2. Materials and Methods

2.1. Bacterial strains

Plasmids were maintained in *E. coli* strain, DH5 alpha [supE44, delta lacU169 (phi 80 lacZ delta M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1]. For electroporation the electrocompetent cells were prepared from the *E.coli* strain DH10B [F? mcrA (mrr-hsdRMS-mcrBC) 80dlacZM15 lacX74 deoR recA1endA1 araD139 (ara, leu)7697 galU galK ? rpsL nupG], and for HMG- constructs were maintained in *E. coli* strain, BL21 [F^-, ompT, hsdSB (r^- B, m^- B), gal, dcm].

Bacterial strains were routinely grown at 37 °C in LB medium (Luria Bertani: 10g bactotryptone, 5g bacto-yeast, and 10g NaCl dissolved in H_2O until 1 liter and sterilized by autoclaving). Supplemented with the appropriate antibiotic.

2.2. DNA purification

Purification of plasmid DNA, restriction fragments and PCR amplicons, were performed using Qiagen kits. All procedures were performed as described by the manufacturer:

- Qiaquick nucleotide removal kit: procedure to remove primers < 10 bases, enzymes, salts, and nucleotides.
- Qiagen gel extraction kit: procedure to purify DNA fragments from agarose or polyacrylamide gels.
- Qiaprep spin miniprep kit: procedure to purify plasmid DNA from small scale bacterial culture (< 5 ml).

2.3. Cell culture and Transfection

All cell lines were grown in DMEM (GIBCO BRL, Germany) supplemented with 10% Fecal Calf Serum Gold (FCS Gold) (PAA, Germany) and 1% Antimycotic antibiotic (Invitrogen, Germany). All Transfections were performed by FuGENE 6 transfection reagent (Roche, Germany).

2.4. Protein expression and purification

Production of N123 was done as described (Ivics et al., 1997). A plasmid expressing a hexahistidine-tagged version of HMGB1 was described in (Aidinis et al., 1999), 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 manufucturer'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-polyacrylamide gel electrophoresis (SDS-PAGE).

The plasmid expressing a maltose binding protein-SB transposase fusion protein (MBP-SB) 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 one liter 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 hours. 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 psi, 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 having the fusion protein were pooled and concentrated to 0.4 mg/ml.

2.5. Electrophoretic mobility shift assay (EMSA)

2.5.1. HMGB1 effect on the binding ability of SB transposase

An *Eco*RI fragment comprising the left inverted repeat of the *SB* element containing both transposase binding sites, an *Afl*II/*Hin*dIII fragment containing only the inner DR, and an *Eco*RI/*Hin*dIII fragment of a modified *SB* element lacking the inner DR, and thus containing only the outer DR (Izsvak et al., 2000), were end-labeled using [α ^{32}P] dATP and Klenow. 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 maltose binding protein-SB transposase fusion (MBP-SB) 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 µM or 1 µM of purified HMGB1. After a 15 minute 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.

2.5.2. Binding ability of mutated IR versus wild type IR

pTneo and pTneo* were digested with *Xba* I /*BamH* I, this gives Right IR (rIR) from pTneo (*Xba* I/*BamH* I fragment) and mutated (m) rIR (*BamH* I fragment) from pTneo*. Both of the fragments were end-labeled using [α ^{32}P] dATP and klenow. Nucleoprotein complexes were formed the same way as mentioned above. But 2ng N123 and 1nM of each DNA probe was used. Gel was dried, exposed to a film over night, and then developed.

2.6. 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 units of T4 DNA ligase (NEB), and incubated at 16 °C. Nine µl aliquots of the reaction mixture were withdrawn at different time points (0, 5, 15, 30, and 60 minutes), 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 hours,

extracted with phenol/chloroform/isoamyl alcohol, and then with chloroform/isoamyl alcohol. 30 µls of the 60 min sample were taken, and digested with 100 units of Exonuclease III (NEB) at 37°C for 30 minutes. 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 one unit/reaction of the T4 DNA ligase was added, and the reaction allowed to proceed for 0, 15, 30, and 60 minutes. Reactions were terminated by the addition of stop solution, the DNA was precipitated, resuspended in TE, and electroporated into DH10B *E. coli* cells.

