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The DNA-bending protein HMGB1 is a cellular cofactor of *Sleeping Beauty* transposition

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ABSTRACT

***Sleeping Beauty* (*SB*) is the most active Tc1/*mariner*-type transposon in vertebrates. *SB* contains two transposase-binding sites (DRs) at the end of each terminal inverted repeat (IR), a feature termed the IR/DR structure. We investigated the involvement of cellular proteins in the regulation of *SB* transposition. Here, we establish that the DNA-bending, high-mobility group protein, HMGB1 is a host-encoded cofactor of *SB* transposition. Transposition was severely reduced in mouse cells deficient in HMGB1. This effect was rescued by transient over-expression of HMGB1, and was partially complemented by HMGB2, but not with the HMGA1 protein. Over-expression of HMGB1 in wild-type mouse cells enhanced transposition, indicating that HMGB1 can be a limiting factor of transposition. SB transposase was found to interact with HMGB1 *in vivo*, suggesting that the transposase may recruit HMGB1 to transposon DNA. HMGB1 stimulated preferential binding of the transposase to the DR further from the cleavage site, and promoted bending of DNA fragments containing the transposon IR. We propose that the role of HMGB1 is to ensure that transposase–transposon complexes are first formed at the internal DRs, and subsequently to promote juxtaposition of functional sites in transposon DNA, thereby assisting the formation of synaptic complexes.**

INTRODUCTION

The use of transposable elements as genetic tools contributed significantly to our understanding of biological systems. In vertebrates, such tools could be applied to both research and therapeutics. Tc1/*mariner* elements are probably the most widespread transposons in nature (1). These elements are able to transpose in species other than their hosts, and are therefore emerging tools for functional genomics in several organisms (1). However, the vast majority of naturally occurring Tc1/*mariner*-like transposons are non-functional due to inactivating mutations. In vertebrates, not a single active element has been found. Based on a comparative phylogenetic approach, we have reconstructed an active Tc1-like transposon from bits and pieces of inactive elements found in the genomes of teleost fish, and named this transposon *Sleeping Beauty* (*SB*) (2).

SB is flanked by ~230 bp terminal inverted repeats (IRs), which contain binding sites for the enzymatic factor of transposition, the transposase. The transposase binding sites (DRs) of *SB* elements are repeated twice per IR in a direct orientation (2). This special organization of IR, termed IR/DR, is an evolutionarily conserved feature of a group of Tc1-like transposons, but not that of the Tc1 element itself (1,3). In addition to the DRs, the left IR of *SB* contains a transpositional enhancer-like sequence, termed HDR (4). Specific binding to the DRs is mediated by an N-terminal, paired-like DNA-binding domain of the transposase (2,4,5). The catalytic domain of the transposase, responsible for the DNA cleavage and joining reactions, is characterized by a conserved amino acid triad, the DDE motif, which is found in a large group of recombinases (6), including retroviral integrases and the RAG1 V(D)J recombinase involved in immunoglobulin gene rearrangements (1).

SB transposes via a DNA intermediate, through a cut-and-paste mechanism. The transposition process can arbitrarily be divided into at least four major steps: (i) binding of the transposase to its sites within the transposon IRs; (ii) formation of a synaptic complex in which the two ends of the elements are paired and held together by transposase subunits; (iii) excision from the donor site; (iv) reintegration at a target site. On the molecular level, mobility of DNA-based transposable elements can be regulated by imposing constraints on transposition. One important form of transpositional control is represented by regulatory 'checkpoints', at which certain molecular requirements have to be fulfilled for the transpositional reaction to proceed. These requirements can operate at any of the four different stages of transposition listed above, and can be brought about by both element- and host-encoded factors.

Several DNA recombination reactions are stimulated by DNA-bending proteins. For example, the transposase binding sites of bacteriophage Mu are brought together by the bending action of the *Escherichia coli* HU protein (7). Hin recombinase-mediated recombination and bacteriophage λ integration are strongly stimulated by HU (8) and integration host factor (IHF) (9), respectively. The eukaryotic high

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mobility group (HMG) proteins can functionally replace HU and IHF in some recombination reactions, indicating some level of exchangeability between these DNA-bending proteins (10). All of these DNA-bending proteins are believed to assist recombinational mechanisms by facilitating the formation of active recombinase–DNA complexes (11,12).

