bind specifically to the inverted terminal repeat of the *Pacmmar* element. Moreover, an in vitro cleavage assay showed that both transposases have the capacity to cleave the transposon. The in vitro cleavage activity of *Pacmmar* transposases appears imprecise, suggesting the requirement of specific host factors or the presence of mutations which have modified the cleavage specificity of the enzyme.

Mariner-like elements (MLEs)[1] are class II transposable elements that belong to the Tc1/*mariner* superfamily. Their sequences are approximately 1.3 kb long and contain a single gene without an intron encoding an ~350-amino acid transposase. The transposase gene is flanked by ~30 bp inverted terminal repeats (ITRs) and is characterized by a DNA binding domain and a catalytic domain. The DNA binding domain is localized at the N-terminal end and extends approximately over the first 150 residues. At the C-terminal end, the transposase exhibits a DD34D signature in the catalytic core sequence (*1*, *2*). MLEs move by a conservative "cut and paste" mechanism catalyzed by the transposase. This process is performed schematically in three steps. First, the transposase binds specifically to its ITRs; second, the enzyme cleaves both strands of DNA at each end of the transposon, and third, the element is inserted into the target site which is a TA dinucleotide duplicated during the insertion process.

Mariner elements are present in a wide variety of genomes, including plants, fungi, invertebrates, and vertebrates (*3*), and most of them lost their activity via an accumulation of mutations. To the best of our knowledge, only 18 sequences were described as potential active elements with no interrupting stop codon (*4*−*15*).

To date, only five MLEs were demonstrated to be active in animals. *Mos1*, *Famar1*, and *Mboumar-9* are naturally active elements isolated from *Drosophila mauritiana* (*16*), *Forficula auricularia* (*17*), and *Messor bouvieri* (*18*), respectively. The *Himar1* active copy is a reconstructed consensus sequence, established from a series of inactive copies present in the genome of the horn fly, *Haematobia irritans* (*19*). The consensus sequence restores an ORF that is considered as the ancestral active copy which invaded the genome. This reconstruction method was also used for the resurrection of the functional Tc1-like elements, *Sleeping Beauty* and *Frog Prince* from eight fish species and the frog *Rana pipiens*, respectively (*20*, *21*). By contrast, *Hsmar1-Ra* is the reconstructed ancestral sequence from *Hsmar1* (*22*), which is one of the two members of the *mariner* subfamily present in the human genome (*23*, *24*). This reconstruction was performed by a computational approach and led to an active transposase (*22*).

Previous studies indicate that transposition of *mariner* elements (e.g., *Mos1* and *Himar1*) can occur in vitro without specific host factors (*19*, *25*, *26*). In this paper, we report the in vitro activity of the crustacean *Pacmmar* element which was previously isolated from the coastal crab *Pachygrapsus marmoratus* (*4*). Two copies of this element, *Pacmmar1.1* and *Pacmmar1.2*, displayed an open reading frame (ORF) encoding *Pac*Tase-1 and *Pac* Tase-2, respectively. Both ORFs encode putative functional transposases which differ only by two amino acids located in the helix−turn−helix domain (*4*).

Although unable to ensure the whole transposition reaction, both *Pac*Tase-1 and *Pac*Tase-2 were able to specifically bind to *Pacmmar* ITR and cleave a plasmid bearing the *Pacmmar*

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[1]Abbreviations: BET, ethidium bromide; DTT, dithiothreitol; Chl[r], chloramphenicol resistance gene marker; EMSA, electrophoretic mobility shift assay; ITR, inverted terminal repeat; MBP, maltose binding protein; MLE, *mariner*-like element; ORF, open reading frame; *Pac*-Tase, *Pacmmar* transposase; SSC, standard sodium citrate buffer; TBE, Tris-borate-EDTA; Tet[r], tetracycline resistance gene marker.

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element. Altogether, our results show that *Pacmmar* transposases are partly functional in vitro, suggesting that they are not the ancestral active copies, or that their functionality requires eukaryotic host factors to complete the transposition mechanisms.

EXPERIMENTAL PROCEDURES

Molecular Reagents. Restriction enzymes and DNA-modifying enzymes were from either New England Biolabs (distributed by Ozyme, Saint-Quentin-en-Yvelines, France) or Promega-France (Charbonnières-les-Bains, France). Plasmids were purified using a Wizard Plus DNA purification system (Promega-France), and PCR fragments were eluted from the gel by using the PCR Clean-up system (Promega-France). Primers and oligonucleotides were synthesized by Operon Biotechnologies GmbH (Cologne, Germany), and all the primers and oligonucleotide sequences used are available as Supporting Information (Tables 1 and 2).

Plasmid Constructions. Both ORFs from *Pacmmar1.1* and *Pacmmar1.2* were amplified by PCR with the primers Pac*Bam*HI and Pac*Hin*dIII (see Table 1 of the Supporting Information) and cloned into the pGEMT easy Vector System (Promega) to generate plasmids pGEMT-ORF1 and pGEMT-ORF2, respectively. These plasmids were then digested by *Bam*HI and *Hin*dIII, and both ORFs were subcloned between the *Bam*HI and *Hin*dIII sites of pMal-c2 (New England Biolabs) to create expression plasmids pMalORF1 and pMalORF2, respectively, allowing the production and purification of the transposases in fusion with the maltose binding protein (MBP). The plasmid pGEMT-*Pac* was constructed by PCR amplification of the full-length *Pacmmar* element from *P. marmoratus* genomic DNA with PacITR primer. The resulting PCR product was cloned using the pGEMT easy Vector System.