2.7. Immunoprecipitation.

Nuclear extracts from approximately $2.0x10^7$ IRES-SB and IRES-K cells were prepared essentially as described (Izsvak et al., 2000). 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, 1x Complete protease inhibitors (Roche)]. For the DNaseI treatment, $MgCl_2$ concentration was increased to 6 mM and 6 units of DNase I (Ambion) was added. The extract was incubated at 23 ^oC for 30 minutes, 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 minutes at 4^oC, and then by adding 50 µl Protein G-Sepharose followed by an overnight incubation at 4 ^oC. 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^oC. 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 cpm) was added, and the reaction continued for a further 45 min. 1 µg

of either anti-HMGB1 or anti-SB was added, and the incubation continued for 2 hours 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. 50 µl of pretreated Protein A-Sepharose was added to the anti-SB samples, and 50 µl of pretreated Protein G-Sepharose was added to the anti-HMGB1 samples, and incubation continued for 3 hours 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.

2.8. Site specific mutations

All PCR products were phosporylated using T4 polynucleotide kinase enzyme (USB) and the phosphorylated products were ligated with T4 DNA ligase (USB). Ligated products were transformed into *E. coli*. Plasmids were recovered by mini-plasmid kit (Qiagen).

2.8.1. Change from acidic to basic amino acids in the SB transposase

Oligonucleotides containing a single amino acid change were used in a PCR to generate a single mutation in the transposase coding plasmid (pCMV-SB10), the names of the oligonucleotides are according to the changes induced in the amino acid, changed amino acids are in a bold text, names and sequences are as the following: E6K: D6K/F (Forward primer) ; 5´..ATC AGC CAA GAC CTC AGA AAA AAA ATT G ...3´; D6K/R (Reverse primer) 5´..**CTT** TTT TGA TTT TCC CAT GAT GTC GGA TC....3´, D10K, D10K/F; 5´..CTC AGA AAA AAA ATT GTA GAC..3´; D10K/R 5´...**CTT** TTG GCT GAT TTC TTT TG..3´. D17K: D17K/F; 5´.. CTC CAC AAG TCT GGT TCA TC ..3´, D17K/R, 5´..**CTT** TAC AAT TTT TTT TCT GAG GTC..3‘. D68K: D68K/F; 5`..GAA CGT ACT TTG GTG CGA AAA G..3´, D68K/R, 5´..**CTT** TCT AGG AGA CAG AAC GCG..3´, D86k: D86K/F, 5´..CTT GTG AAG ATG CTG GAG...3‘, D86K/R; 5´...**CTT** CTT TGC TGT TGT TCT G..3‘ , E92K: E92K/F; 5‘..GAA ACA GGT ACA AAA GTA TC..3‘,E92K/R; 5´..**CTT** CAG CAT CTT CAC AAG G…3´. E93K: E93K/F; 5´..ACA GGT ACA AAA GTA TCT ATA TC..3´. E93K/R; 5´..**CTT** CTC CAG CAT CTT CAC AAG..3´. D140K. D140K/F; 5´.AAA GAT CGT ACT TTT TGG AGA AAT G..3´, D140K/R;5'..**CTT** CCC ATG TGC AGT TGC AAA C..3'. D142K: D142K/F; 5´...CGT ACT TTT TGG AGA AAT GTC C..3´. D142K/R; 5´..**CTT** TTT GTC CCC ATG TGC AG..3´. E158K: E158K/F; 5'..CTG TTT GGC CAT AAT GAC CAT C..3', E158K/R; 5'..**CTT** TAT TTT TGT TTC ATC AGA CCA G..3', D164K: D164K/F;

5´...CAT CGT TAT GTT TGG AGG AAG..3´. D164K/R, 5‘...**CTT** ATT ATG GCC AAA CAG TTC..3´. E174K: E174K/F; 5´..GCT TGC AAG CCG AAG AAC ACC..3´. E174K/R: 5´...**CTT** CCC CTT CTT CCT CCA AAC A..3´., D260K: D260K/F; 5´…AAG **AAG** AAC AAA GTC AAG GTA TTG GAG TGG…3´ D260K/R: 5´…AAG CCA TTT TGC CAC AAC TTT GG…3´. E216K: E216K/F, 5'.. AAT TAT GTG GAT ATA TTG AAG CAA C..3', E216K/R; 5'..**CTT** CTT CCT CAT GAT GCC ATC…3'. E321R: E321R/F; 5´…ATC CGA AAC GTT TGA CCC AAG TTA AAC…3´. E321R/R; 5´…ATC C**TC T**CA CAA GCT TCC CAC AAT AAG..3`.

