6. Sleeping Beauty Transposase

6.1. Results

The main structure-function analysis was focused on the N-terminal DNA binding domain of the transposase and the purification of a soluble form of the full-length transposase. A schematic representation of three N-terminal constructs is given in the figure below.

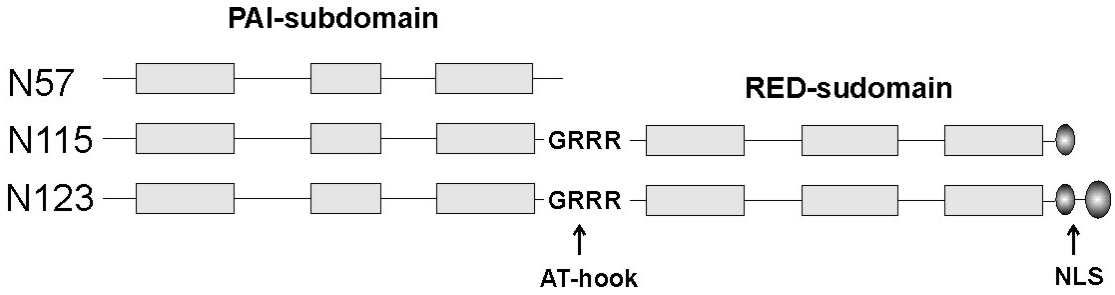

Figure 34. Histidine-tagged SB transposase variants. Boxes are the predicted α-helices making the putative helix-turn-helix motif. Of these N57 (has PAI subdomain, the N-terminal HTH of the paired domain), N115 (contains the two predicted HTH motifs of the complete bipartite DNA-binding domain) and N123 (contains the complete DNA-binding domain and the NLS) were purified to homogeneity and used in crystallization trials.

6.1.1. Protein expression and purification

The N-terminal 123 residues (14.9 kDa and pI of 11.47) of SB transposase were expressed in *E. coli* BL 21 DE3 (Novagen) strain with a C-terminal 6-His tag from the pET21a vector. Cells were grown in L broth at 37 °C to an OD_{600} of 0.5 and then transferred to 30 °C till an OD_{600} of 0.6 was reached and then induced with 0.5 mM IPTG for further 3 hours. The cells were harvested and lysed by passage through a French Press in 25 % glycerol, 1 % Tween 20, 5 mM β-mercaptoethanol, 0.2 mg/ml Pefabloc, 1 M NaCl, 25 mM HEPES pH 7.4. The lysate was centrifuged, and the supernatant was loaded on a column of Ni^{2+}-NTA (Qiagen) resin. Bound protein was eluted with 300 mM imidazole, 200 mM NaCl, 25 mM HEPES pH 7.4. Eluted protein was dialysed for 3 hours against 25 mM HEPES (pH 7.6), and 100 mM NaCl in a 3.5 kDa MWCO membrane. The second step in

the purification involved a Mono-S a cation exchange column (Pharmacia), where a linear gradient was run from 50 to 1000 mM NaCl over 20 mls. Protein eluted at about 500 mM NaCl. Overnight dialysis was carried out against 25 mM HEPES (pH 7.6) and 50 mM NaCl in a membrane with molecular weight cutoff of 3.5 kDa. N-terminal sequencing showed that the first terminal methionine was missing.

Other N-terminal DNA-binding domains purified were N57 (7.4 kDa and pI of 10.6) and N115 (14 kDa and pI of 11.35) (Fig.34). The purification protocol was essentially the same as described for N123 (Fig. 35).