The suicide donor plasmid *Pac*TetR6K, used for the in vitro transposition assay, was constructed as follows. The oriR6K was amplified by PCR from pRC704 (gift from J. Bischerour) with primers R6K1 and R6K2 and cloned into the pGEMT easy Vector System to generate pGEMT-R6K as described previously (*27*). The tetracycline resistance cassette was obtained by digestion of pBR322 (Fermentas, distributed by Ozyme) by *Eco*RI and *Ava*I. Cohesive ends of the resulting fragment were treated with T4 DNA polymerase to generate blunt ends and inserted into pGEMT-*Pac* digested by *Ava*I and treated with T4 DNA polymerase to generate pGEMT-*Pac*Tet. Both pGEMT-R6K and pGEMT-*Pac*Tet were then digested by ApaI and *Pst*I and ligated together with a T4 DNA ligase to form the *Pac*TetR6K plasmid. This plasmid was then amplified in *Escherichia coli* strain RC5081 (gift from J. Bischerour) containing the *pir* factor as previously described (*27*).

Transposase Production and Purification. The chemically competent Rosetta 2 *E. coli* cells (gift from J. Bischerour) were transformed with either pMalORF1 or pMalORF2 expression vectors, and a culture of transformed cells was grown in 900 mL of LB medium, at 37 °C until the OD_{600} reached ~0.5. The production of the MBP−*Pac*Tase fusion protein was then induced by the addition of 0.3 mM isopropyl β-D-thiogalactopyranoside in the culture medium overnight at 28 °C. Cells were harvested and resuspended in 90 mL of buffer A [20 mM Tris (pH 9) and 100 mM NaCl] and then lysed with lysozyme (400 μg/mL) in the presence of 43 mg/mL protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France). The soluble fraction, retrieved by centrifugation, was loaded onto a 15 mL tube with 1 mL of amylose resin (New England Biolabs) previously washed with 20 mL of buffer A. After incubation for 1 h at 4 °C while being shaken, the resin was washed at 4 °C with 200 mL of buffer A, and the protein was eluted with 2 mL of buffer A supplemented with 10 mM maltose. Purified proteins were aliquoted by 10 μL and stored at −80 °C. The proteolytic cleavage of the MBP fusion protein was performed by digestion with factor Xa (New England Biolabs) for 6 h at room temperature.

Electrophoretic Mobility Shift Assay (EMSA). To generate pGEMT-5′ITR and pGEMT-3′ITR plasmids, both 5′ITRs and 3′ITRs were cloned into the pGEMT easy Vector after adenylation of the 3′ end. Plasmids pGEMT-5′ITR and pGEMT-3′ITR were digested by *Eco*RI to excise the ITRs sequences. Digestions were followed by a Klenow fill-in reaction using [α-^{32}P]dATP. The standard EMSA was conducted using 0.1 pmol of ^{32}P-radiolabeled probe and 100 nM transposase in a 20 μL volume of reaction buffer B [20 mM Tris (pH 7.9), 100 mM NaCl, 250 μg/mL bovine serum albumin, 2 mM dithiothreitol (DTT), 10% glycerol, and 5 mM $MgCl_2$]. The mixture was incubated for 2 h at 37 °C. Products were separated on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer. The gel was then dried before autoradiography.

To assess binding specificity, we performed the EMSA with the addition of an increasing amount of unlabeled competitor DNA in excess (5−500-fold excess). The competitor DNA was obtained by annealing complementary oligonucleotides (sequences available in Table 2 of the Supporting Information) for 10 min at 95 °C followed by a slow decrease in temperature. Both unlabeled and radiolabeled DNAs were present in the reaction mixture before the addition of the transposase.

In Vitro Cleavage Assay. The pGEMT-*Pac* plasmid (400 ng) was incubated with 10 nM tranposase in a 20 μL volume of reaction buffer B [20 mM Tris (pH 7.9), 100 mM NaCl, 250 μg/mL bovine serum albumin, 2 mM DTT, 10% glycerol, and 5 mM $MgCl_2$]. The mixture was incubated at 37 °C for times varying between 10 min and overnight. The reaction was stopped by heat inactivation of the protein at 65 °C for 10 min. The cleavage products were separated in a 1% agarose gel and analyzed by ethidium bromide (BET) staining. DNA was then transferred onto a positively charged nylon membrane. Fragments containing *Pacmmar* sequences were detected by hybridization with the full-length *Pacmmar* sequence. The preparation of the probe and conditions of hybridization and washing of the membrane were as previously described (*4*).

For the determination of biochemical conditions of cleavage, 400 ng of pGEMT-*Pac* was incubated for 2 h with 10 nM tranposase in a 20 μL volume of reaction buffer B at various temperatures (4, 15, 25, 37, 50, and 65 °C) or pHs (6,7, 8, 9, and 10). The transposase concentration effect was assayed by using 0.1, 0.5, 1.0, 10, 100, 200, 500, and 1000 nM transposase incubated with 400 ng of pGEMT-*Pac* in buffer B for 2 h at 37 °C. Several divalent cations were tested by incubating 400 ng of pGEMT-*Pac* with 10 nM transposase in 20 μL of reaction buffer B [20 mM Tris (pH 7.9), 100 mM NaCl, 250 μg/mL bovine serum albumin, 2 mM DTT, and 10% glycerol] supplemented with either $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $SrCl_2$, or $ZnSO_4$ (each at 5 mM) for 2 h at 37 °C.