HMG proteins are classified into three subfamilies, HMGB1/2 (formerly known as HMG1/2), HMGA1a/b (formerly known as HMGI/Y) and HMGN1/2 (formerly known as HMGB14/17, that share many physical characteristics, but differ in their main functional domains (13). Both the HMGB and HMGA1 group proteins are known to bind A/T-rich DNA through interactions with the minor groove of the DNA helix (12). HMGB1 is an abundant (~10^6 molecules/cell), non-histone, nuclear protein associated with eukaryotic chromatin (12). Through its DNA-binding domain, termed the HMG-box, HMGB1 binds DNA in a sequence-independent manner, but with preference for certain DNA structures including four-way junctions and severely undertwisted DNA (13–16). HMGB1 has low affinity to B-form DNA, and is thought to be recruited by other DNA-binding proteins through protein–protein interactions, and induce a local distortion of the DNA upon binding. The ability of HMGB1/2 proteins to bend DNA was demonstrated *in vitro* (13). These proteins facilitate self-ligation of short DNA fragments (17,18), and can bridge linear DNA fragments thereby enhancing multimerization of longer DNAs (19). Together with the closely related HMGB2 protein, HMGB1 has been implicated in a number of eukaryotic cellular processes including gene regulation, DNA replication and recombination (12,20). HMGB1/2 directly interact with a number of proteins, including some HOX (21) and POU domain (22) transcription factors and the TATA-binding protein (23), and facilitate their binding through protein–protein interactions. HMGB1/2 were shown to enhance immunoglobulin V(D)J recombination by enforcing specific DNA recognition (24) through their interaction with the RAG1/2 recombinase complex (25), and facilitating cleavage (24). In addition, HMGB1 was found to promote Rep protein-mediated site-specific cleavage of adeno-associated virus DNA (26). The production of retroviral cDNA does not require an excision step, but the downstream events of retroviral integration are highly similar to other transpositional reactions (27). Interestingly, HMGA1 family members, but not HMGB1/2, are required for retroviral cDNA integration (28,29). Both V(D)J recombination and retroviral integration have common features with *SB* transposition. RAG-mediated cleavage at the ends of recombination signal sequences (RSSs) in V(D)J recombination is probably analogous to the excision step of transposition, whereas the biochemical steps leading to insertion of signal molecules, retrovirus integration and DNA transposition are essentially the same (27).

SB mediates transposition in a variety of vertebrate species (30), and is more active than other members of the Tc1/*mariner* family (31). Because there is substantial interest in developing transposon technology for gene therapy (32) and gene discovery (31), it is of importance to dissect the molecular mechanisms involved in transposition and its regulation. In particular, differential interactions between the transposon and host-encoded factors may result in limitation of host range. In this work, we evaluated HMG proteins as cellular host factors of *SB* transposition in mammalian cells. We have found that HMGB1 is required for efficient *SB* transposition. *SB* transposition was significantly reduced in HMGB1-deficient mouse cells. This effect was fully complemented by expressing HMGB1, partially by expressing HMGB2, but not with HMGA1. Interestingly, transient over-expression of HMGB1 in wild-type mouse cells enhanced transposition, indicating that HMGB1 is a limiting factor of transposition. SB transposase was found to interact with HMGB1 *in vivo*, suggesting that the transposase may actively recruit HMGB1 to transposon DNA via protein–protein interactions. HMGB1 enhanced preferential binding of the SB transposase to the internal transposase binding sites within the transposon IRs, and promoted bending of DNA fragments comprising the transposon IRs. These data are consistent with a role of HMGB1 in synaptic complex formation in transposition.

MATERIALS AND METHODS

Protein expression, purification and electrophoretic mobility shift assay (EMSA)

Production of N123 was done as described (2). A plasmid expressing a hexahistidine-tagged version of HMGB1 was described in Aidinis *et al*. (25), and was kindly provided by M. Bianchi, Milan, Italy. Protein expression was induced by the addition of 0.4 mM IPTG in *E.coli* BL21 cells. Purification was done using a nickel resin (Qiagen), according to the manufacturer's protocol. The purified protein was dialyzed against 25 mM HEPES pH 7.4, 10% glycerol, 1 M NaCl and 2 mM β-mercaptoethanol, and its concentration determined by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis.

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). Protein purification protocol was as described by the manufacturer of the amylose resin (NEB). 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 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 was added. Cell lysis was done by 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 vol 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 having the fusion protein were pooled and concentrated to 0.4 mg/ml.

An *Eco*RI fragment comprising the left IR of the *SB* element containing both transposase binding sites, an *Afl*II/*Hin*dIII fragment containing only the inner DR (IDR), and an *EcoR*I/*Hin*dIII fragment of a modified *SB* element lacking the IDR, and thus containing only the outer DR (ODR) (30), were end-labeled using [α^{32}P]dATP and Klenow. Equal amounts of DNA fragments were used for labeling. Nucleoprotein complexes were formed in 20 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.1 mg/ml BSA, 150 mM NaCl, 1 mM DTT in a total volume of 10 µl. Reactions contained ~0.08 nM DNA probe, 1 µg poly(dI)(dC), 75 nM HMGB1 and 0.2, 0.1 or 0.05 ng N123. Reactions with MBP–SB transposase fusion contained ~0.1 nM of left IR probe (*Eco*RI fragment) and either 2 nM or 20 nM of purified MBP–SB with or without 0.1 or 1 µM of purified HMGB1. After a 15 min incubation on ice, 5 µl of loading dye containing 50% glycerol and bromophenol blue was added, and the samples loaded onto a 5% polyacrylamide gel. Radioactive bands were quantified using a Molecular Dynamics PhosphorImager System.

