3. Results

3.1. The DNA-bending Protein HMGB1 is a Cellular Cofactor of *Sleeping Beauty* Transposition

3.1.1 HMGB1 is required for efficient *Sleeping Beauty* transposition in mouse cells

The importance of HMGB1 for *SB* transposition has been assessed by applying an *in vivo* transposition assay (Ivics et al., 1997) on wild-type and HMGB1-deficient mouse cells (Fig. 5). The assay is based on cotransfection of a donor plasmid carrying an antibiotic resistance gene (*zeo*)-marked transposon and a transposase-expressing helper plasmid into cultured cells (Fig. 5A). In control experiments, a plasmid expressing β-galactosidase (CMVβ) substitutes for the transposase helper plasmid. Cells were placed under antibiotic selection, and the numbers of resistant colonies counted. The ratio between numbers obtained in the presence versus the absence of transposase is the readout of the assay, and is a measure of the efficiency of transposition. Consistent with earlier findings (Izsvak et al., 2000), transposition was readily detectable in the wild-type mouse embryonic fibroblast (MEF) cell line VA1 (about 3-fold increase in colony number in the presence of transposase) (Fig. 5B). However, colony numbers obtained in HMGB1-deficient C1 cells were not significantly different in the presence and absence of transposase, indicating a severe drop in transposition efficiency.

In order to confirm that the effect is specific for the lack of HMGB1 protein in C1 cells, a plasmid expressing human HMGB1 was cotransfected together with the transposon vectors. Exogenous overexpression of HMGB1 increased colony numbers about 4-fold (Fig. 5B), which not only rescues but exceeds wild-type transpositional rates. The effect of HMGB1 in this experiment is specific for the transposition reaction, because in the absence of transposase HMGB1 did not increase the number of zeo-resistant colonies in the C1 cell line (data not shown). We tested the specificity of complementation by cotransfecting plasmids expressing two other members of the HMG family, HMGB2 and HMGA1. HMGB2 showed partial complementation, consistent with its structural similarity and functional overlap with HMGB1 (Bustin, 1999; Thomas and Travers, 2001), whereas HMGA1 had no significant effect on the efficiency of transposition (Fig. 5B).

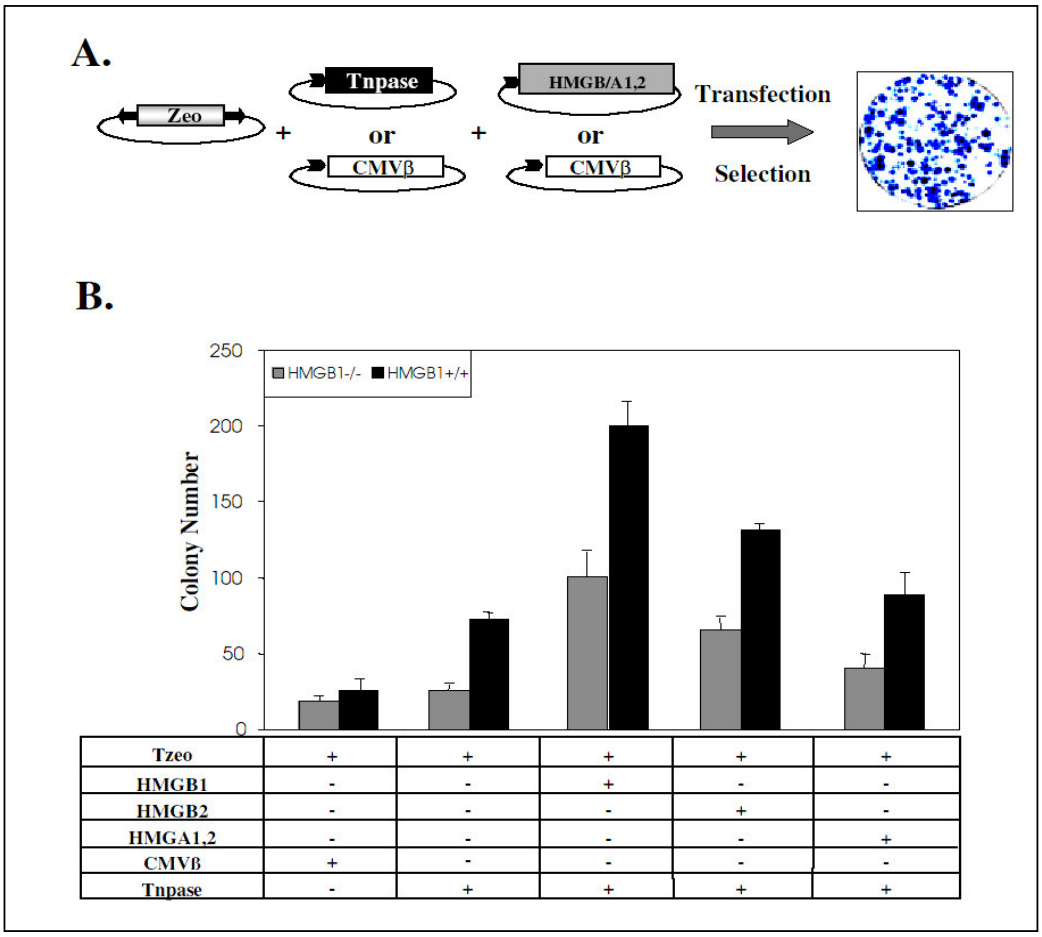


Fig. 5. Efficient *Sleeping Beauty* transposition requires HMGB1. (A) Schematic representation of the *in vivo* transposition assay. Constructs expressing HMG proteins are cotransfected with transposon donor and transposase-expressing helper plasmids into cultured cells. In control transfections, a plasmid expressing β-galactosidase is cotransfected. Cells were placed under zeocin selection, and resistant colonies are counted. The ratio of colony numbers in the presence versus in the absence of transposase is a measure of the efficiency of transposition. Arrows flanking the zeocin gene in the transposon donor construct represent the terminal inverted repeats. **(B) The effect of HMG proteins for transposition.** HMG protein expressing constructs were cotransfected into either wild-type (black columns) or HMGB1-deficient (gray columns) mouse cells. The indicated constructs were used either to complement or to overexpress different HMG proteins. The efficiency of transgene integration was estimated by counting zeo-resistant colonies. The numbers on the left represent the numbers of colonies per 10^5 cells plated.

Transient overexpression of HMGB1 in wild-type cells was shown to enhance the biological activity of several proteins that interact with HMGs, including the V(D)J recombinase RAG1/2 (Aidinis et al., 1999). Therefore, wild-type MEFs were cotransfected with the transposon system together with plasmids expressing HMGB1, HMGB2 or HMGA1. A pattern of transpositional enhancement similar to that in the HMGB1-deficient C1 cell line

was observed: HMGB1 had the most pronounced effect by increasing transposition about 2.5-fold (Fig. 5B). Overexpression of HMGB2 had a smaller effect, and increased the numbers of resistant colonies by about 1.5-fold, whereas overexpressing HMGA1 resulted in no change in colony numbers (Fig. 5B). Taken together, these results establish that HMGB1 is required for efficient DNA transposition in mouse cells, and that HMGB1 is a limiting factor of transposition in wild-type cells.

3.1.2. HMGB1 enhances bending of the *SB* transposon terminal inverted repeat and the full length transposon

Upon binding to DNA, HMG proteins induce conformational changes in the DNA, thereby facilitating juxtaposition of distantly bound proteins and assembly of multiprotein complexes (Paull et al., 1993). *SB* has two transposase binding sites per terminal inverted repeat, separated by about 160 bp spacer regions (Fig. 2A). We hypothesized that the bending activity of HMGB1 could contribute to bringing the DRs and/or the complete inverted repeats closer in space, thereby assisting the formation and/or stabilization of a synaptic complex.