The specificity of cleavage was investigated by a competition assay using 10 nM transposase incubated with 400 ng of pGEMT-*Pac* plasmid and 400 ng of competitor plasmid (pUC18) for 2 and 6 h. After heat inactivation, cleavage products were separated by electrophoresis on a 1% agarose gel.

In Vitro Transposition Assay. Suicide plasmid *Pac*TetR6K (100 ng) and target plasmid pBC (100 ng) were incubated in the presence of a transposase concentration varying from 1 to 1500 nM (1, 5, 10, 100, 500, 1000, and 1500 nM) in buffer B for 2 or 16 h at 37 °C. The reaction was stopped when the mixture was heated for 5 min at 65 °C, followed by a 30 min incubation at 37 °C with proteinase K (0.13 μg/mL). Then DNA was purified using phenol/chloroform extraction. Purified DNA was used to transform One Shot Top 10 chemically competent cells (Invitrogen, Cergy-Pontoise, France), and transformed cells were selected on LB medium containing either chloramphenicol (150 μg/mL) or a mixture of tetracycline (20 μg/mL) and chloramphenicol (150 μg/mL). The transposition level was then calculated as the ratio between bacteria with transposition events (number of tetracycline and chloramphenicol resistant bacteria) and total transformed bacteria (chloramphenicol resistant bacteria).

In Vitro Cleavage Site Determination. pGEMT-*Pac* (400 ng) was incubated with 10 nM tranposase in 20 μL of reaction buffer B containing 5 mM $MgCl_2$. The mixture was incubated 4 h at 37 °C. For the positive control, the pGEMT-*Pac* plasmid was cleaved by the restriction enzyme *Apa*I or *Pst*I. The reaction was stopped by heat inactivation of the protein at 65 °C for 10 min, and cleavage products were separated by electrophoresis on a 1% agarose gel. After purification, 100 ng of linear products was mixed with 40 pmol of the single-stranded oligo Linker (5′-TGCACAACTGCAGAACCAATGCATTGCCTTGGGGG-CCCAT-3′), heated to 95 °C for 3 min, and chilled on ice. Ligation was then performed overnight at room temperature using 40 units of T4-RNA ligase in a 50 μL final volume. After heat inactivation at 65 °C for 10 min, approximately 100 ng of DNA was used as a template for two rounds of PCR. In the first PCR, the following primers were used: linker1 and pGEMT-*Pac* 4 for the 5′ side and linker1 and pGEMT-*Pac*7 for the 3′ side. The nested PCR was then performed using 1 μL of the previous PCR products and the following primers: linker2 and pGEMT *Pac*9 for the 5′ side and linker2 and pGEMT-*Pac*7 for the 3′ side (see Table 1 of the Supporting Information). After the second amplification, PCR products were separated on a 1.5% agarose gel, purified, cloned, and sequenced.

RESULTS

In Vitro Transposition Assay. In a first attempt, the transposition activity of *Pac*Tase-1 and *Pac*Tase-2 was assayed as a whole by using an in vitro strategy derived from previous reports on *Mos1* and *Hsmar1* activity (*25*, *27*). To test the activity of *Pacmmar* transposases, both *Pacmmar1.1* and *Pacmmar1.2* ORFs were cloned in the pMAL expression vector and expressed in *E. coli* to produce MBP−*Pac*Tase-1 and MBP−*Pac*Tase-2 recombinant fusion proteins, respectively. To ensure that the MBP part of the fusion protein did not interfere with experimental results, controls were conducted with either MBP alone or *Pac*Tase without MBP, obtained after proteolytic cleavage of the MBP tag. In addition, since all results presented below were identical for both MBP−*Pac*Tase-1 and MBP−*Pac*Tase-2, we mainly referred only to MBP−*Pac*Tase.

A tetracycline resistance (Tet^r) marker was inserted into the consensus *Pacmmar* sequence carried on a suicide donor plasmid bearing an R6K replication origin, which requires the *pir* gene product to replicate. The target plasmid, bearing a chloramphenicol resistance (Chl^r) marker and a ColE1 replication origin,