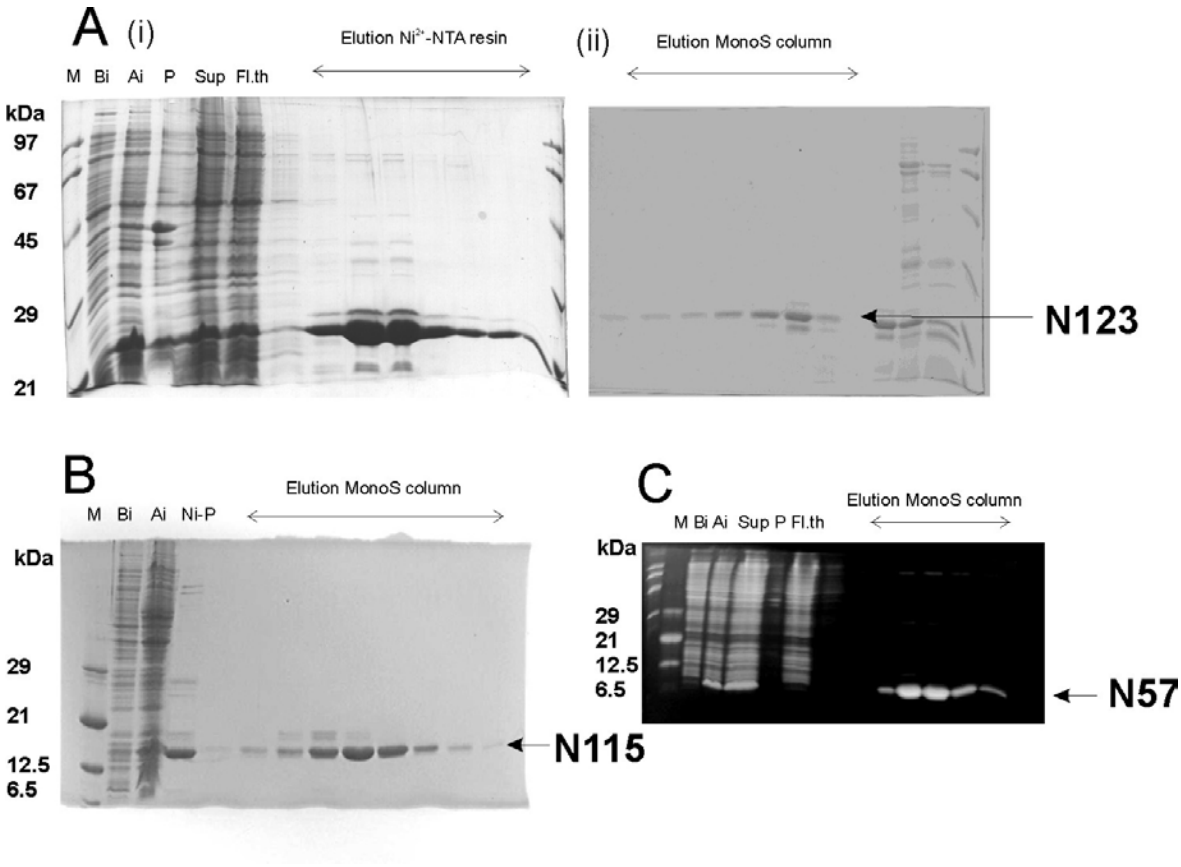

Figure 35. Purification of N-terminal variants of the transposase Sleeping Beauty. SDS PAGE gels showing the purification process (M: marker, Bi: before induction, Ai: after induction, P: pellet, Sup: supernatant after cell lysis and centrifugation, Fl.th: flow through from the Ni^{2+}-NTA resin and Ni-P: pooled fractions after Ni^{2+}-NTA step). (**A**) Purification of N123 construct (i) affinity purification over Ni^{2+}-NTA resin (ii) cation exchange over MonoS column. (**B**) Purification of N115 and (**C**) N57 construct.

6.1.2. Oligonucleotide preparation

Oligonucleotides based on the SB binding site (Table 7) were designed differing in length (30, 34, 36, 37 base pair for N123/N115 and 27, 26 base pair for N57) and were tested for their DNA binding (see next section).

The different oligomers were synthesized on a 1 µmol scale (Biotez) and resuspended in 10 mM Tris pH 7.6, 100 mM NaCl, and 1 mM EDTA. The two strands were mixed in equimolar amounts, annealed by heating to 94 °C and gradual cooling overnight. The double stranded DNA was purified on a Mono Q column (Pharmacia) in 10 mM Tris pH 7.6 with a NaCl gradient.