To address this question, a ligase-mediated circularization assay (Stros, 1998) was performed on a DNA fragment comprising the left IR of the transposon. This assay measures the effect of HMGB1 on intramolecular ligation (circularization) of relatively short, and thus rigid, linear DNA molecules. The bending activity of HMGB1 results in enhanced juxtaposition of DNA ends, and therefore in enhanced circle formation by ligation. The radioactively labeled transposon IR fragment was incubated with T4 DNA ligase for different periods of time. The experiment was performed using a low concentration of ligase, and under these conditions, no ligation products were detected in the absence of HMGB1 even after 60 minutes of incubation (Fig. 6, lane 5). In contrast, in the presence of HMGB1, ligation products began to appear after 15 minutes of incubation (lanes 8-10 in Fig. 6). Production of DNA circles was verified by treating the 60-minute sample with Exonuclease III (ExoIII), which cleaves linear DNA, but leaves circular DNA intact. Two ligation products were resistant to ExoIII treatment (Fig. 6, lane 11), confirming the presence of circular DNA molecules. These results show that HMGB1 has a profound ability to bend a DNA fragment containing the transposon inverted repeat.

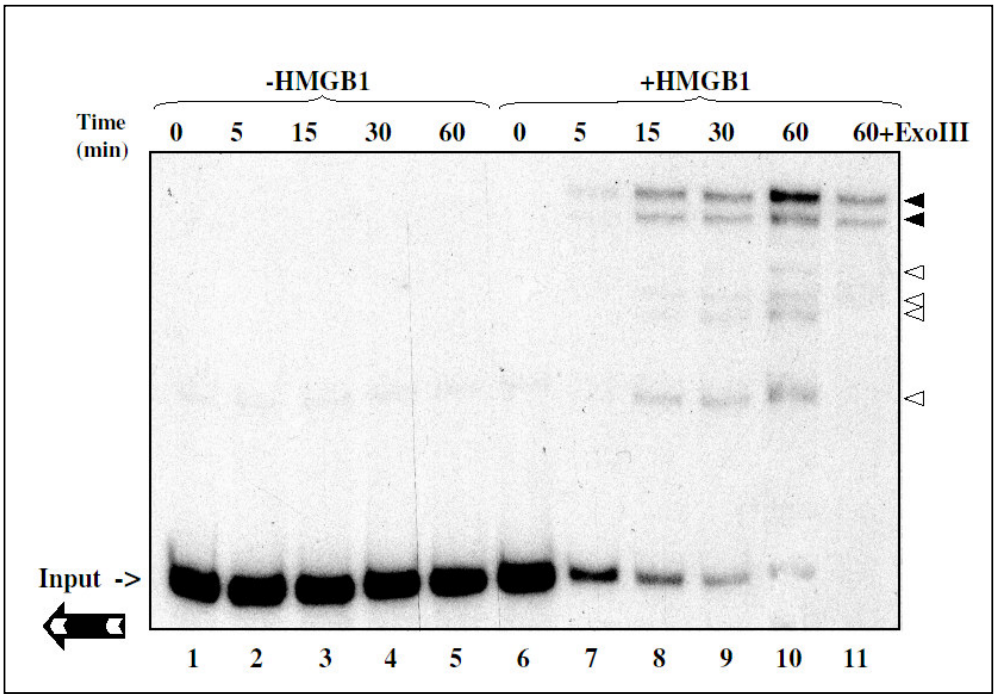


Fig. 6. HMGB1 enhances bending of the transposon inverted repeat. Intramolecular ligation (circularization) assay was performed to monitor the effect of HMGB1 on bending of the left IR of the transposon. The probe was ligated by T4 DNA ligase in the absence or presence of HMGB1 for the time periods indicated. Lane 11 is the same as lane 10, except treated with Exonuclease III. Empty triangles indicate linear ligation products. Filled triangles point to circular ligation products resistant to ExoIII digestion.

A different assay was utilized to investigate HMGB1-induced bending of a complete transposon. This assay is based on circularization of linear DNA molecules by T4 DNA ligase, and subsequent transformation into bacteria. Because of the enhanced ability of circular DNA to transform *E. coli*, the number of bacterial colonies serves as a measure of the efficiency of the ligation reaction. An *SB* transposon containing a zeocin-resistance gene (*zeo*) and an origin of replication was used for this experiment (Fig. 7A). The linear transposon was treated with T4 ligase in the absence and presence of HMGB1 for different periods of time. As shown in Fig. 7B, the average number of bacterial colonies was significantly higher in samples containing HMGB1. This shows that HMGB1-induced bending has the potential to assist the SB transposase during synaptic complex formation either by bringing the transposon binding sites and/or the terminal repeats physically closer to each other.

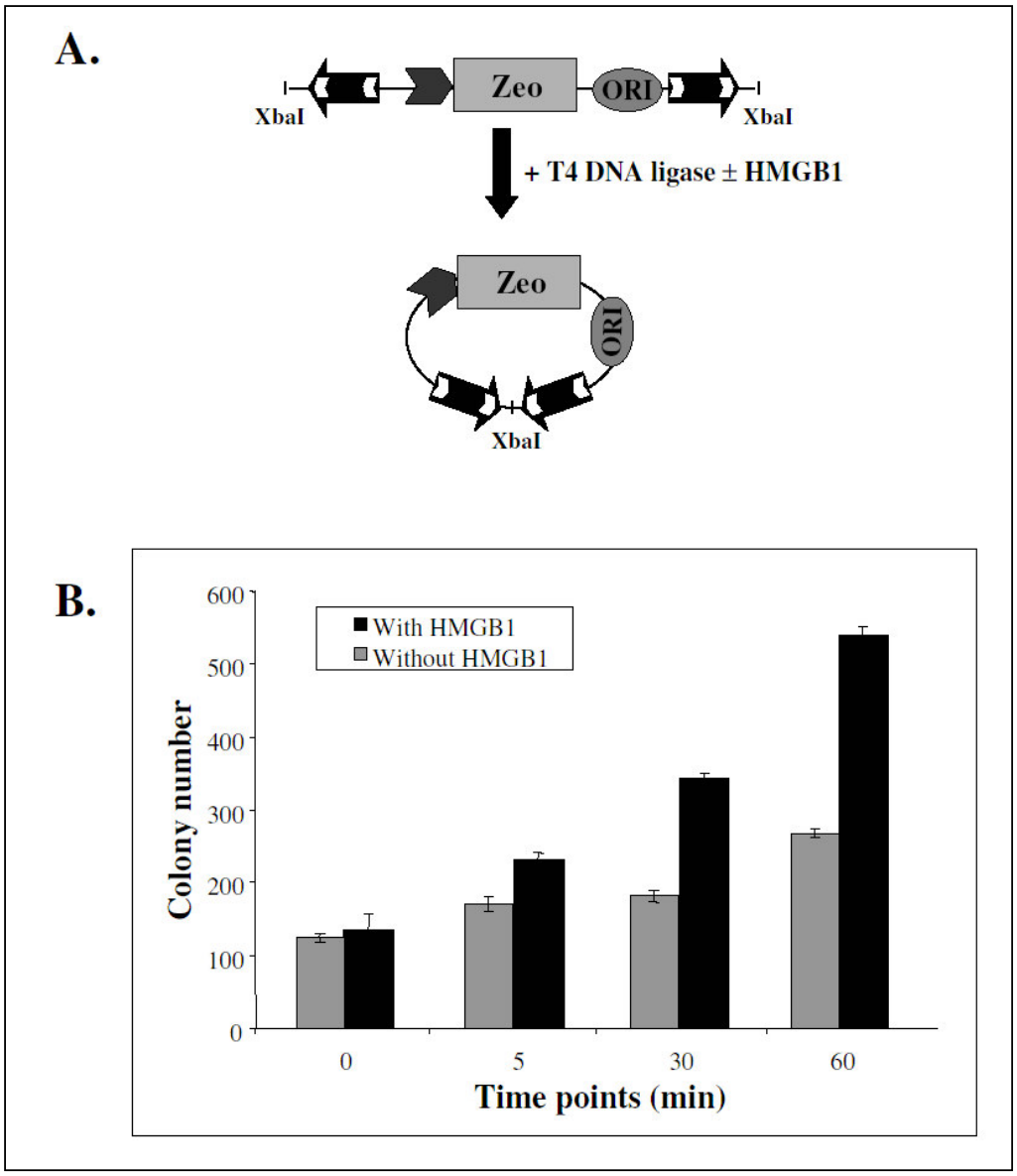


Fig. 7. Bending effect of HMGB1 on a complete *Sleeping Beauty* transposon. (A) Schematic representation of the circularization assay. The *SB* transposon contains a zeocin resistance gene (*zeo*) and a bacterial origin of replication (ORI). Black arrows flanking the element are the terminal inverted repeats, white arrows inside the IRs are the transposase binding sites. T4 ligase circularizes the linear transposon. The effect of HMGB1 on T4 ligase-mediated circularization is measured by transformation into *E. coli* cells, and counting bacterial colonies. **(B) Effect of HMGB1 on circle formation of *SB* transposon DNA.** Shown are numbers of bacterial colonies after transformation of DNA incubated with T4 ligase in the presence and absence of HMGB1, for the time periods indicated. Numbers are average of three individual experiments.

