Plant Chromosomal HMGB Proteins Efficiently Promote the Bacterial Site-Specific β-Mediated Recombination in Vitro and in Vivo †

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Plant Chromosomal HMGB Proteins Efficiently Promote the Bacterial Site-Specific β-Mediated Recombination in Vitro and in Vivo†

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ABSTRACT: In the presence of an accessory DNA bending protein, the bacterial site-specific β recombinase catalyzes resolution and DNA inversion. Five different maize high mobility group B (HMGB) proteins were examined for their potential to facilitate β recombination in vitro using DNA substrates with different intervening distances (73−913 bp) between two directly oriented recombination (*six*) sites. All analyzed HMGB proteins (HMGB1 to HMGB5) could promote β recombination, but depending on the DNA substrate with different efficiencies. The HMGB1 protein displayed an activity comparable to that of the natural promoting protein Hbsu, whereas the other HMGB proteins were less effective. Phosphorylation of the HMGB1 protein resulted in an increased efficiency of HMGB1 to promote β recombination. Analyses of DNA substrates with closely spaced *six* sites demonstrated that in the presence of HMGB1 the recombination rate was correlated to the distance between the *six* sites, but independent of the helical orientation of the *six* sites. Using a *Bacillus subtilis* strain defective in Hbsu, the coexpression of β recombinase and HMGB1 (or a truncated HMGB1 derivative) revealed that a plant HMG-box domain protein is sufficient for assisting β to catalyze recombination in vivo. Our results using β recombination as a model system suggest that the various plant HMGB proteins (and their posttranslationally modified versions) have the potential of forming a repertoire of different DNA structures, which is compatible with the idea that the HMGB proteins can act as architectural factors in a variety of nucleoprotein structures.

Many DNA interactions are regulated by communication of proteins bound to adjacent sites on DNA, which usually requires the DNA to be folded into specific conformations. When the intervening DNA segment is shorter than ∼150 bp (persistence length of DNA), the interaction of DNA-bound proteins may be markedly inhibited, due to the inherent inflexibility of the DNA. Architectural DNA bending factors often assist the formation of productive nucleoprotein structures by folding the DNA into the appropriate shape (*1*−*4*). Well-studied examples of reactions that are stimulated by accessory DNA bending proteins are prokaryotic site-specific recombination reactions, which are promoted by the DNA binding and bending proteins HU, IHF (integration host factor), or Fis in *Escherichia coli* (*4*). In several cases, structurally unrelated eukaryotic proteins belonging to the high mobility group B (HMGB) protein family [previously termed HMG1/2 proteins, (*5*)] can functionally replace the bacterial DNA bending proteins in the site-specific recombination reactions in vitro, which makes these recombination reactions attractive models to study the architectural function of eukaryotic HMGB proteins in the formation of complex nucleoprotein structures (*6*−*8*).

The chromatin-associated HMGB proteins contain one or two copies of a common DNA binding motif termed the HMG-box domain, which has a conserved L-shaped fold consisting essentially of three α-helices (*9*−*11*). The HMGB proteins interact in a sequence-independent manner with the minor groove of linear DNA, but bind with high affinity to certain distorted DNA structures. Mediated by the HMG-box domain, they can severely bend DNA and can constrain supercoils in plasmid DNA (*9*−*11*). The HMGB proteins can facilitate the formation of a variety of specific nucleoprotein structures, and therefore they may act in various DNA-dependent processes such as transcription and recombination (*1, 3, 9, 10, 12*). In contrast to other eukaryotes, which usually express two or three different HMGB proteins, plants have at least five different HMGB proteins (*12*). Plant HMGB proteins display a single HMG-box domain, which is flanked by a basic N-terminal domain and an acidic C-terminal domain. In maize, there are five different HMGB proteins (HMGB1, HMGB2/3, HMGB4, and HMGB5 proteins, previously termed HMGa, HMGc1/2, HMGd, and HMGe, respectively), which have relatively conserved HMG-box domains, but they differ substantially outside the DNA binding domains (*12*). The HMGB1 protein can functionally complement the defect of the essential chromatin-associated Hbsu protein in *B. subtilis*, as the expression of HMGB1 in the mutant strain lacking a fully functional Hbsu could overcome the increased sensitivity of the mutant cells to the lethal effects of DNA damaging agents (*13*).