Table 7. Oligonucleotides used in the co-crystallization experiments with the SB constructs

Base pairs	Oligonucleotide Sequence
30bp	5' CCT AAG TGT ATG TAA ACT TCC GAC TTC AAC 3' 3' GGA TTC ACA TAC ATT TGA AGG CTG AAG TTG 5'
36bp	5' CAA CCT AAG TGT ATG TAA ACT TCC GAC TTC AAC TGT 3' 3' GTT GGA TTC ACA TAC ATT TGA AGG CTG AAG TTG ACA 5'
34bp	5' CAA CCT AAG TGT ATG TAA ACT TCC GAC - - C AAC TGT 3' 3' GTT GGA TTC ACA TAC ATT TGA AGG CTG - - G TTG ACA 5'
27bp	5' GGT ACG TTT ACA TAC ACT TAG GAG ACC 3' 3' CCA TGC AAA TGT ATG TGA ATC CTC TGG 5'

6.1.3. Oligonucleotide binding behaviour of N123 and N57

The oligonucleotide binding of the N-terminal constructs was checked in a band shift assay (Fig. 36). A relative decrease is seen in the intensity of the DNA band upon increasing the protein concentration. As judged by these experiments, the stoichiometry of the N123-36bp/N57-27bp complex is 2:1.