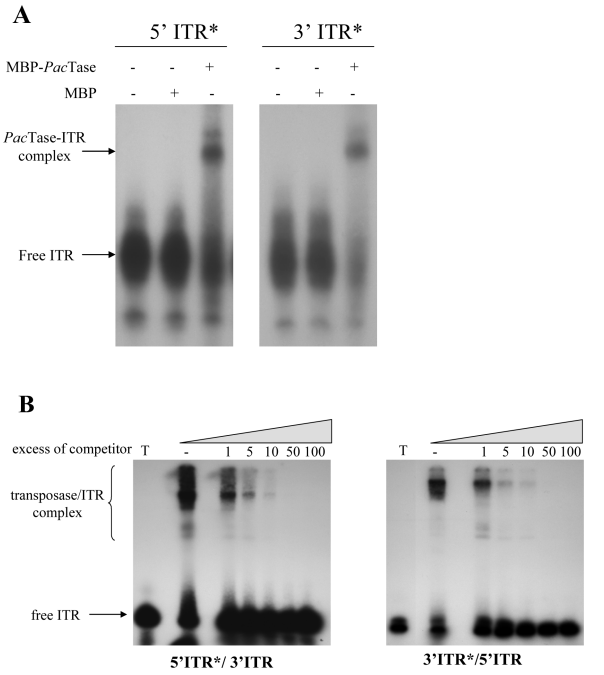

FIGURE 1: Binding of MBP−*Pac*Tase to *Pacmmar* 5′ITR and 3′ITR. (A) Electrophoretic mobility shift assays were performed as described in Experimental Procedures using either radiolabeled *Pacmmar* 5′ITR* (left) or 3′ITR* (right) and with either MBP−*Pac*Tase or MBP as indicated. (B) DNA binding experiments using radiolabeled *Pacmmar* 5′ITR* (left) or 3′ITR* (right) as a probe in competition with a 1−100-fold excess of unlabeled 3′ITR or 5′ITR, respectively. After electrophoresis, dried gels were exposed to X-ray film for 5 h. Results are from one independent experiment representative of three.

was incubated with the donor plasmid and transposase before transformation of *pir*$^-$ *E. coli* chimiocompetent cells. Double-resistant colonies would appear only if transposition occurred between Tet^r donor and Chl^r target plasmids. In such assays, no transposition events have been observed with MBP−*Pac*Tase, even when the level of transposase was increased to 1500 nM (1, 5, 10, 100, 500, 1000, and 1500 nM) and the incubation time was as long as 16 h. However, when using the *Mos1* sequence in the donor plasmid, and 80 nM *Mos1* transposase as a positive control, we were able to mobilize the Tet^r marker with a transposition rate of approximately 2×10^{-4} as previously reported (*25*, *26*). Since this in vitro transposition assay suggests that MBP−*Pac*Tase was not able to catalyze the full transposition reaction in such conditions, the different steps of the transposition reaction were investigated one by one, to identify which step of the transposition failed.

DNA Binding Specificity of Pacmmar Transposase. The electrophoretic DNA mobility shift assay (EMSA) with the *Pacmmar* ITR sequences as targets showed that MBP−*Pac*Tase was able to form a nucleoproteic complex with either 3′ITR or 5′ITR, whereas MBP alone was not (Figure 1A). Interestingly, the affinity of MBP−*Pac*Tase was similar for both ITRs (Figure 1B). To show the specificity of this DNA binding, competition assays were performed with 0.1 pmol of radiolabeled *Pacmmar* 5′ITR and an increasing excess of several competitors sharing more or less identity with the *Pacmmar* 5′ITR target (Table 1). As shown in Figure 2A, DNA binding was strongly inhibited in the presence of as little as a 10-fold excess of unlabeled *Pacmmar* 5′ITR. Similar results were obtained with *Pacmmar* 3′ITR as unlabeled competitor (data not shown) despite the four nucleic differences between them (Table 1).

Conversely, DNA binding of MBP−*Pac*Tase to *Pacmmar* 5′ITR was resistant to a large excess of unrelated DNA (Figure 2B) or *Mos1* 5′ITR (Figure 2C). Nevertheless, when using *Bytmar1* 5′ITR which is 72% identical to *Pacmmar* 5′ITR (Table 1), as a competitor a slight inhibition could be observed (Figure 2D), indicating that the affinity of MBP−*Pac*Tase for DNA is correlated with the sequence identity with respect to *Pacmmar* 5′ITR. Similar results were obtained in competition assays using radiolabeled *Pacmmar* 3′ITR instead of radiolabeled 5′ITR (data not shown). This obviously shows the ability of *Pac*Tase to bind specifically *Pacmmar* ITRs.

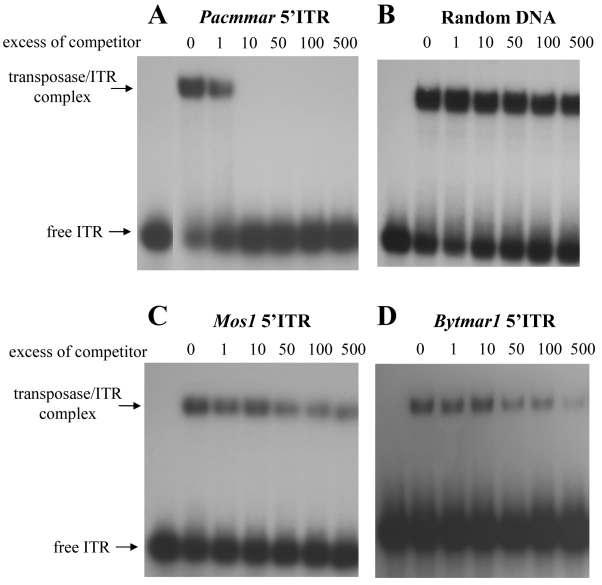

FIGURE 2: DNA binding specificity of MBP−*Pac*Tase. Electrophoretic mobility shift assays were performed using 0.1 pmol of the radiolabeled 5′ITR of *Pacmmar* together with 100 nM MBP−*Pac*Tase. Binding specificity was assayed by adding an unlabeled competitor from 0.1 to 50 pmol. The competitors tested were *Pacmmar* 5′ITR (A), a 35 bp random DNA sequence (B), *Mos1* 5′ITR (C), and *Bytmar1* 5′ITR (D). Results are from one independent experiment representative of three.