Ligase-mediated circularization assay

A ^{32}P-labeled left IR fragment (~0.04 nM) with cohesive *Eco*RI ends was pre-incubated with 6 nM HMGB1 on ice for 20 min in T4 DNA ligase buffer, in a final volume of 50 µl. The ligation reaction was initiated by the addition of 0.025 U of T4 DNA ligase (NEB), and incubated at 16°C. Aliquots of 9 µl of the reaction mixture were withdrawn at different time points (0, 5, 15, 30 and 60 min), and were added to 41 µl of stop solution (0.5% SDS, 10 mM EDTA, 1 mg/ml Proteinase K). The reactions were incubated at 50°C for 2 h, extracted with phenol/chloroform/isoamyl alcohol, and then with chloroform/isoamyl alcohol. Aliquots of 30 µl of the 60 min sample were taken, and digested with 100 U of Exonuclease III (ExoIII) (NEB) at 37°C for 30 min. Aliquots of 25 µl of each extracted sample were run on a 4% non-denaturing polyacrylamide gel, gels were dried and autoradiographed at –80°C.

For bacterial transformations, ~0.1 nM linearized transposon DNA was pre-incubated with 12 nM HMGB1, then 1 U/reaction of the T4 DNA ligase was added, and the reaction allowed to proceed for 0, 15, 30 and 60 min. Reactions were terminated by the addition of stop solution, the DNA was precipitated, resuspended in TE, and electroporated into DH10B *E.coli* cells.

Immunoprecipitation

Nuclear extracts from ~2.0 × 10^7 IRES-SB and IRES-K cells were prepared essentially as described previously (30). The nuclear extract was diluted to contain 100 mM NaCl with binding buffer [25 mm HEPES–KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mm KCl, 0.5 mM EDTA, 0.2 mM DTT, 0.1 mM NP-40, 12% glycerol, 1× complete protease inhibitors (Roche)]. For the DNase I treatment, $MgCl_2$ concentration was increased to 6 mM and 6 U of DNase I (Ambion) was added. The extract was incubated at 23°C for 30 min, followed by the addition of 2 µl of 500 mM EDTA. The extract was precleared in two steps, first by adding 1 µg mouse IgG and 20 µl Protein G-Sepharose (50% slurry in PBS) for 60 min at 4°C, and then by adding 50 µl Protein G-Sepharose followed by an overnight incubation at 4°C. Immunoprecipitation was performed using 1 µg HMGB1 antibody (Santa Cruz Biotech.), or actin antibody (clone ACTN05, Neomarkers), or p15 antibody (R-20, Santa Cruz Biotech.), or goat preimmune serum (Sigma) and 15 µl Protein G-Sepharose (50% slurry in PBS). The tubes were rotated overnight at 4°C. The beads were washed four times in PBS, resuspended in SDS sample buffer, and subjected to western hybridization with a rabbit polyclonal antibody against the SB transposase.

Co-immunoprecipitation was done using purified HMGB1 (1 µM) and purified MBP–SB (0.2 µM), either alone or together in 20 µl final volume in binding buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2% glycerol, 0.25 µg BSA, 50 mM KCl, 0.01% NP-40 for 20 min at 4°C. Then ~1 nM of a ^{32}P-labeled transposon fragment containing the left IR (~120 000 c.p.m.) was added, and the reaction continued for a further 45 min. One microgram of either anti-HMGB1 or anti-SB was added, and the incubation continued for 2 h at 4°C with rotation. Protein A- and Protein G-Sepharoses were pretreated with the binding buffer containing 500 µg/ml of herring sperm DNA overnight. An aliquot of 50 µl pretreated Protein A-Sepharose was added to the anti-SB samples, 50 µl of pretreated Protein G-Sepharose was added to the anti-HMGB1 samples, and incubation continued for 3 h at 4°C. The immunoprecipitate was washed three times with the binding buffer. Radioactivity of the DNA that remained bound to the beads was quantified with a liquid scintillation counter.

Cell culture and *in vivo* transposition assay

Cell lines were grown in DMEM supplemented with 10% fetal bovine serum. Wild-type (VA1) and HMGB1-deficient (C1) mouse embryonic fibroblast (MEF) cell lines have been described previously (33), and were kindly provided by M. Bianchi, Milan, Italy. All DNA transfections were done with FUGENE6 transfection reagent (Roche, Germany). Cells were cotransfected with 90 ng each of pCMV-SB (2) and pT/zeo, (30) and 500 ng plasmid expressing HMGB1 (25), HMGB2 (25), HMGA1 (34) or β-galactosidase. 10^5 transfected cells were plated out for selection, using 100 µg/ml zeocin (Invitrogen). After 3 weeks of selection, colonies were stained and counted as described previously (2). Transfectability of the C1 cell line is lower than that of the VA1 line, which was determined by transfection of a GFP-expressing plasmid. Therefore, transpositional efficiencies in the two cell lines presented in Figure 1B have been normalized to transfection efficiencies.