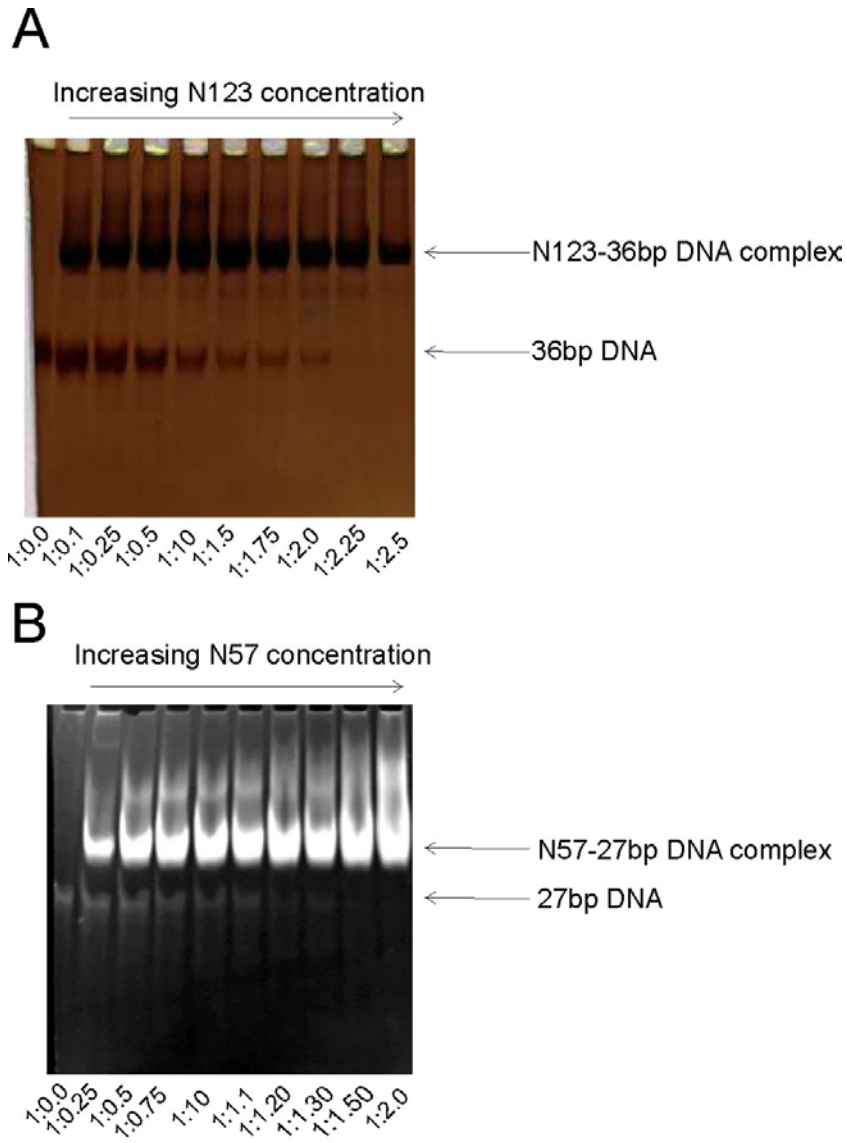

Figure 36. Band shift assay for determining the binding of oligonucleotides with N123 and N57. The two proteins and the oligonucleotides were purified as described in the earlier section. All samples were incubated at 4 ºC for 30 min before running on a 15 % native gel, which was subsequently stained with silver nitrate. All ratios are with constant amount of DNA and the varying factor is the protein. (**A**) N123 protein with 36bp oligonucleotide (0.12 nanomoles). (**B**) N57 protein with 27bp oligonucleotide (0.05 nanomoles).

6.1.4. Purification of a full-length active transposase (MBP-SB)

Protein insolubility is a major problem when trying to purify full-length transposases expressed in *E. coli* and Sleeping Beauty is no different. A procedure was developed to purify large amounts of full-length transposase suitable for biochemical and crystallographic studies. The plasmid expressing a maltose-binding protein (MBP)–SB transposase fusion protein was made by cloning the SB transposase gene into the *Xmn*I/*Eco*RI sites of pAML-c2X (NEB). The plasmid was transformed into the BL21-CodonPlus-RIL *E.coli* strain (Stratagene).

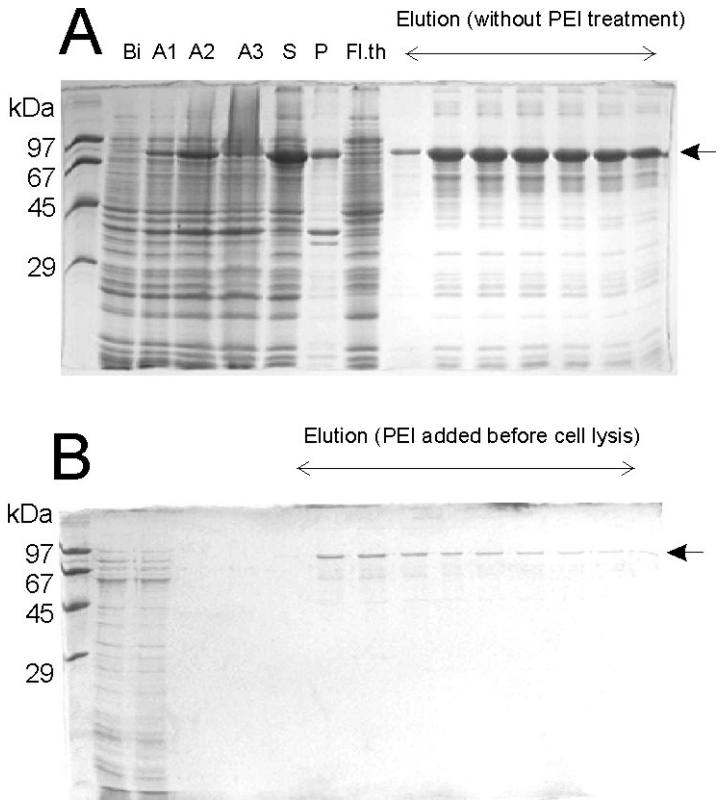

Figure 37. Purification of a full-length active transposase (MBP-SB). An important step in purification was addition of polythyleneimine (PEI) to precipate the nucleic acids. Arrow marks the position of the fusion protein, MBP-SB (95 kDa). (**A**) Though large amounts of protein could be purified without PEI treatment, but the eluted fractions were heavily contaminated with nucleic acids (Bi: before induction, A1-A3: 1, 2 and 3 hours after induction, S and P: supernatant and pellet after cell lysis, respectively. (**B**) Use of PEI before before cell lysis removed the nucleic acid impurities.

A 1 l bacterial culture was grown to OD (A_{600}) ≈0.5, IPTG was added to a final concentration of 0.3 mM, and the culture was further incubated at 37 °C for 2 h. Cells were harvested and resuspended in 30 ml of column buffer (CB, 20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT). Before cell lysis, 0.6 mg DNase I and 0.5% (v/v) polyethyleneimine (PEI) was added (Fig. 37).