3.1.3. HMGB1 enhances the DNA-binding activity of the SB transposase

DNA transposition is a complex process that begins with sequence-specific binding of the transposase to sites within the transposon inverted repeats (IRs). We hypothesized that, in addition to its DNA-bending activity shown in Figs 6 and 7, HMGB1 stimulates transposition by enhancing transposase binding to the IRs.

Histidine-tagged versions of both the N-terminal DNA-binding domain of the SB transposase (N123), and HMGB1 were purified from *E. coli* using affinity chromatography. The left IR, containing two transposase binding sites, was radioactively labelled and used as a probe in an electrophoretic mobility shift assay (EMSA) (Fig. 8A). As shown earlier (Ivics et al., 1997), N123 produced two shifted bands, representing complexes in which either one (complex C1) or both sites (complex C2) are bound (Fig. 8A, lane 2). HMGB1 enhanced binding of N123, indicated by a more prominent formation of C2 (lane 3). The enhancement was inversely dependent on the concentration of N123 relative to that of HMGB1; stimulation of binding was about two-fold at 30 nM N123 (lane 3), five-fold at 15 nM N123 (lane 5), and more than seven-fold at 7.5 nM N123 (lane 7), as judged by comparing the total bound radioactivity (sum of bands C1 and C2) to the unbound, free probe in the presence and absence of HMGB1. In this assay, HMGB1 alone did not shift the probe when added at 75 nM concentration (data not shown). HMGB1 did not produce a supershift either, indicating that a ternary complex containing the DNA probe, N123 and HMGB1 is unstable, at least under the conditions used in the assay.

Next, the effect of HMGB1 on transposase binding to IR probes carrying only a single transposase binding site was tested in EMSA. The outer DR (ODR) is located next to the transposase cleavage site, whereas the inner (IDR) is about 200 bp from the end of the transposon. The inner and outer transposase binding sites are not identical, they share about 80% sequence identity and the IDR is shorter by two base pairs (Fig. 2C). There appeared to be a clear preference for the IDR in transposase binding, because N123 bound to it approximately three-fold stronger than to the outer site (compare lanes 2 and 6 in Fig. 8B). The presence of HMGB1 appeared to further emphasize this preference for IDR binding: at 75 nM concentration of HMGB1, N123 shifted approximately 90% of the IDR probe (lane 8), but only about 50% of the ODR probe (lane 4). HMGB1 alone did not shift either probe (lanes 3 and 7). Taken together, these data show that HMGB1 stimulates transposase binding

to the transposon inverted repeats, and that it has a more pronounced effect on binding to the internal transposase binding site.

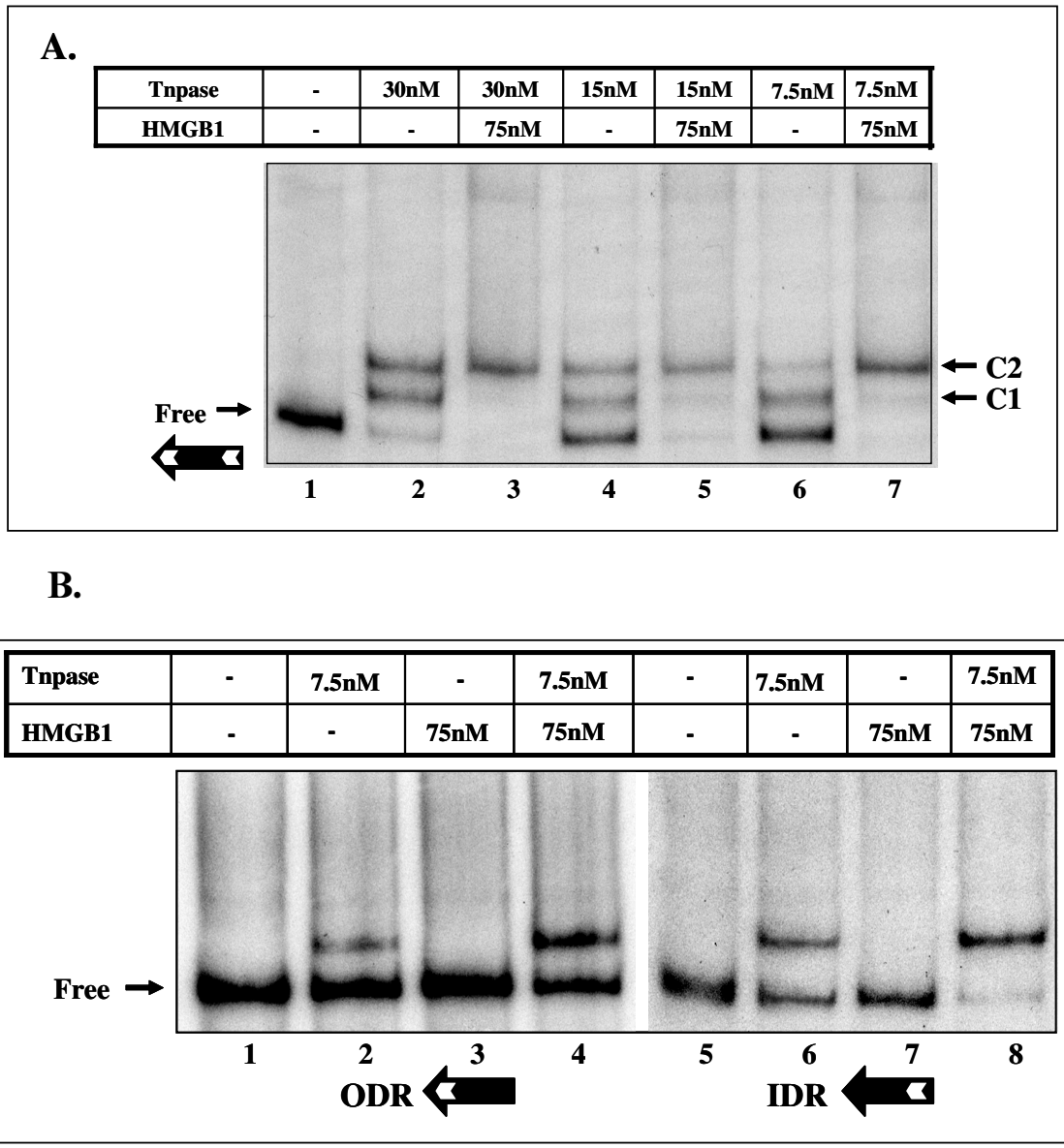


Fig. 8. HMGB1 stimulates specific binding of Sleeping Beauty transposase to the transposon inverted repeats. (A) The effect of HMGB1 on transposase binding to the left IR. Electrophoretic mobility shift assay was performed using the left inverted repeat of *SB*, containing two binding sites for the transposase, as a probe and N123, an N-terminal derivative of SB transposase containing the specific DNA-binding domain. C1 and C2 indicate the two DNA-protein complexes formed in the assay. **(B) The effect of HMGB1 on binding to either the outer or the inner transposase binding sites in the context of the left IR.** Electrophoretic mobility shift assay showing the stimulatory effects of HMGB1 on binding of N123 to the outer (ODR) and inner (IDR) binding sites.