DNA Cleavage Activity of MBP−PacTase. To test the cleavage efficiency of MBP−*Pac*Tase, an in vitro kinetic cleavage assay was performed with a target plasmid bearing the full-length *Pacmmar* sequence. The cleavage activity of both the MBP−*Pac*Tase fusion protein (Figure 3, lanes 3−8) and MBP-free *Pac*Tase (lanes 9−14) was characterized by a concomitant decrease in relaxed and supercoiled forms of the plasmid, and an increase in the level of linear plasmid. The amount of linear plasmid increases with time, but no excision of the *Pacmmar* element could be observed (Figure 3). To ensure that the absence of excision product was not due to a lack of sensitivity of BET staining, DNA was transferred from an agarose gel to a nylon membrane and hybridized with a full-length *Pacmmar* radiolabeled probe. However, no excision product could be detected (data not shown). A control test with MBP alone, produced and purified under the same conditions as MBP−*Pac*Tase, resulted in the absence of cleavage activity, ruling out the possibility of DNA nuclease copurification during the MBP−*Pac*Tase production procedure (Figure 3, lane 15).

As described above, MBP−*Pac*Tase was able to cleave the pGEMT-*Pac* plasmid bearing *Pacmmar* ITRs (Figure 3 and Figure S1A of the Supporting Information). In addition, MBP−*Pac*Tase was able to linearize a plasmid deprived of any ITR (pBC), but with slower kinetics (Figure S1A of the Supporting Information). It should be noted that the well-known *Mos1* transposase was also able to cleave the pBC plasmid in the absence of any ITR (Figure S1B of the Supporting Information). The specificity of MBP−*Pac*Tase toward the cleavage target was further investigated by using an empty plasmid (pUC 18) as a competitor of the *Pacmmar*-bearing plasmid. As shown in Figure 4C (lanes 3 and 7), both plasmids were cleaved in 2 h when alone. By contrast, when both plasmids were in competition, the linear form of the pUC18 plasmid remained absent after 2 h, whereas the pGEMT-*Pac* plasmid was linearized (Figure 4, lane 5). This suggests that MBP−*Pac*Tase preferentially cleaved the *Pacmmar*-bearing plasmid rather than the empty plasmid.

The conditions of cleavage were also explored to determine if some conditions allowed the complete excision of *Pacmmar*. The

Table 1: Comparison of *Pacmmar* 5′ITR and Competitor Oligonucleotides Used in the DNA Binding Competition Assay

Oligonucleotide	DNA sequence	Identity [a]
Pacmmar 5′ITR	TACGAGGGGTGATCAGAAAGTAATGACAGTCGGAC	88.6%
Pacmmar 3′ITR	TACGAGGGGTGATCGAAAAGTAATGACAGTGAGAC	
Pacmmar 5′ITR	-TACGAGGGGTGATCAGAAAGTAATGACAGTCGGAC	72.0%
Bytmar1 5′ITR	CTACGAGGGGCGGTCAGAAAGTTCTGCAATTCGGTA	
Pacmmar 5′ITR	TACGAGGGGTGATCAGAAAGTAATGACAGTCGGAC	57.1%
Mos1 5′ITR	TACCAGGTGTAC-----AAGTAGGGAATGTCGGTT	
Pacmmar 5′ITR	TACGAGGGGTGATCAGAAAGTAATGACAGTCGGAC	38.9%
Random DNA	TGCGTCAGACCGCCCGCGATAATCCTAGTTGTT-	

[a] Sequence identity with respect to *Pacmmar* 5′ITR. Identical nucleotides are in black boxes.

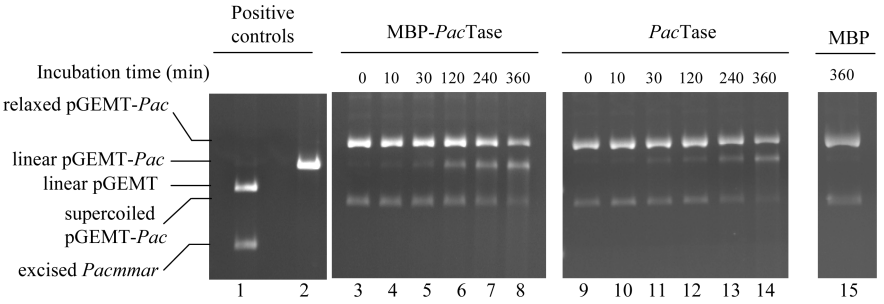

FIGURE 3: Kinetics of *Pac*Tase cleavage activity. The pGEMT-*Pac* plasmid was incubated with 10 nM MBP−*Pac*Tase fusion protein (lanes 3−8), 10 nM *Pac*Tase after cleavage of the fusion protein by factor Xa (lanes 9−14), or 10 nM MBP alone (lane 15). Incubations were run as indicated from 10 to 360 min at 37 °C. The agarose gel electrophoresis of cleavage products is presented with positive controls: lane 1, excision control (pGEMT-*Pac* digested by *Apa*I and *Pst*I); and lane 2, linear control (pGEMT-*Pac* digested by *Apa*I). Results are from one independent experiment representative of three.