RESULTS

HMGB1 is required for efficient *SB* transposition in mouse cells

We assessed the importance of HMGB1 for *SB* transposition by applying an *in vivo* transposition assay (2) on wild-type and HMGB1-deficient mouse cells (Fig. 1). The assay is based on cotransfection of a donor plasmid carrying a zeocin resistance gene (*zeo*)-marked transposon and a transposase-expressing helper plasmid into cultured cells (Fig. 1A). In control experiments, a plasmid expressing β-galactosidase (CMVβ) substitutes for the transposase helper plasmid. Cells are placed under antibiotic selection, and the numbers of resistant colonies counted. The ratio between numbers obtained in the

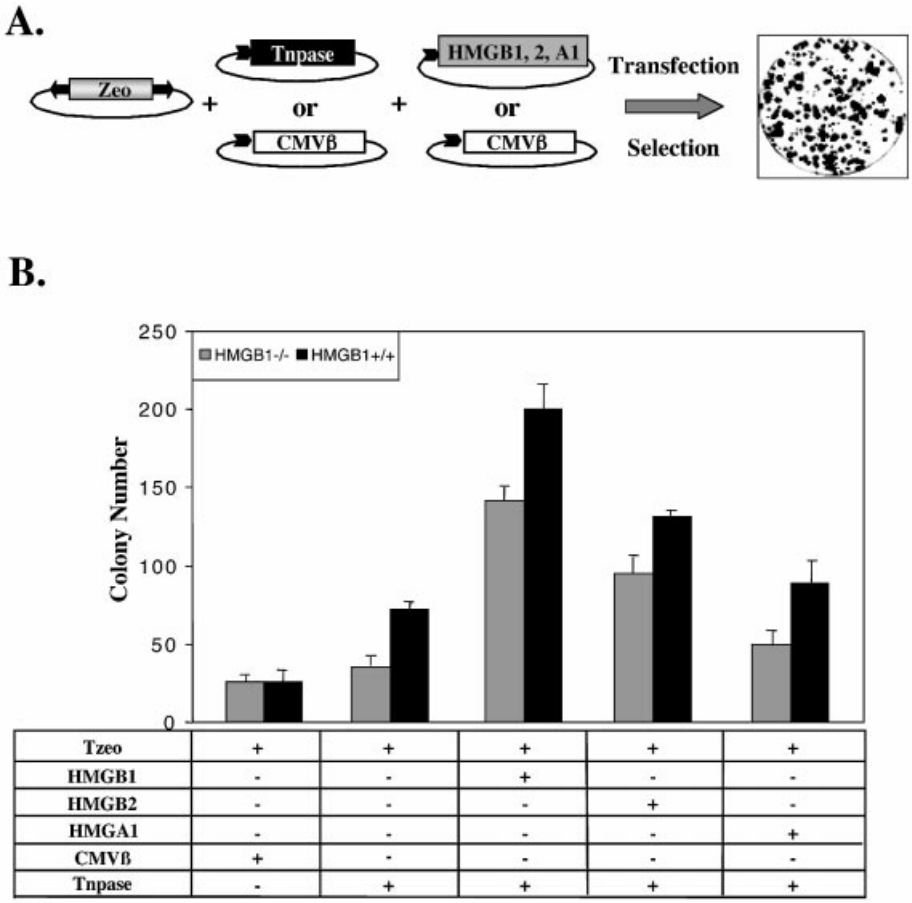

Figure 1. Efficient *SB* transposition requires HMGB1. (**A**) Schematic representation of the *in vivo* transposition assay. Constructs expressing HMG proteins are cotransfected with transposon donor and transposase-expressing helper plasmids into cultured cells. In control transfections, a plasmid expressing β-galactosidase is cotransfected. Cells are 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 IRs. (**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 over-express 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.

presence versus the absence of transposase is the read-out of the assay, and is a measure of the efficiency of transposition. Consistent with our earlier findings (30), transposition was readily detectable in the wild-type MEF cell line VA1 (~3-fold increase in colony number in the presence of transposase) (Fig. 1B). 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 over-expression of HMGB1 increased colony numbers ~4-fold (Fig. 1B), 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 (12,13), whereas HMGA1 had no significant effect on the efficiency of transposition (Fig. 1B).

Transient over-expression 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 (25). Therefore, we cotransfected wild-type MEFs 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 ~2.5-fold (Fig. 1B). Over-expression of HMGB2 had a smaller effect, and increased the numbers of resistant colonies by ~1.5-fold, whereas overexpressing HMGA1 resulted in no change in colony numbers (Fig. 1B). 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.

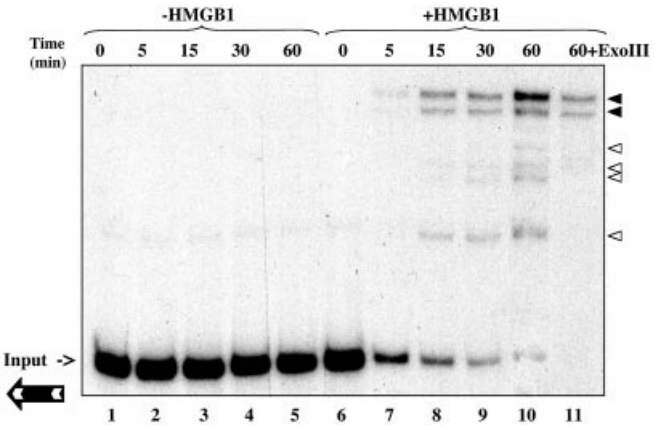

Figure 2. HMGB1 enhances bending of the transposon IR. Intramolecular ligation (circularization) assay was performed to monitor the effect of HMGB1 on bending of the left IR of the transposon. The probe was ligated by T4 DNA ligase in the absence or presence of HMGB1 for the time periods indicated. Lane 11 is the same as lane 10, except treated with ExoIII. Empty triangles indicate linear ligation products. Filled triangles point to circular ligation products resistant to ExoIII digestion.