3.1.4. SB transposase physically interacts with HMGB1

Since HMGB1 has no sequence specificity on its own, it has to be actively recruited to specific sites by other DNA-binding proteins (Bustin, 1999). To investigate possible physical interactions between the SB transposase and HMGB1, an immunoprecipitation experiment was performed (Fig. 9). Nuclear extracts were prepared from HeLa cells constitutively expressing the SB transposase. An antibody against human HMGB1 or a matched preimmune serum was used for immunoprecipitation. Precipitated proteins were subsequently blotted and hybridized with a polyclonal antibody against SB transposase. SB transposase was coprecipitated with the HMGB1 antibody, but not with the preimmune serum (Fig. 9, lanes 1 and 2). Treating the nuclear extract with DNaseI did not influence the formation of the immunocomplex (compare lanes 2 and 3 in Fig. 9) indicating that the detected signal was not due to nonspecific, simultaneous binding of SB and HMGB1 to genomic DNA.

Furthermore, interaction between HMGB1 and SB transposase is not dependent on the presence of transposon DNA, because immunoprecipitation in the presence or absence of DNA gave similar results (data not shown). Control nuclear extracts did not produce a signal (lane 4); thus, immunoprecipitation is dependent on the presence of SB transposase. Treatment of nuclear extracts with actin and p15 antibodies failed to immunoprecipitate SB transposase (lanes 5 and 6), indicating that the interaction observed is specific for HMGB1. Similar results were obtained when purified HMGB1 protein was immobilized on agarose beads, and incubated with purified SB protein (data not shown). This shows that the transposase actively interacts with HMGB1.

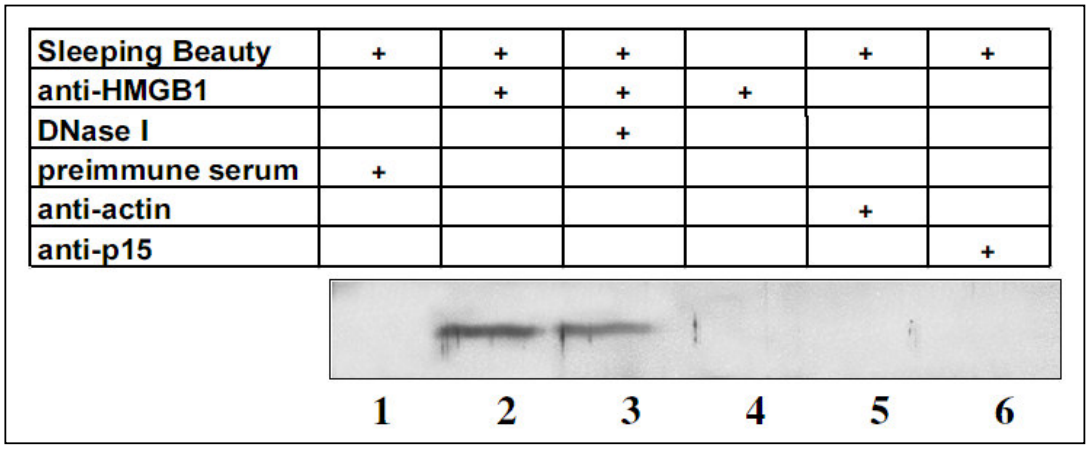
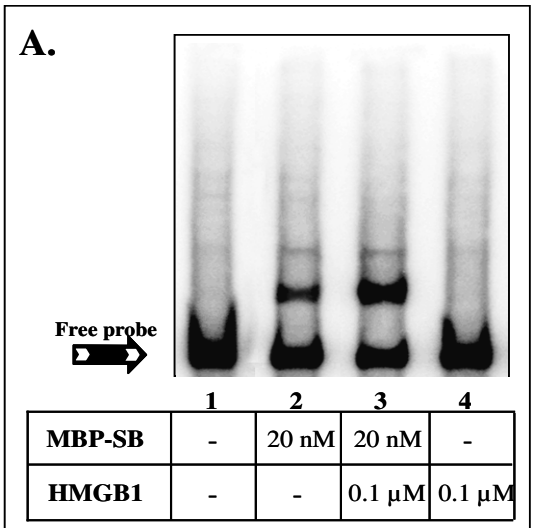


Fig. 9. Sleeping Beauty transposase interacts with HMGB1. Immunoblot of nuclear extracts of HeLa cells expressing SB transposase, and control cells, after incubation with antibodies against human HMGB1, actin and p15 proteins or a preimmune serum, with or without DNaseI treatment. The blot was hybridized with an anti-SB antibody.

3.1.5. Formation of a ternary complex of transposon DNA, SB transposase and HMGB1

The activity of HMGB1 at the transposon inverted repeats necessitates the temporary existence of a nucleoprotein complex containing the transposon IRs, the transposase and HMGB1. We considered that the full-length transposase protein is required for either the formation or stability of such a complex. Because 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, using the same inverted repeat probe as in Fig. 8A. HMGB1 enhanced the binding efficiency of MBP-SB more than two times (Fig. 10A, compare lanes 2 and 3). HMGB1 alone did not shift the probe (lane 4). The most efficient enhancement of DNA-binding was observed when HMGB1, MBP-SB and DNA were added to the reaction at a molar ratio of 5:1:0.05 (Fig. 10A, lane 3 and data not shown). We concluded that the MBP-SB fusion protein was active in binding to the transposon IRs, and that, as observed before, HMGB1 stimulated this binding.

Next evidence for a ternary complex was obtained by using MBP-SB in a coimmunoprecipitation experiment. Towards this end, radioactively labelled transposon IR DNA was incubated with MBP-SB and HMGB1, and coimmunoprecipitated with either anti-SB or anti-HMGB1 antibodies. Fig. 10B shows that the anti-SB antibody precipitated about three times more DNA-transposase complexes when HMGB1 was present in the reaction, consistent with our findings that HMGB1 enhances binding of the transposase to transposon DNA. The anti-HMGB1 antibody did not coimmunoprecipitate DNA when MBP-SB or HMGB1 were added alone to the probe. However, the anti-HMGB1 antibody did coimmunoprecipitate DNA in the presence of both MBP-SB and HMGB1 (Fig. 10 B). In contrast, N123 was not able to form a ternary complex (data not shown). These results show that HMGB1 can form a ternary complex with MBP-SB and transposon DNA. Because the catalytic steps of DNA transposition require Mg^{2+} as a cofactor (Craig, 1995), and because ternary complex formation in this experiment was observed in a Mg^{2+}-free buffer, we conclude that a likely role of HMGB1 in transposition is realized prior to catalysis, most likely during synaptic complex assembly.



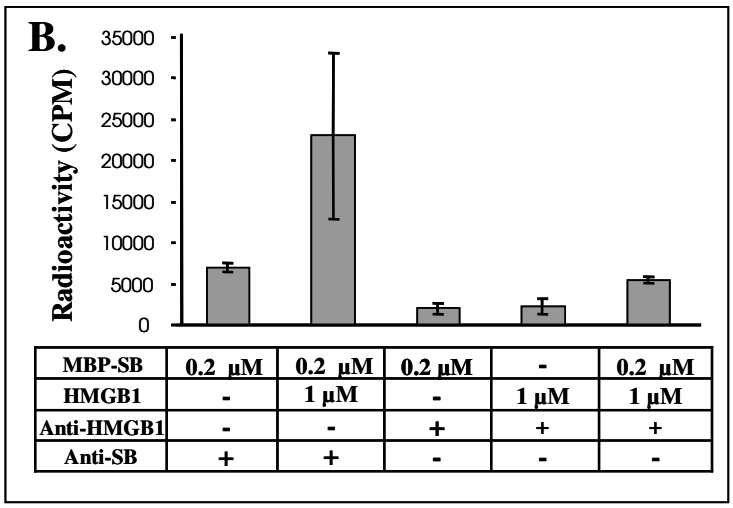


Fig. 10. Formation of a ternary complex of the full-length Sleeping Beauty transposase, HMGB1, and transposon DNA. (A) HMGB1 stimulates specific binding of a maltose binding protein-SB transposase fusion to the transposon inverted repeats. Electrophoretic mobility shift assay was performed using the left inverted repeat of *SB*, containing two binding sites for the transposase, as a probe, and MBP-SB. The radioactively labeled 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. The arrow denotes the free probe. **(B) Coimmunoprecipitation of transposon-transposase complexes with SB and HMGB1 antibodies.** Purified HMGB1 (1 µM) and purified MBP-SB (0.2 µM) were individually or together incubated with a radioactively labeled inverted repeat probe. Anti-SB and anti-HMGB1 antibodies were used to coimmunoprecipitate labeled DNA after incubation with MBP-SB and HMGB1 alone or together. After extensive washing, the radioactivity of DNA bound to immunoabsorbent agarose was measured by scintillation counting. The average cpm values obtained with the anti-SB antibody are the following: MBP-SB 6946; MBP-SB plus HMGB1 23033; the values with the anti-HMGB1 antibody are: MBP-SB 2010; HMGB1 2304 and MBP-SB plus HMGB1 5473.