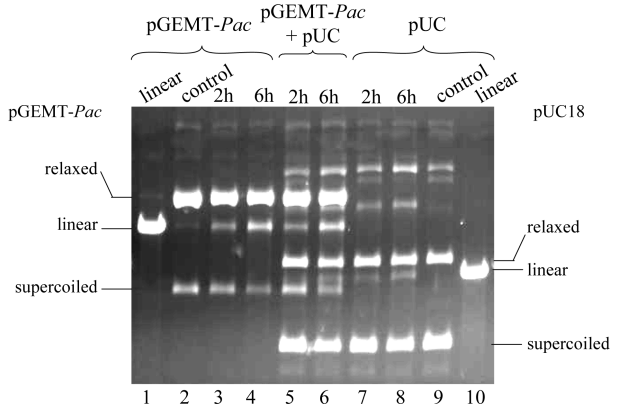

FIGURE 4: Specificity of the cleavage activity of MBP−*Pac* Tase. MBP−*Pac*Tase (10 nM) was incubated for 2 or 6 h at 37 °C in the presence of either pGEMT-*Pac* alone, pUC18 alone, or both plasmids. Controls are pGEMT-*Pac* or pUC18 alone without MBP−*Pac*Tase (control) and pGEMT-*Pac* or pUC18 digested by *Pst*I (linear). DNA fragments were separated in an agarose gel stained with ethidium bromide. Results are from one independent experiment representative of three.

effect of transposase concentration on cleavage activity was investigated using various concentrations of transposase (0.1, 0.5, 1.0, 10, 100, 200, 500, and 1000 nM) for 2 h. Results showed that a minimum of 10 nM MBP−*Pac*Tase was required to produce the linearization of the plasmid in 2 h (Figure 5A), whereas concentrations of MBP−*Pac*Tase from 0.1 to 1.0 nM were inefficient (data not shown). Unspecific DNA degradation could be observed with a MBP−*Pac*Tase concentration ≥100 nM.

Although all previous experiments were conducted in the presence of Mg^{2+}, the MBP−*Pac*Tase cleavage activity was also determined in the presence of various divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Sr^{2+}, and Zn^{2+}). These divalent cations have been tested because of their presence in the seawater and their potential presence in the intracellular fluid of the crab *P. marmoratus*. Results confirmed that MBP−*Pac*Tase activity is cation-dependent and that only Mg^{2+} and Mn^{2+} allowed the appearance of a linear plasmid (Figure 5B). In addition, MBP−*Pac*Tase was active for cleavage over a wide range of temperatures [25−50 °C (Figure 5C)] and at pHs ranging from 6 to 10, with an optimum between pH 7 and 9 (Figure 5D).

Determination of the MBP−PacTase Cleavage Site. To identify the sites of cleavage of the *Pacmmar* transposase, the linear product of pGEMT-*Pac* incubated with MBP−*Pac*Tase was purified, and a linker was ligated at the cleavage site. After amplification and cloning of DNA with flanking primers, the cleavage sites were identified. Two positive controls were performed by digestion of pGEMT-*Pac* with *Pst*I and *Apa*I, which cut at the 5′ and 3′ ends of the transposon, respectively. After cloning and sequencing had been conducted, four sequences were obtained, one for the cleavage at the 5′ side of the MLE and three at the 3′ end of the MLE. As depicted in Figure 6, results showed that cleavage occurred outside the transposon, 53−83 bp from the ITR. These results could explain the smeary PCR products obtained from amplification of the linear product when cleavage occurred around the 3′ITR (Figure 6A) and suggest that MBP−*Pac*Tase has an imprecise cleavage activity.

DISCUSSION

The *Pacmmar* element is a *mariner* element isolated from the coastal crab *P. marmoratus* and belongs to a novel *mariner* subfamily called *marmoratus* (*28*). Two copies of this element, i.e., *Pacmmar1.1* and *Pacmmar1.2*, display open reading frames that encode two putative active transposases, *Pac*Tase-1 and *Pac*Tase-2, respectively. These transposases displayed all the hallmarks of *mariner* transposases such as a DD34D catalytic core, a well-conserved YSPDLAP motif, and a WIPHLL sequence, which was slightly modified from the WVPHEL motif described by Robertson (*29*). At the secondary structure level, the *Pacmmar* transposase binding domain displayed three α-helices and one helix−turn−helix segment as previously described for other *mariner* transposases (*2*). The *Pacmmar1.1* and *Pacmmar1.2* sequences were 99 and 99.5% identical at the nucleic and amino acid levels, respectively. The two transposases differed by only two amino acids located in the helix−turn−helix DNA-binding domain, where the W_{102} and R_{109} residues found in *Pac*Tase-1 were substituted with G_{102} and H_{109} in *Pac*Tase-2. The identity between *Pacmmar1.2* and the consensus sequence (*4*), established by the majority rule from 14 *Pacmmar* elements, suggests that *Pacmmar1.2* could be the ancestral active element, as described for the transposable elements *Himar1* (*19*), *Sleeping Beauty* (*20*), and Frog Prince (*21*). Then *Pacmmar1.2* could be an active element naturally present in the genome of its host. Therefore, the aim of this work was to investigate the in vitro activity of the transposases encoded by the *Pacmmar* elements, *Pacmmar1.1* and *Pacmmar1.2*. We showed that both transposases are defective for in vitro transposition but are able to perform some specific activities required for transposition, such as specific binding to ITR and DNA cleavage.