HMGB1 enhances bending of the *SB* transposon terminal IR 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 (11,12). *SB* has two transposase-binding sites per terminal IR, separated by ~160 bp spacer regions. We hypothesized that the bending activity of HMGB1 could contribute to bringing the DRs and/or the complete IRs closer in space, thereby assisting the formation and/or stabilization of a synaptic complex.

To address this question, a ligase-mediated circularization assay (18) 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 min of incubation (Fig. 2, lane 5). In contrast, in the presence of HMGB1, ligation products began to appear after 15 min of incubation (lanes 8–10 in Fig. 2). Production of DNA circles was verified by treating the 60 min sample with ExoIII, which cleaves linear DNA but leaves circular DNA intact. Two ligation products were resistant to ExoIII treatment (Fig. 2, 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 IR.

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 *zeo* and an origin of replication was used for this experiment (Fig. 3A). The linear transposon was treated with T4 ligase in the absence and presence of HMGB1 for different periods of time. As shown in Figure 3B, the average number of bacterial colonies was significantly higher in samples containing HMGB1.

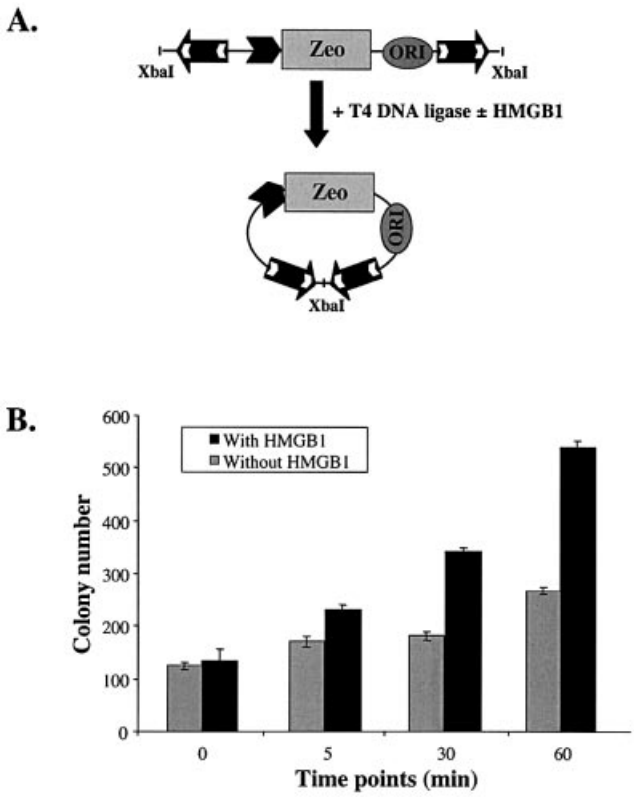

Figure 3. Bending effect of HMGB1 on a complete *SB* transposon. (**A**) Schematic representation of the circularization assay. The *SB* transposon contains *zeo* and a bacterial origin of replication (ORI). Black arrows flanking the element are the terminal IRs, white arrows inside the IRs are the transposase 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 the average of three individual experiments.

We conclude 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.

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 IRs. We hypothesized that, in addition to its DNA-bending activity shown in Figures 2 and 3, 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

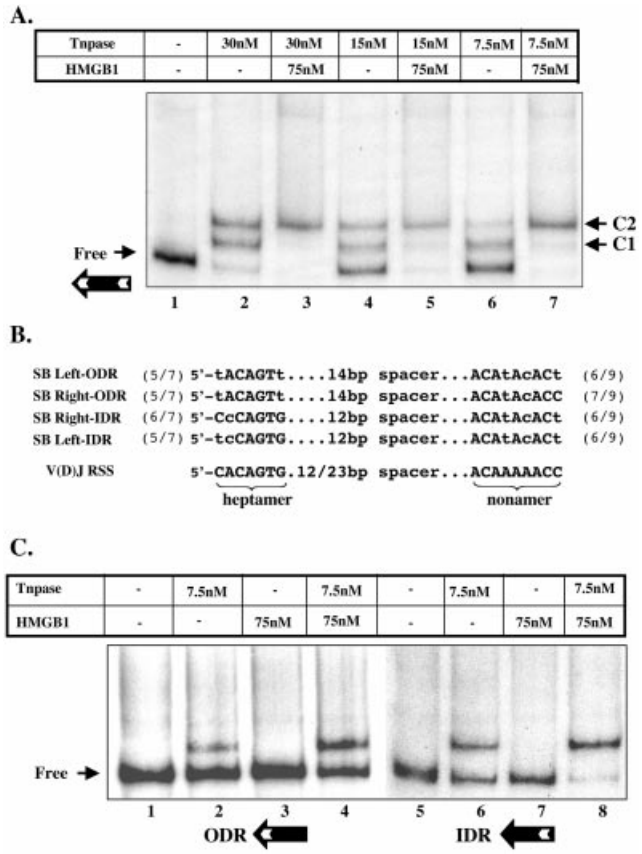

Figure 4. HMGB1 stimulates specific binding of SB transposase to the transposon IRs. (**A**) The effect of HMGB1 on transposase binding to the left IR. EMSA was performed using the left IR of *SB*, containing two binding sites for the transposase, as a probe, and N123, an N-terminal derivative of SB transposase containing the specific DNA-binding domain. C1 and C2 indicate the two DNA–protein complexes formed in the assay. (**B**) Comparison of *SB* transposase binding sites and the RAG1/2 recognition signal sequences. The degrees of similarities to the heptamer and nonamer motifs are indicated. (**C**) The effect of HMGB1 on binding to either the outer or the inner transposase binding sites in the context of the left IR. EMSA showing the stimulatory effects of HMGB1 on binding of N123 to the ODR and IDR.