Cells were lysed by passage through a French press at 1200 p.s.i., and the pellet obtained after centrifugation was resuspended in 50 ml CB containing 750 mM NaCl. In the higher ionic strength buffer, MBP–SB was dissolved, but nucleic acids and some other proteins remained in the pellet. The supernatant was diluted 1:5 with CB and loaded on an amylose resin column (12 ml of resin equilibrated with column buffer) with a flow rate not exceeding 1 ml/min. Washing was done with 12 column volumes of wash buffer (CB with 750 mM NaCl). The fusion protein was eluted with elution buffer (20 mM Tris pH 7.4, 750 mM NaCl, 1 mM EDTA, 1 mM DTT and 10 mM maltose), 25 fractions of 2 ml each were collected; the fractions containing the fusion protein were pooled and concentrated to 0.4 mg/ml.

6.2. Discussion

6.2.1. Crystallization trials

Crystallization trials were done with all three N-terminal constructs of the transposase, N57, N115 and N123. Though all three constructs were able to bind the oligonucleotides tested (Table 7), however no suitable crystals could be obtained either with the protein alone or in presence of DNA. Crystallization attempts with purified N123 bound to a 36 bp oligonucleotide yielded in crystals but these were mostly unsuitable for data collection (Fig. 38)

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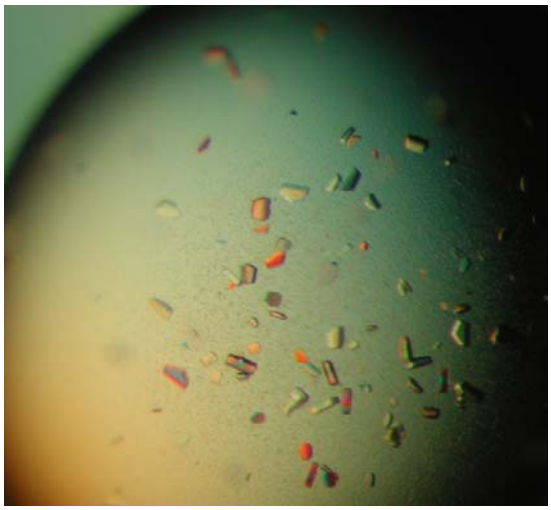

Figure 38. Small crystals of N123-DNA complex. Small crystals were obtained but these never diffracted beyond 10 Å resolution.

6.2.2. SB Transposase forms a tetrameric complex with DNA

Sedimentation equilibrium experiments were carried out to analyze the stoichiometry of complex formation between an oligonucleotide containing the transposase-binding site and N123. A prerequisite for such analysis is knowledge of the molecular masses of the reactants. The values obtained for the oligonucleotide and N123 protein (Fig. 39) indicate that both the oligonucleotide and the protein are in a monomeric state in solution.

Mixtures consisting of 1.4 µM oligonucleotide and variable amounts of N123 protein were centrifuged until reaching the sedimentation equilibrium, and analyzed with respect to complex formation. Although the oligonucleotide was monomeric in solution, it appeared to dimerize in the presence of a small amount of N123. The best fit of radial absorbance curves is reached assuming an oligonucleotide dimer binding four molecules of N123 (Fig. 39). This is also supported by the M_r values, which have a maximum at a 4:2 ratio of N123 to oligonucleotide, and drop at higher ratios because of the excess of free N123. Taken together, the transposase can take up a tetrameric form in solution in the presence of DNA, and the N-terminal DNA-binding region is sufficient to mediate tetramerization (Izsvak *et al*., 2002).