2.8.2. Combination of the hyperactive mutations

Combined mutations was induced according to instruction of QuickChange ^{TM} Site directed Mutagenesis kit (Stratagene). Primers used for D260K were: D260K/2/F; 5´..G GCA AAA TGG CTT AAG AAG AAC AAA GTC AAG GTA TTG GAG TGG CC..3´. D260K/2/R 5´..GG CCA CTC CAA TAC CTT GAC TTT GTT **CTT** CTT AAG CCA TTT TGC C..3´. R115H was used as a template for PCR to obtain D260K/R115H. R115H was as well used as a template for the R143C primers generate a double mutant of R115H/R143C; the R143C oligonucleotides used in the PCR were: R143C/F; 5´...GCA CAT GGG GAC AAA GAT **TGT** ACT TTT TGG AGA AAT GTC CTC TGG...3´, R143C/R; 5´..CCA GAG GAC ATT TCT CCA AAA AGT **ACA** ATC TTT GTC CCC ATG TGC...3´. R143C/D260K double mutant was also induced using D260K mutant construct as template for PCR using R143C primers mentioned above. D260K/R115H double mutant was used as a template for PCR using the R143C primers to generate the triple mutant R115H/260/R143C (3M).

2.8.3. Proline mutagenesis of the helix motif between the DNA-binding and catalytic domains

For L132P mutations, PCR was done using the primers: L132P/F; 5´..CGG TTT GCA ACT GCA CAT GGG...3´. L132P/R; 5´...**AGG** TCT GGC TTT CTT ATG TCG G..3´. F134P: F134P/F; 5´.. GCA ACT GCA CAT GGG GAC AAA G..3´. F134P/R; 5´..**AGG** CCG TAG TCT GGC TTT CTT ATG..3´. T136P: T136P/F; 5´..GCA CAT GGG GAC AAA GAT CGT AC..3´., T136P/R; 5‘..**GGG** TGC AAA CCG TAG TCT GGC..3´. H138P: H138P/F; 5´..GGG GAC AAA GAT CGT ACT TTT TG..3´. H138P/R; 5´...**AGG** TGC AGT TGC AAA CCG TAG..3´. D140P: D140P/F; 5´...AAA GAT CGT ACT TTT TGG AGA AAT

G..3‘. D140P/R; 5´.. **AGG** CCC ATG TGC AGT TGC AAA C..3´. AT Hook mutation, R61P: R61P/F; 5´...CGC GTT CTG TCT CCT AGA GAT G...3´, R61P/R; 5´...**AGG** CCT TCC TGA GCG GTA TGA C..3´.

2.9. Construction of the sandwich vector (SA)

Using the CAGT as a target for mutations: CA→ GC change in rIR was induced in pT and pTneo plasmids using the primers: for pT: FTC-3 (5´ AAC AAC ATG GGA CCA CGC AGC CGT CA 3´) and SB-SAND (5´ TTG CA**T CTA GAG CGG CCG C**GT TGA AGT CGG AAG TTT ACA TAC ACC TTA GCC 3`). and for pTneo: PR-Neo ( 5` CCT TGC GCA GCT GTG CTC GAC G 3´) and SB-SAND (5´ TTG CA**T CTA GAG CGG CCG C**GT TGA AGT CGG AAG TTT ACA TAC ACC TTA GCC 3`). SB-SAND primer introduces *Xba* I restriction site (bold underlined), and *Not* I (bold). The mutated (m) rIR was subcloned in pT and pTneo instead of the wild type rIR. Clones were sequenced and checked with restriction digestion analysis. The mutations were verified by DNA sequencing.

pEGFP-C1 was linearized with *Ase* I resriction enzyme, klenow filled, and ligated with *Not* I klenow filled Pt*, to have a fragment of 4.7kb adjacent to the Pt* transposon (T*-pEGFP-C1). Klenow filled *Ase* I PEGFP was also cloned in *Blp* I Pt fragment to be located between two wild type inverted repeat inside the transposon (left IR-pEGFP-C1-Right IR). I call this construct AB7.