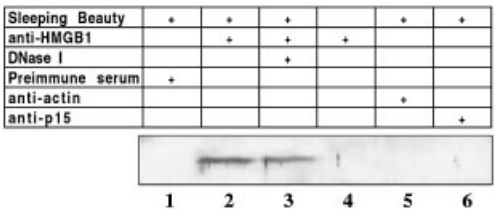

Figure 5. SB transposase interacts with HMGB1. Immunoblot of nuclear extracts of HeLa cells expressing SB transposase, and control cells, after incubation with antibodies against human HMGB1, actin and p15 proteins or a preimmune serum, with or without DNase I treatment. The blot was hybridized with an anti-SB antibody.

were purified from *E.coli* using affinity chromatography. The left IR, containing two transposase-binding sites, was radioactively labeled and used as a probe in an EMSA (Fig. 4). As shown previously (2), N123 produced two shifted bands, representing complexes in which either one (complex C1) or both sites (complex C2) are bound (Fig. 4A, lane 2). HMGB1 enhanced binding of N123, indicated by a more prominent formation of C2 (Fig. 4A, lane 3). The enhancement was inversely dependent on the concentration of N123 relative to that of HMGB1; stimulation of binding was ~2-fold at 30 nM N123 (Fig. 4A, lane 3), 5-fold at 15 nM N123 (Fig. 4A, lane 5), and >7-fold at 7.5 nM N123 (Fig. 4A, 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 an EMSA. The ODR is located next to the transposase cleavage site, whereas the IDR is ~200 bp from the end of the transposon. The IDR and ODR are not identical, they share ~80% sequence identity and the IDR is shorter by 2 bp (Fig. 4B). There appeared to be a clear preference for the IDR in transposase binding, because N123 bound to it ~3-fold stronger than to the ODR (compare lanes 2 and 6 in Fig. 4C). The presence of HMGB1 appeared to further emphasize this preference for IDR binding: at 75 nM concentration of HMGB1, N123 shifted ~90% of the IDR probe (Fig. 4C, lane 8), but only ~50% of the ODR probe (Fig. 4C, lane 4). HMGB1 alone did not shift either probe (Fig. 4C, lanes 3 and 7). Taken together, these data show that HMGB1 stimulates transposase binding to the transposon IRs, and that it has a more pronounced effect on binding to the IDR.

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 (12). To investigate possible physical interactions between the SB transposase and HMGB1, an immunoprecipitation experiment was performed (Fig. 5). 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. 5, lanes 1 and 2). Treating the nuclear extract with DNase I did not influence the formation of the immunocomplex (compare lanes 2 and 3 in Fig. 5), indicating that the detected signal was not due to non-specific, 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 (Fig. 5, 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 (Fig. 5, lanes 5 and 6), indicating that the interaction observed is specific for

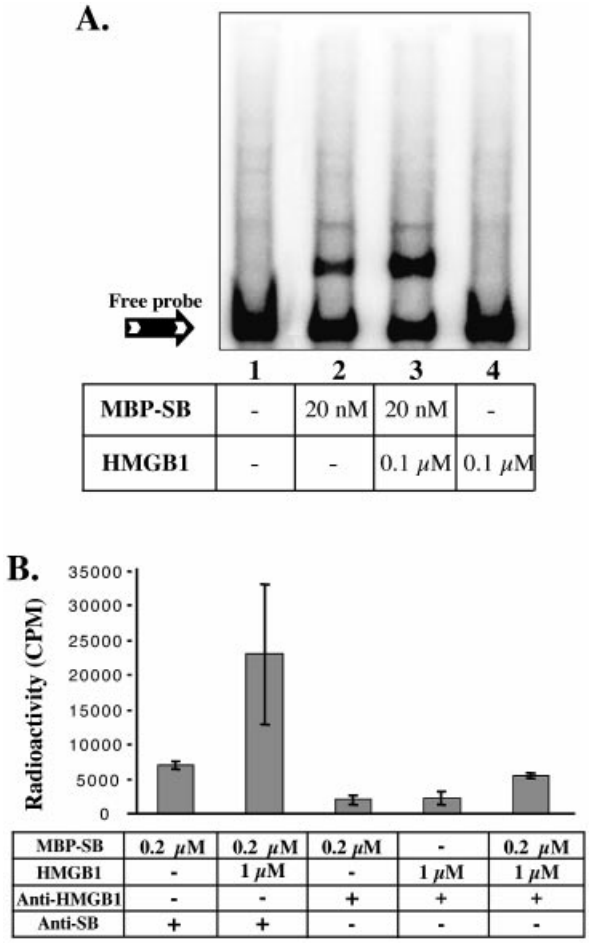

Figure 6. Formation of a ternary complex of the full-length SB transposase, HMGB1, and transposon DNA. (**A**) HMGB1 stimulates specific binding of a MBP–SB transposase fusion to the transposon IRs. EMSA was performed using the left IR of *SB*, containing two binding sites for the transposase, as a probe, and MBP–SB. The radioactively 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 incubated individually or together with a radioactively labeled IR 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 c.p.m. values obtained with the anti-SB antibody are the following: MBP–SB, 6946; MBP–SB plus HMGB1, 23 033. The values with the anti-HMGB1 antibody are: MBP–SB, 2010; HMGB1, 2304; and MBP–SB plus HMGB1, 5473.