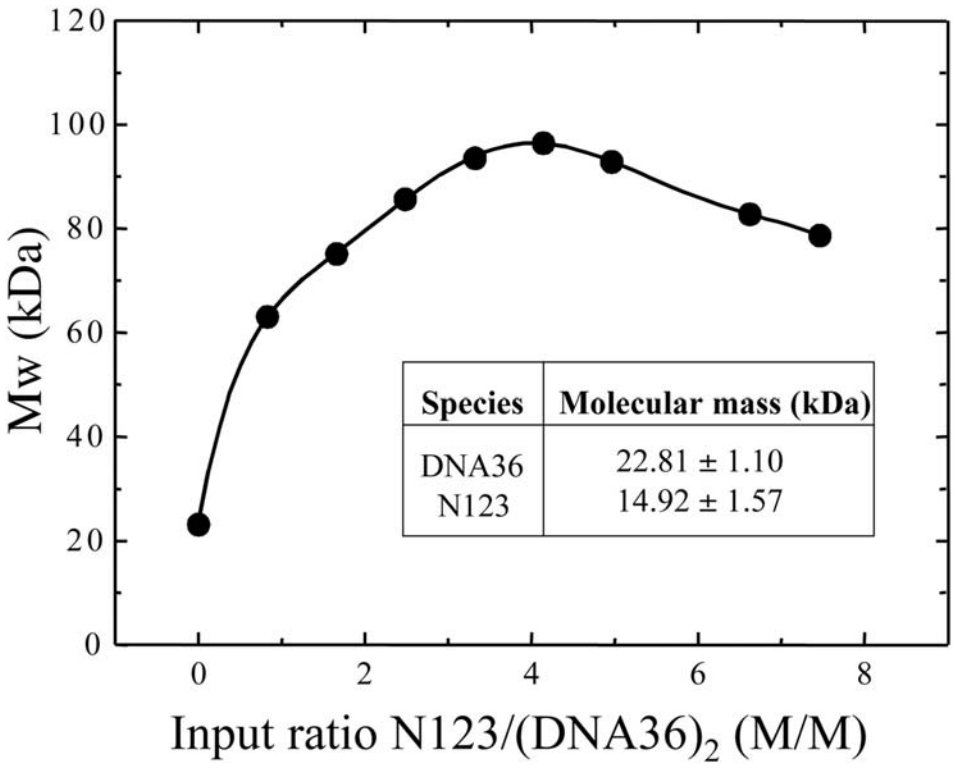

Figure 39. Sleeping Beauty transposase forms a tetrameric complex with DNA in solution. Figure shows influence of N123 to oligonucleotide ratio on complex formation. The loading concentration of oligonucleotide was 1.4 µM.

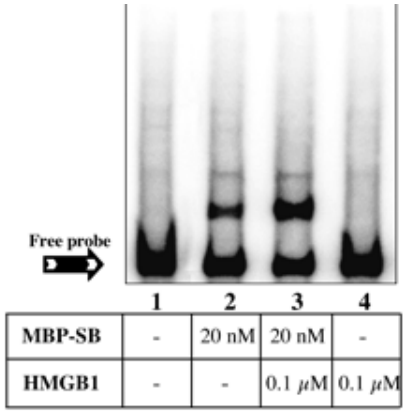

Figure 40. Formation of a ternary complex of the full-length SB transposase, HMGB1, and transposon DNA. HMGB1 stimulates specific binding of a MBP–SB transposase fusion to the transposon IRs. EMSA was performed using the left IR of *SB*, containing two binding sites for the transposase, as a probe, and MBP–SB. The radioactively labelled IR fragment was incubated with buffer only (lane 1), or with 20 nM MBP–SB alone (lane 2), or together with 0.1 μM HMGB1 (lane 3). Lane 4 contained 0.1 μM HMGB1 alone. (Adopted from Zayed *et al*., 2003).

6.2.3. The purified MBP-SB transposase is functionally active

Since the production of recombinant, full-length SB transposase is difficult due to insolubility problems, a maltose-binding protein–SB transposase fusion protein (MBP–SB) was expressed in *E. coli*, and purified. MBP–SB was first tested for DNA-binding activity in an EMSA experiment. HMGB1 enhanced the binding efficiency of MBP–SB more than two-fold (Fig. 40). HMGB1 alone did not shift the probe (Fig. 40, lane 4). The most efficient enhancement of DNA binding was observed when HMGB1, MBP–SB and DNA were added to the reaction (Fig. 40, lane 3). The conclusion was that the MBP–SB fusion protein was active in binding to the transposon IRs, and that, as observed before, HMGB1 stimulated this binding (Zayed *et al*., 2003).