The ITR sequences of MLEs are imperfectly conserved in most elements as for *Bytmar1* or *Mos1*, which display three and four different nucleotides, respectively, between 3′ITR and

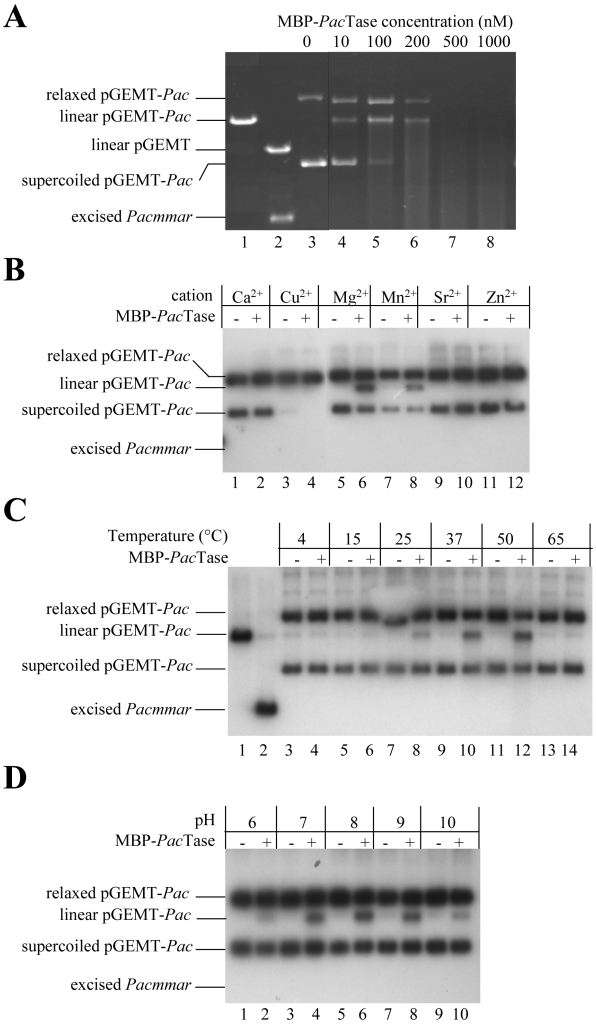

FIGURE 5: Enzyme concentration effect (A), cation requirement (B), temperature (C), and pH (D) dependence of MBP−*Pac*Tase cleavage activity.

(A) The pGEMT-*Pac* plasmid was incubated with an increasing concentration of MBP−*Pac*Tase as indicated for 2 h at 37 °C before analysis of the cleavage products on an ethidium bromide-stained agarose gel. Lanes 1 and 2 are linear control (pGEMT-*Pac* digested by *Apa*I) and excision control (pGEMT-*Pac* digested by *Apa*I and *Pst*I), respectively. (B) The pGEMT-*Pac* plasmid was incubated for 2 h with (+) or without (−) 10 nM MBP−*Pac*Tase in the presence of the indicated divalent cations (5 mM). Then cleavage products were separated in an agarose gel and analyzed by hybridization using a full-length *Pacmmar* probe. (C) The pGEMT-*Pac* plasmid was incubated for 2 h with (+) or without (−) 10 nM MBP−*Pac*Tase at various temperatures ranging from 4 to 65 °C as indicated. Lanes 1 and 2 are the same controls as in panel A. (D) Same as panel C with the incubation temperature set to 37 °C and the pH varying from 6 to 10 as indicated. Results are from typical experiments representative of three.

5′ITR (*13*, *26*). A difference in binding affinity has been reported for *Mos1* transposase, which has a 10-fold higher affinity for the 3′ITR than for the 5′ITR (*26*, *30*). By contrast, the *Pacmmar* transposase exhibited the same affinity for either the 5′ITR or the 3′ITR of *Pacmmar* despite four different nucleotides at positions 13, 14, 29, and 30. One of these nucleotides (i.e., that at position 14) is the first one of the conserved region 5′T/A-A-A/G-A/G-T/G3′ which has been previously defined by a sequence logo analysis of the ITR of various subfamilies of *mariner* elements (*31*). With regard to *Pacmmar*, this nucleotide stretch was conserved in the 3′ITR, whereas there was a G instead of T/A

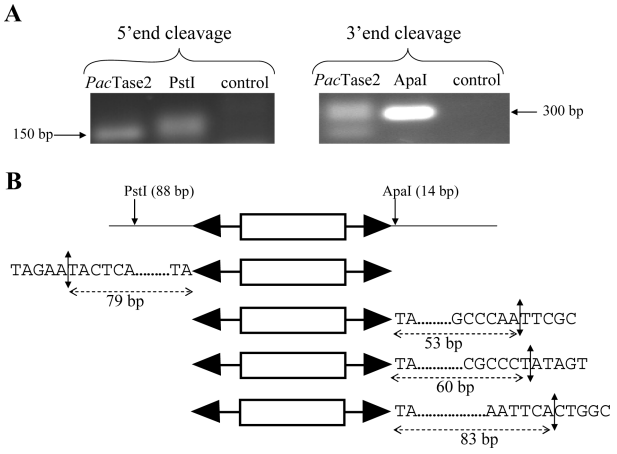

FIGURE 6: Determination of the MBP−*Pac*Tase cleavage site. (A) The pGEMT-*Pac* plasmid was incubated with 10 nM MBP−*Pac*Tase at 37 °C for 4 h. After separation and purification, the cleavage products were ligated to oligo linkers and amplified by linker-mediated PCR as described in Experimental Procedures. Each PCR reaction used a linker-specific primer and a primer specific for either the 5′ end of *Pacmmar* (left) or the 3′ end of *Pacmmar* (right). PCRs performed with *Pst*I- or *Apa*I-digested DNA were positive controls, and the PCR performed with uncleaved DNA was a negative control. (B) Cleavage positions identified after cloning and sequencing of PCR products. Filled line double arrows indicate the cleavage sites of MBP−*Pac*Tase, and the distance of the cleavage site from ITR is indicated in base pairs (bp).

in the 5′ITR. Despite an imperfect repeat of ITRs, our results show that the binding affinity remains strong for both ITRs, suggesting that ITR binding is not the defective step of *Pac*Tase transposition.