HMGB1. Similar results were obtained when purified HMGB1 protein was immobilized on agarose beads, and incubated with purified SB protein (data not shown). We conclude that the transposase actively interacts with HMGB1.

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

The activity of HMGB1 at the transposon IRs 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 IR probe as in Figure 4A. HMGB1 enhanced the binding efficiency of MBP–SB more than two times (Fig. 6A, compare lanes 2 and 3). HMGB1 alone did not shift the probe (Fig. 6A, 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. 6A, 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 we sought evidence for a ternary complex using MBP–SB in a coimmunoprecipitation experiment. Radioactively labeled transposon IR DNA was incubated with MBP–SB and HMGB1, and coimmunoprecipitated with either anti-SB or anti-HMGB1 antibodies. Figure 6B 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. 6B). 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 (27), and because ternary complex formation in our experiments 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.

DISCUSSION

In this work we presented evidence that HMGB proteins are cellular cofactors of *SB* transposition. In HMGB1 knockout cells, transpositional activity was found to be marginal (Fig. 1B). This residual activity can probably be accounted for by the presence of HMGB2 in these cells (33). HMGB2 was found to partially or fully complement the absence of HMGB1 in some reactions (12), so these two proteins are interchangeable to a certain degree. Indeed, transient over-expression of HMGB2 partially complemented the HMGB1 deficiency in our transposition assays (Fig. 1B). Mammalian cells contain significant amounts of HMGBs; there might be one molecule of HMGB1 for every 2 kb of the human genome (35). Therefore, our finding that transient over-expression of HMGBs in wild-type mouse cells enhances transposition (Fig. 1B) was unexpected. However, this phenomenon is not without precedent: transient over-expression of HMGB1 by transfection enhances the activity of certain HMGB1 interactors, such as RAG1/2 (24), some Hox proteins (21), and nuclear hormone receptors (36). Our findings suggest that HMGB1 can be a limiting factor of *SB* transposition, and that

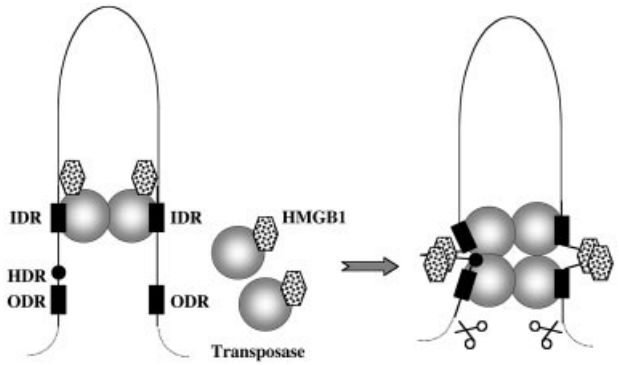

Figure 7. A proposed model for the role of HMGB1 in *SB* synaptic complex formation. SB transposase (gray spheres) recruits HMGB1 (dotted hexagons) to the transposon IRs. First, HMGB1 stimulates specific binding of the transposase to the IDRs. Once in contact with DNA, HMGB1 bends the spacer regions between the DRs, thereby assuring correct positioning of the ODRs for binding by the transposase. Cleavage (scissors) proceeds only if complex formation is complete. The complex includes the four binding sites (black boxes), the HDR enhancer sequence (black circle) and a tetramer of the transposase.

different cellular levels of HMGB1 might modulate the efficiency of transposition in different tissues or species.

Possible roles of HMGB1 in transposition

In prokaryotes, the DNA-bending proteins HU and IHF bind directly to DNA, and no protein–protein interactions are required for their targeting (7). In contrast, HMGs have low affinity for standard, B-form DNA, and interactor proteins usually guide them to certain sites. We have shown that the SB transposase is an HMG interactor (Figs 5 and 6). The interaction was detectable in the absence of DNA, suggesting that SB might actively recruit HMGB1 to sites of transposition. At which step is HMGB1 required for transposition? We considered the following, not mutually exclusive, possibilities: (i) HMGB1 induces a structural change in transposon DNA, which is required for efficient transposition; (ii) HMGB1 enhances binding of the transposase to the transposon IRs; (iii) HMGB1 induces a conformational change of the transposase that makes the transposase more active; (iv) HMGB1 plays a role in transposon integration by making contacts with chromatin components and/or by DNA-bending at target sites (37). In this paper we provided evidence that HMGB1 promotes circle formation of transposon DNA (Figs 2 and 3), that it significantly enhances specific transposase binding to the transposon IRs (Fig. 4), and that it can form a ternary complex with the transposase and transposon DNA (Fig. 6). Thus, although a role of HMGB1 in transposon integration cannot be ruled out, our results are consistent with a role of HMGB1 in the early steps of transposition, prior to catalysis.