3.2. Specific modification of *SB* transposon system to improve its transpositional activity

3.2.1 Site-specific mutations in the transposase and transposition assay

Based on findings with the Tn5, Tn10 and Himar1 transposases, we hypothesized that acidic to basic amino acid replacements in the SB transposase have the potential to increase transpositional activity. There are altogether 28 aspartic acid (D) and glutamic acid (E) residues in the SB transposase, they are listed in Table 1. We categorized these amino acids with respect to their conservation in the Tc1/*mariner* family (Table 1). For example, the DDE residues of the catalytic domain are absolutely conserved in the transposases, and are required for transposition (Doak et al., 1994; Plasterk et al., 1999). Therefore, conserved D or E residues were not subjected to mutagenesis. The remaining 15 acidic amino acids which are not conserved between the Tc1/*mariner* elements were subjected to site-specific mutagenesis as described in the Materials and Methods. These amino acids were replaced by either lysine (K) or arginine (R) residues (Table 1).

Next, we tested the transpositional activities of the mutant transposases relative to the wild-type transposase in an *in vivo* transposition assay (Ivics et al., 1997). The same numbers of Hela cells were cotransfected with expression plasmids containing the mutant transposase genes together with a transposable element that carries a neomycin-resistance gene (pTneo). The wild-type transposase plasmid (pCMV-SB) was used as a positive control in a parallel experiment. In the negative control experiment, a plasmid expressing β-galactosidase (pCMVβ) instead of the transposase was used. Cells were placed under antibiotic (G418) selection and the numbers of resistant colonies were counted. Colony numbers obtained with the wild-type SB transposase was set to 100%, to which the values of the mutant constructs were compared as a measure of transpositional activity relative to wild-type SB transposase. Mutations including E6K, D10K, D17K, D68K, D86K, E92K, E93K, E158K, D164K, E174K, E216K; E321R reduced transposition frequency to background levels (Fig. 11B). These results suggest that these amino acid replacements are detrimental for SB transposase activity. D140K reduced transposition to nearly 60% and D142K to about 50%, this indicates that these amino acids play important roles in SB transposase activity. Transposition activity of D260K was about 30% higher than the wild type (Fig. 11B), this may indicate that the acidic-to-basic change in this position increases the charge interaction between the negative phosphate backbone of the DNA and the SB transposase, thereby slightly improving the function of the transposase.

Amino acid residue in SB transposase	Status in other Tc1/mariner transposase	Change made
E6	Non-conserved	K
D10	Non-conserved	K
D17	Non-conserved	K
D68	Non-conserved	K
E69	Conserved	--
D86	Non-conserved	K
E92	Non-conserved	K
E93	Non-conserved	K
D140	Non-conserved	K
D142	Non-conserved	K
D153	Conserved	--
E154	Conserved	--
E158	Non-conserved	K
D164	Non-conserved	K
E174	Non-conserved	K
D210	Conserved	--
E216	Non-conserved	K
D220	Conserved	--
D244	Conserved	--
D246	Conserved	--
D260	Non-conserved	K
E267	Conserved	--
D274	Conserved	--
E279	Conserved	--
E284	Conserved	--
E306	Conserved	--
E307	Conserved	--
E321	Non-conserved	R

Table 1. Changing of the conserved acidic amino acids to basic amino acids in the SB transposase. List of acidic amino acid residues in the SB transposase, their conservation status within the Tc1/mariner family, and their mutations. The non-conserved amino acids were changed to lysine or arginine residues.

The production of an N-terminal truncated version of the transposase (N115, the first 115 amino acids of the SB transposase) can have a repressing effect on *SB* transposition (Izsvak et al., unpublished). The mechanism of N115 production is not known, but we reasoned that amino acid changes of R115 might interfere with this process, thereby reducing the repressing effect on transposition. In order to preserve the positive charge in position 115 of the transposase polypeptide, a histidine residue was introduced to replace the arginine. The activity of R115H was indeed found higher than that of the wild-type transposase by about

35% (Fig. 11B). During the process of SB transposase gene reconstruction (Ivics et al., 1997), there was a version containing a single, naturally occurring mutation, R143C, compared to the consensus transposase sequence. This version was tested against the wild-type transposase, and its activity was consistently found slightly higher than the wild-type by about 25% (Fig. 11B).