EcoR I/*Xba*I fragment of pT* containing the transposon carrying mrIR was sub-cloned instead of *Eco*RI/*Xba*I fragment of Puc18. Puc18 vector containing the mrIR was digested with *Xba*I/*Sca*I. This fragment was ligated to the previous 4.7kb in the construct T*-pEGFP-C1, having this piece of DNA between two complete transposons (T*-4.7kb-T*) (Fig. 13 A) which are opposite in direction. This construct is called the Sandwich Vector (SA). Clones have been extensively checked with restriction digestion analysis.

SA was linearized with *Bgl* II, which it is a unique enzyme cuts one time in the middle of the SA. The linearized SA was klenow filled and ligated with the β-galactosidase gene (4.5 Kb *Pst* I fragment, of pCMVβ) forming 14.7kb SA-gal vector (T*-pEGFP-C1+β-galactosidase gene-T*), in other words (T*-9.2kb-T*) (Fig. 14A). Clones were selected based

on the blue-white selection and then were further confirmed with the restriction digestion analysis.

2.10. *In vivo* transposition assay

2.10.1. HMG-Constructs

Cell lines were grown in DMEM supplemented with 10% fetal bovine serum. Wild-type (VA1) and HMGB1-deficient (C1) mouse embryonic fibroblast cell lines have been described (Calogero et al., 1999), and were kindly provided by M. Bianchi, Milan, Italy. Cells were cotransfected with 90 ng each of pCMV-SB (Ivics et al., 1997) and pT/zeo (Izsvak et al., 2000) and 500 ng plasmid expressing HMGB1 (Aidinis et al., 1999) or HMGB2 (Aidinis et al., 1999) or HMGA1 (Melillo et al., 2001) or β-galactosidase. 10^5 transfected cells were plated out for selection, using 100 μg/ml zeocin (Invitrogen). After three weeks of selection, colonies were stained and counted as described (Ivics et al., 1997). 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 Fig. 5B have been normalized to transfection efficiencies.

2.10.2. Mutant-constructs

The plasmids encoded the different mutation constructs (120ng each): E6K, D10K, D17K, D68K, D86K, E92K, E93K, E158K, D164K, E174k, E216K; E321K, L132P, F134P, T136P, H138P, D140P, D140K, R143C, R115H, R115H/260, R115H/R143C, R143C/260, and R115H/260/R143C, were individually cotransfected together with the same concentration of SB10 coding plasmids (90ng). In the control experiments; pCMVβ was used.

2.10.3. Sandwich vector constructs

For checking the transposition activity of pTneo*; 90ng of either ptneo* or pTneo was cotransfecting together with 90ng from pCMV-SB10, pCMVβ was used as a non-related plasmid in the control experiment.

For checking the activity of the SA vector in an *in vivo* transposition assay: five sets of equal numbers of Hela cells were co-transfected with the following plasmids, the first: 90 ng of SA-containing plasmid and 30 ng of the transposase coding plasmid (SB10), second: 90ng AB7 and 30ng SB10, third: 90ng pTneo 7.5 and 30ng SB10, fourth: 90ng SA-gal and 30ng SB10, and the fifth: pT/10.3 and 30ng SB10. For each transfection a negative control was included where a pCMVβ were cotransfected with each individual substrate construct instead of SB10. For confirming the success of the assay a positive control was always included where pTneo is cotransfected with SB10. 10^5 transfected cells were selected and grown under neo-resistance. Colonies were fixed using formaldehyde and stained using bromophenol blue.

2.11. Chemicals and Materials

Chemical	Company
All plasmid kits	Qiagen
All the oligonucleotides	Biotez
bromophenol blue	Sigma
BL21-CodonPlus-RIL *E. coli* strain	Stratagene
Complete protease inhibitors	Roche
Dulbecco´s medium	Sigma
Exonuclease III	NEB
Geneticin (G418)	Gibico
Glycerol	Sigma
2-Mercaptoethanol	Merck
Ammonim persulphate	Gibico
Nickel resin	Qiagen
Penicillin/Streptomycin	Invitrogen
Phosphate-Buffered Saline (PBS)	Gibico
Phenol	Gibico
DNase I	Ambion
goat preimmune serum	Sigma
actin antibody	Neomarkers
p15 antibody	Santa Cruz Biotech
HMGB1 antibody	Santa Cruz Biotech
FUGENE6 transfection reagent	Roche
zeocin	Invitrogen
T4 polynucleotide kinase enzyme	USB
T4 DNA ligase	USB
QuickChange ™ Site directed Mutagenesis kit	Stratagene