Considering the significant drop of transposition activity in HMGB1-deficient cells (Fig. 1B), the role of HMGB1 in transposition has to be a critical one. *SB* has four transposase-binding sites, directly repeated at the ends of the terminal IRs. We have previously shown that: (i) presence of the four transposase binding sites is absolutely required for transposition (30) and (ii) SB transposase forms tetramers in complex with transposase-binding sites (4). These observations are consistent with an interaction between the IR/DR structure and a transposase tetramer during transposition. We propose that one of HMGB1's roles is to bring the two binding sites closer to each other during synaptic complex formation (Fig. 7). HMGB1 might promote communication between DNA motifs that are otherwise distant to each other, including the DRs, the transpositional enhancer and the two IRs (Fig. 7). Similar to *SB* transposition, a DNA-bending protein, HU, is involved in looping out the linker DNA between transposase binding sites during Mu-transpososome assembly (7). If the only role of HMGB1 is to extrude the spacer region between the DRs, thereby bringing them close to each other in space, then deleting the spacer would rescue transposition activity even in the absence of HMGB1. We have constructed transposons, in which the DRs were 10, 20 and 50 bp from each other. None of these transposons had any detectable activity (data not shown), indicating that physical proximity of the DRs is not sufficient for transposition, and that the correct geometrical configuration of the IRs and the binding sites is crucial.

These observations indicate that a highly specific configuration of functional DNA elements within the IRs has a critical importance in *SB* transposition (Fig. 7). This complex needs to be very precisely assembled, and probably includes the four DRs, the HDR enhancer motif, four transposase molecules (4) and HMGB1 (Fig. 7). Because transposase has higher affinity to the internal binding sites within the transposon IRs (Fig. 4C), it appears that the order of events that take place during the very early steps of transposition is binding of transposase molecules first to the inner sites, and then to the outer sites. The pronounced effect of HMGB1 on binding of the transposase to the inner sites suggests that HMGB1 enforces ordered assembly of a catalytically active synaptic complex. If any of these molecular requirements is not fulfilled properly, the transposition reaction is hampered or does not proceed at all. Indeed, replacement of the outer transposase binding sites with the inner sites, i.e. increasing binding at the outer sites, abolishes SB transposition (38). An assembly pathway similar to the one we propose for *SB* has been described for bacteriophage λ. The integrase protein, together with IHF, first assembles on a high affinity attachment site (attP) on the phage genome (39), and then captures another, low affinity site on the bacterial chromosome (attB) (40). In this system, the order of assembly is determined by the difference in affinity of the integrase for the attP and attB sites.

HMGB1 has overlapping, but distinct roles in V(D)J recombination and *SB* transposition

In V(D)J recombination, the RAG1/2 complex specifically binds to the nonamer and heptamer motifs of the RSSs (Fig. 4B), which are separated by 12 or 23 bp spacer regions (12/23-RSS). V(D)J recombination preferentially takes place between a 12-RSS and a 23-RSS, which is termed the 12/23 rule (24,25,41). HMGB1/2 significantly stimulates the binding of both RSSs, but this stimulation is especially pronounced at the 23-RSS (24). This selective enhancement of binding is thought to enforce the specificity of the subsequent cleavage step (24,25). Recent results indicate that the RAG1/2 complex first assembles on a single RSS, and that the partner RSS is later incorporated into the complex as naked DNA (42). Initial binding of RAG1/2 to the 12-RSS results in more faithful adherence to the 12/23 rule. Because there is no substantial

difference in the binding affinity of RAG1/2 for naked 12- and 23-RSSs in the presence of HMGB proteins, it has been suggested that chromatin structure may influence whether RAG1/2 binds first to a 12- or a 23-RSS *in vivo* (42).

The transposase-binding sites of *SB* resemble the RSSs in their sequence (Fig. 4B). Similarly to the RSSs, the spacing between the nonamer and heptamer-like motifs within the transposase-binding sites is different, 12 and 14 bps, in the internal and external DRs, respectively. We have found that SB transposase preferentially binds the IDR (12DR) (Fig. 4C). The 2 bp difference in spacer length between 12DR and 14DR might not be sufficient for HMGB1 to assert its DNA-bending activity to promote transposase binding. More likely, the helical phasing of the heptamer- and nonamer-like sequences in 14DR might be less favorable for transposase binding. In contrast to V(D)J recombination, the original preference of the SB transposase for binding to the 12DR is not altered, but even further emphasized in the presence of HMGB1 (Fig. 4C). In conclusion, HMGB1 seems to have overlapping, but distinct roles in SB transposition and in V(D)J recombination.

The IR/DR-type organization of IRs is an evolutionarily conserved feature of many transposons in the Tc1 family (1), but its function in transposition has been enigmatic. Our results suggest that the IR/DR introduces a higher level regulation into the transposition process: the repeated transposase binding sites, their dissimilar affinity for the transposase, and the effect of HMGB1 to differentially enhance transposase binding to the inner sites are all important for a geometrically and timely orchestrated formation of synaptic complexes, which is a strict requirement for the subsequent catalytic steps of transposition.

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