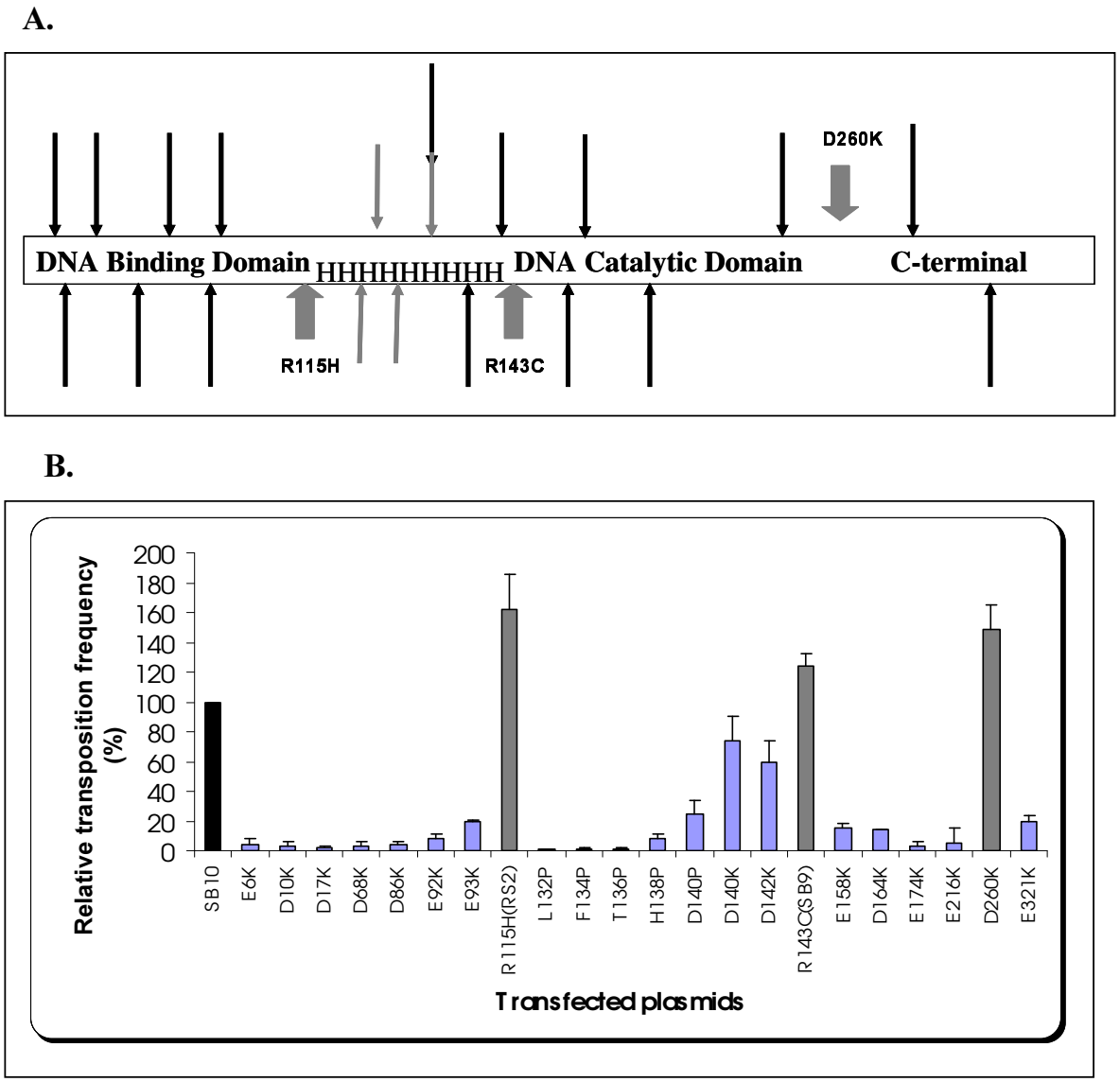


Fig 11. Effects of amino acid changes in the transposase on the efficacy of *Sleeping Beauty* transposition. **A. Schematic representation of some domains of SB transposase showing the positions of the induced mutations**. Multiple Hs refer to the helix spanning the area between the DNA binding domain and the DNA catalytic domain. Black arrows refer to the position of the proline amino acid changes. Gray arrows refer to the positions of change from acidic to basic amino acids. The small fat gray arrows refer to the position of the hyperactive mutations. **B. The result of amino acid changes on transpositional efficiency.** Plasmids encoding the wild type and mutant SB transposase proteins were cotransfected using pTneo as a substrate plasmid. In control experiments pCMVβ was used as a negative control instead of pCMV-SB10. The wild type (SB10) was set at 100%.

Next, the question was asked whether combinations of the hyperactive mutations would result in an additive or a synergistic effect. Towards that end, R115H was combined with R143C and D260K, and R143C was combined with D260K to give the three possible double mutants. An additional mutant combining all three single mutations (3M) was also engineered. R115H/D260K showed a 3.7-fold, R115H/R143C a 3.5-fold- , R143C/D260K a 2.6-fold-, and the 3M combination nearly 2.5-fold increase in transposition activity compared to the wild-type transposase SB10 (Fig. 12). These results indicate that the R115H mutation acts synergistically with both D260K and R143C.

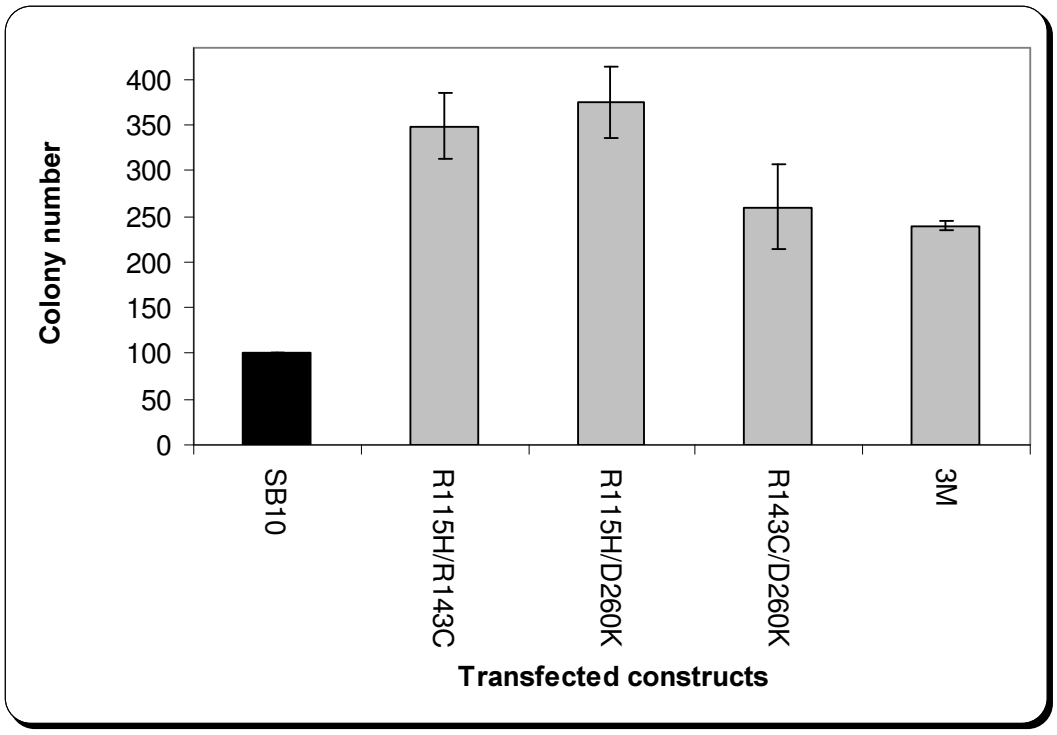


Fig. 12. **Synergistic effects of combinations of individual hypertransposing mutations.** The mutations were combined as explained in the Materials and Methods and compared with the wild type similar as described in Fig 11. Standard deviations were derived from multiple indpendent experiments.

In Tn5 transposase, there is interference between the C-terminal region and the N-terminus during interaction with the transposon DNA (Davies et al., 2000). Introduction of a proline residue helps to break this interference leading to a hyperactive transposase which has higher affinity to the transposase binding site (Davies et al., 2000). Similarly, experiments have shown that the region immediately following the N-terminal DNA-binding domain in the SB transposase has a negative impact on transposase binding to the transposon inverted

repeats (Izsvak et al., unpublished). This region is predicted to assume a helical conformation, and is conserved in the Tc1/*mariner* transposon family (Fig. 13). Based on the Tn5 observations, we reasoned that introduction of proline residues in the predicted helix motif between the DNA-binding- and catalytic domains could lead to a change in transposase structure, thereby allowing better access of the transposase to its binding sites. Towards that end, the mutations L132P, F134P, T136P, D140P (only one at a time) were introduced into the transposase, and their transposition activity determined as above. The first three mutations essentially abolished transposition, whereas D140P showed about 17% of the wild-type activity (Fig. 11B). These results indicate that these amino acid replacements are detrimental for the activity of the SB transposase, possibly because changing the spatial arrangement of the transposase leads to a non-functional structure of the transposase.

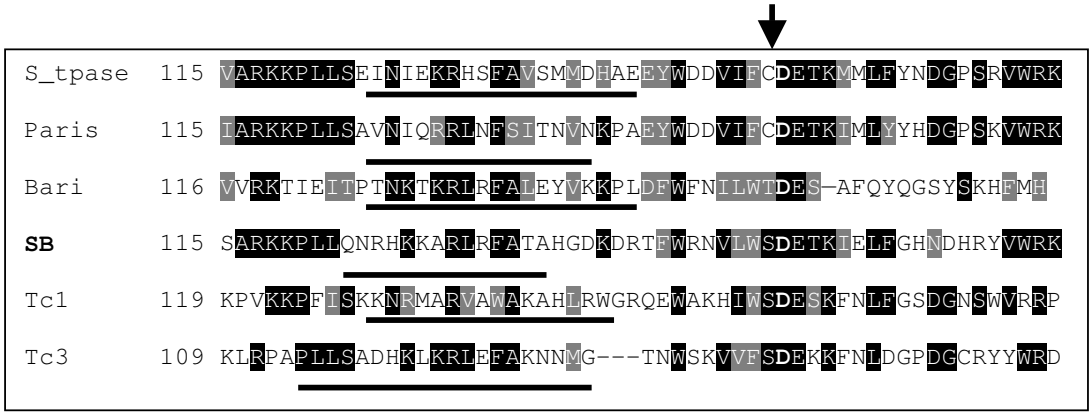


Fig. 13. Conservation of a predicted helix between the DNA binding and catalytic domains in Tc1/mariner transposases. Alignment of segments of different transposases belonging to the Tc1/*mariner* elements shows the conservation of a predicted helix spanning the region between the DNA-binding domain and the catalytic domain of the Sleeping Beauty transposase. The underlined protein sequences indicate the corresponding helices in each of the indicated transposases according to the PredictProtein program (Rost and Sander, 1993). The first conserved D in the DDE domain is shown through the whole transposase sequences where the arrow indicates.