The transposase−ITR recognition is thought to be specific of the transposable element (*21*, *31*). Accordingly, we have demonstrated that the MBP−*Pac*Tase-1 and MBP−*Pac*Tase-2 fusion proteins bind specifically to the ITR sequences of *Pacmmar*. Binding of *Pacmmar* transposases to ITR from other elements (i.e., *Mos1* and *Bytmar1*) was less efficient than the binding to *Pacmmar* ITR. Despite the two amino acid differences between *Pac*Tase-1 and *Pac*Tase-2 (i.e., W vs G at position 102 and R vs H at position 109) located within the HTH motif, which is a DNA-binding domain (*1*, *2*, *32*), the same results were obtained with these two transposases. This indicates that the specificity of binding to ITR was unaltered by the two different amino acids. Probably, these two amino acids are not directly involved in transposase−ITR interaction. In some case, a unique change in a residue of the HTH sequence may abolish the transposase−ITR interaction, as observed with *Mos1* (*30*), *Himar1* (*33*), and *Metnase* (*34*).

After the step that involves binding to ITRs, the next step of the transposition mechanism is the excision of the transposon involving DNA cleavage at both sides of the element. By using an in vitro cleavage assay, we showed that *Pacmmar* transposases are able to cleave DNA, but only at one side of the transposon. This suggests that *Pac*Tase may require a specific host factor to ensure the transposon excision and then the full transposition mechanism.

To optimize reaction conditions, some biochemical parameters of the cleavage reaction have been investigated, including transposase concentration, divalent cation requirement, pH, and temperature. A high concentration of transposase has been reported to decrease the transposition activity of *Mos1* and *Himar1*, and this phenomenon has been called "overproduction inhibition" (*30*, *35*, *36*). The mechanism of overproduction

inhibition has been explained as a competition between multimeric transposase complexes that avoid excision of the transposon. In the case of *Pacmmar* transposase, a cleavage was observed from 10 to 200 nM. Higher concentrations seem to decrease the specificity of the transposase which seems to act like a nuclease.

Both Mg^{2+} and Mn^{2+} were identified as cofactors of *Mos1*, *Himar1*, and *Hsmar1* activity (*27*, *35*, *37*). In this study, MBP−*Pac*Tase cleavage capacity was tested with some divalent cations which are naturally present in seawater, the natural environment of *P. marmoratus*. These experiments pointed out the requirement for Mg^{2+} or Mn^{2+} as previously described, whereas other divalent cationic metals (i.e., Ca^{2+}, Cu^{2+}, Sr^{2+}, and Zn^{2+}) were unable to replace the former at the concentration used. It was reported that a high pH (9 or 10) increased the binding activity of *Mos1* transposase (*26*) and that *Himar1* transposase has a maximum of activity at 28 °C (*35*). Besides, the cleavage activity of *Pacmmar* transposase was not influenced by pH in the range of 7−9 and only slightly affected by the temperature ranging from 25 to 50 °C. These results indicate that *Pac*Tase cation, temperature, and pH requirement agree with that already reported for other MLE transposases. Nevertheless, some parameters such as ionic strength may also influence the *Pac*Tase activity but have not been tested here.

Cleavage sites of MLE transposases, including *Mos1*, *Himar1*, and *Hsmar1*, have been mapped precisely at each end of the transposon, even if the transferred strand is cleaved predominantly two or three nucleotides within the element (*19*, *27*, *38*). By contrast, the identification of four cleavage sites showed that *Pac*Tase generates imprecise cleavages, far from the MLE end. However, a preference of MBP−*Pac*Tase for the ITR-bearing plasmid rather than the ITR-free plasmid was shown by a competition experiment, suggesting that the binding domain is functional while the catalytic region catalyzes a noncanonical cleavage. Several explanations are conceivable. The presence of MBP linked to the transposase may hinder the formation of the cleavage complex, resulting in aberrant cutting off. Very small amounts of transposase-truncated forms may act as inhibitors of the reaction as previously described for IS911 (*39*). The requirement of specific host factors interacting with the transposase to stimulate the transposition reaction has been previously demonstrated in the case of the *Sleeping Beauty* transposase (*40*), and such a phenomenon may be necessary to ensure a precise cleavage with *Pacmmar* transposase. Finally, some mutations in the *Pacmmar* sequence may affect the functionality of the enzyme and particularly its ability to make a precise and specific cutting off. In addition, such discrepancies have been already reported for *Metnase*, a *Hsmar*-related transposase, which displays uncoupling between DNA binding and cleavage activity (*34*).

Altogether, the results presented here confirm the putative activity of *Pacmmar* transposase, which should now be explored in vivo to check the influence of host factors, especially of eukaryotic origin.

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SUPPORTING INFORMATION AVAILABLE

Sequences of PCR primers (Table 1) and of oligonucleotides used in electrophoretic mobility shift assays (Table 2) and additional results of the cleavage assay with both *Pac*Tase and *Mos1* transposase (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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