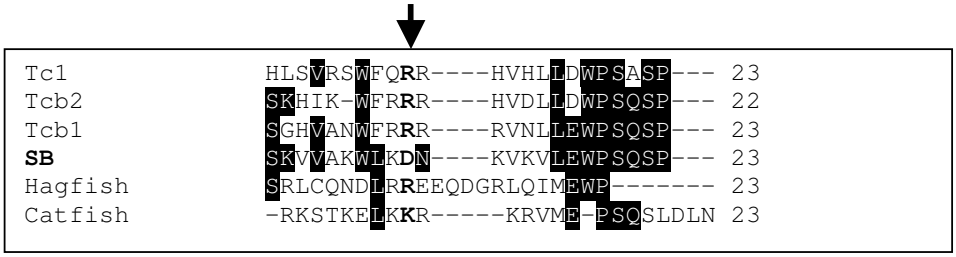


Fig. 14. **Aspartic acid residue (D) in SB transposase is either lysine (K) or arginine (R) in other Tc1/mariner transposases**. A part of alignment between some Tc1/mariner transposases shows that the D260 in SB transposase is either D or K (shown in bold), and arrow indicates.

3.2.2 Construction of the sandwich vector

Tn10 is a composite bacterial transposon, it comprises a pair of 1.4-kb IS10 insertion sequences located in opposite orientation flanking about 6.7 kb of unique sequence (Kleckner et al., 1996). Transposition has been observed not only for Tn10 but also for both IS10-right and IS10-left individually. As mentioned before, the Tc1/*mariner* element *Paris* was also shown to form composite elements by flanking long DNAs in an inverted orientation (Petrov et al., 1995). Based on this knowledge, designing a similar version of the *Sleeping Beauty* transposon by flanking a relatively large piece of DNA carries hope for boosting the capacity of SB transposase to mobilize long transgenes. A requirement for such a transposon to work is the inability of the individual *SB* units to transpose on their own. Towards this end, mutations were induced in the right inverted repeats (rIR) of the *SB* transposons in both pT and pTneo plasmids (Ivics et al., 1997), taking the conserved 5'-CAGT sequence between Tc1/*mariner* elements as a target of mutations. Mutations induced a CA-to-GC change. The mutated (m)rIR was subcloned back to either plasmid resulting in the two transposons with mutated right ends. I called the plasmids carrying the mutations pT* (Fig. 15A) and pTneo*.

3.2.2.1. Induction of mutations in the right inverted repeats of *SB* interfere with transposition, but not with the binding capacity of the transposase

To answer the question whether the induced mutations interfere with transposition, pTneo* and pTneo were used in an *in vivo* transposition assay in which the transposase expressing plasmid, pCMV-SB was cotransfected with either plasmid as described in the Materials and Methods. The results of the transfection showed that these mutations interfere with transposition SB transposase, forming a defective transposable element (Fig. 15B).

A further (suspected) requirement for the sandwich transposon to work is that the transposase can bind to all of its binding sites within the composite element. Next, the question was asked whether the mutations that were induced at the end of the element, and which abolish transposition, affect the binding ability of the transposase. Both of the rIR and (m)rIR fragments were isolated from plasmids, radiolabeled, and their ability to be bound by the transposase examined in a mobility shift EMSA experiment, using N123 (the DNA-binding domain of SB transposase). The results showed that there is no difference between the wild-type and the mutant IR fragments in terms of binding to N123 (Fig 15C, compare lane 2

and 4). These results therefore demonstrate that the induced mutations interfere only with transposition, but not with SB transposase binding.

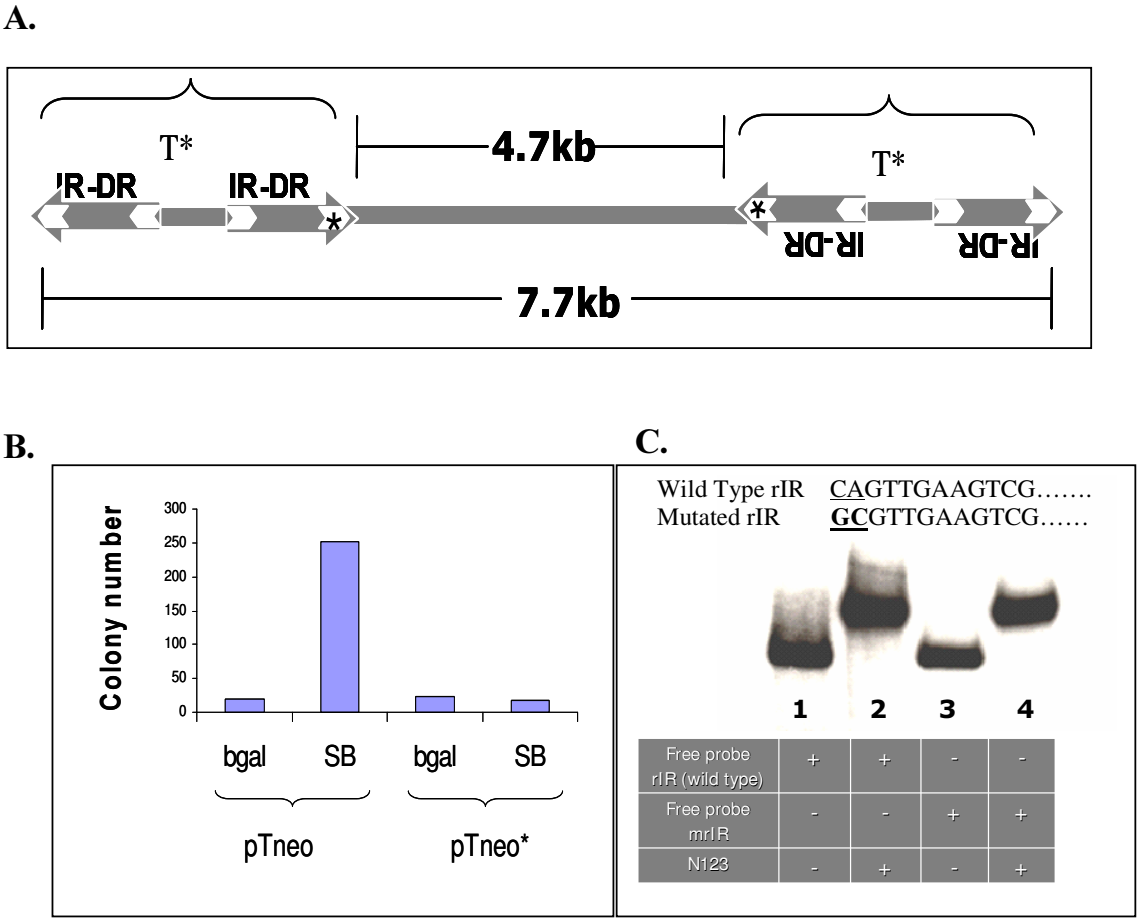


Fig. 15. Induction of mutations in the right IR interfere with transposition, but not with the binding capacity of the transposase. A. Schematic representation of the sandwich vector. Asterisks indicate the positions of the induced mutations. **B.** Graph showing that induction of a mutation in the right inverted repeat of pTneo abolishes the transposition. **C**. Electrophoretic mobility shift assay was performed using either the ^{32}P-radiolabeled wild-type or the mutated rIR, as a probe and N123, an N-terminal derivative of SB transposase containing the specific DNA-binding domain of the SB transposase.

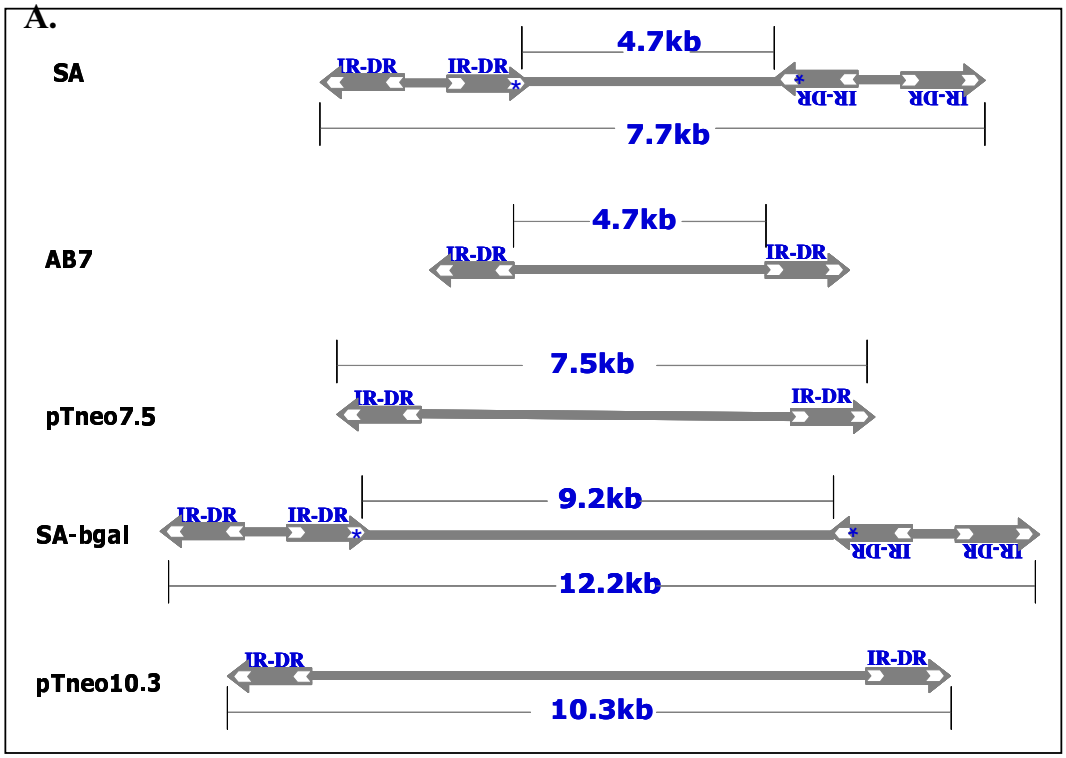
3.2.2.2. Sandwiching DNA pieces between two complete *SB* transposons in an inverted orientation

As detailed in Materials and Methods, a 4.7-kb piece of DNA was subcloned between two T* transposons in an inverted orientation forming a plasmid of 10.3 kb carrying a transposon fragment of about 7.7 kb (Fig 15A and 16A), this construct is called the sandwich vector

(SA). The SA construct is a head-to-head transposon construct comprising (intact 5‘-end)-body of T element-(disabled 3‘-end)-4.7 kb insert with a selection marker-(disabled 3‘-end)-body of T element-(intact 5‘-end). The same piece was subcloned in between the wild-type inverted repeats of pT (Fig. 16A), and I called this construct AB7. This construct carries a transposon segment of 5.5 kb (Left IR-4.7 kb insert with a selection marker-Right IR). An additional piece of DNA of 4.5 kb containing the *lacZ* gene from pCMVβ was subcloned in the body of the SA fragment between the two T* transposon fragments, to give a total transposon length of 12.2 kb (T*-9.2-T*) (Fig. 16A). I call this construct SA-βgal.

3.2.3. The sandwich vector transposes more efficiently than wild-type *SB* transposon in tissue culture

The efficiency of transposition of the SA and its derivative SA-βgal (Fig. 16A) was tested using the *in vivo* transposition assay. Other marker genes of similar size were used as controls in the same assay. pTneo 7.5 (Fig. 16A) (a plasmid that contains the *neo* resistance gene within a 7.5 kb transposon), and AB7 (a construct in which the same 4.7 kb DNA piece as in SA is subcloned between two wild-type IRs) were used as controls for SA, and pTneo 10.3 (Fig. 16A) (a plasmid that contains the *neo* gene within a 10.3-kb transposon) was used as a control for SA-βgal. For each construct, the ratio between colony numbers in the presence versus in the absence of transposase was calculated as a measure of the transposition efficiency. The SA carrying a 7.7-kb-long transposon jumped about 3-fold more efficiently than a similar size marker transposon, pTneo 7.5 (Fig. 16B), and 2.2-fold more efficiently than AB7. This result indicates that the sandwich vector is indeed more efficient in transposing relatively long DNA fragments than wild-type *SB*. However, transposition of SA-βgal, which has a 12.2-kb transposon was only slightly more efficient than that of the 10.3-kb-long wild-type transposon (Fig. 16B). Thus, SA abides the same rule as wild-type *SB*, namely, that transposition rates are inversely proportional to the length of the transposon (Izsvak et al., 2000). These results suggest that increasing the numbers of binding sites for the transposase can partially restore efficient transposition of large size transposable elements.



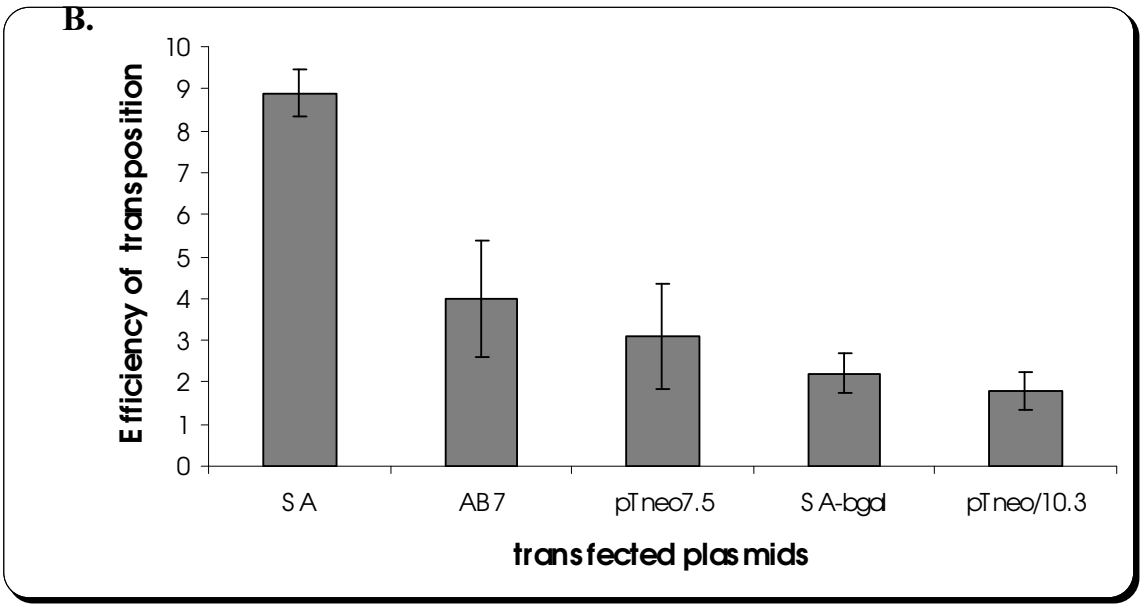


Fig. 16. Sandwich Vector (SA) functions better than similar size marker. A. Constructs respresenting different substrate transposons; SA, AB7, pTneo 7.5, SA-βgal, and pTneo10.3. **B.** Cotransfections were performed in Hela cells, where each substrate plasmid was cotransfected with CMV-SB10, but in control transfections, a plasmid expressing β-galactosidase was co-transfected instead. Cells were placed under neomycin selection, and resistant colonies were counted. The ratio of colony numbers in the presence versus in the absence of transposase is a measure of the efficiency of transposition. The graphs show that the SA vector jumps better than either AB7 or pTneo 7.5. SA-βgal jumps slightly better than pTneo 10.3.