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Gram-positive broad host range plasmid pSM19035-encoded β recombinase catalyzes both DNA resolution, when the specific *six* recombination sites are directly oriented, and DNA inversion, when the *six* sites are inversely oriented, provided that a chromatin-associated DNA bending protein is present in the reaction mixture (e.g., Hbsu or the structurally unrelated HMGB protein) (*14*). The *six* recombination site is a 90 bp DNA region that contains two adjacent binding subsites (I and II) for binding of β dimers, with the crossing over point localized to the center of subsite I (*15*). β dimers bind to an inverted repeat motif at subsite I and to a direct repeat motif at subsite II (*16*). Each protomer of a β dimer interacts with both sides of the DNA helix and with each half-site of the imperfect palindrome at subsite I, but only one protomer of a β dimer seems to interact with sequences located in both the major and minor groove of the DNA at subsite II [(*16*); Figure 1]. β recombinase alone is unable to synapse two distant *six* sites, and it relies on Hbsu or HMGB proteins to activate strand exchange (*14*). A similar observation was reported for another member of this subfamily of serine recombinases (Sin recombinase) (*17, 18*). In this case, the Hbsu (termed HU in *E. coli*) binding site has been mapped between subsites I and II of the Sin recombinase recognition sequence (*18*).

To analyze the potential of the plant HMGB proteins in facilitating the formation of β recombinase synaptic complex, we have comparatively studied here the different maize HMGB proteins. The HMGB-induced structural changes in the DNA promote β synaptic complex formation in vitro between two directly repeated minimal 90 bp *six* sites separated by intervening sequences of 84−913 bp. The HMGB1 protein and the individual HMG-box domain were expressed in an Hbsu-defective *B. subtilis* strain to examine their ability to facilitate β synaptic complex formation in vivo.

EXPERIMENTAL PROCEDURES

Construction of Strains and Plasmids. The 90 bp *six* sites were amplified by PCR in two different reactions creating different restriction enzyme recognition sites flanking the *six* sites. For PCR reactions, 50 ng of pCB106 (*15*) was used as template, and the reactions contained 1 μM of each primer (*six*1: 5′-AATTGAGCTCTATAGGTCAATAGAGTATACTTAT; *six*2: 5′-AATTCCGCGGTATTATGCTCAACTTAAATGACCT; *six*3: 5′-AATTCTCGAGTATAGGTCAATAGAGTATACTTAT; *six*4: 5′-AATTGGTACCTATTATGCTCAACTTAAATGACCT), 0.1 mM deoxyribonucleoside triphosphates, and 1 unit of *DeepVent*$_R$ DNA polymerase (New England Biolabs). After restriction enzyme digestion either with *Sac*I/*Sac*II or with *Xho*I/*Kpn*I, the obtained fragments were purified and cloned in two steps into pBC-SK (Stratagene), yielding pBC*six*85. To obtain the plasmid pBC*six*103, a double-stranded oligonucleotide (5′-CTAGAAAAGGTGGCACTA) having *Xba*I restriction sites on both ends was cloned into the *Xba*I site of pBC*six*85. The other pBC*six* plasmids were derived from pBC*six*85 or pBC*six*103 by restriction enzyme digestion with either *Eco*RI or *Pst*I, followed by filling-in the obtained sticky ends or removing them by treatment with T4 DNA polymerase (MBI Fermentas). Finally, these plasmids were religated, yielding pBC*six*107, pBC*six*99, pBC*six*88, pBC*six*86, pBC*six*84. Plasmid pBC*six*73 was generated by digesting pBC*six*85 with *Sma*I/*Eco*RI, followed by a treatment with T4 polymerase and religation of the DNA. All plasmid constructions were checked by DNA sequencing.

A DNA segment containing two directly repeated 90 bp *six* sites spaced by 2.2 kb (*six*-*xyl*E-*six*) or 103 bp (*six*-103-*six*) was integrated, as a single chromosomal copy, in the *amy* locus of *B. subtilis* wt and its isogenic derivative *hbs*4755 cells (*19*) to render BG571 and BG569 (wt background) and BG599 and BG597 (*hbs*4755) strains, respectively. The plasmid-borne HMGB1(G35-Y109) [pHMGB1 (G35-Y109)], HMGB1(M1-E157) [pHMGB1 (M1-E157)], or *hbs* gene (pHbsu) were all placed under control of the *cat* promoter. *B. subtilis* BG571 [(*six*-*xyl*E-*six*) or BG569 (*six*-103-*six*)] and BG599 [(*hbs*4755, *six*-*xyl*E-*six*) or BG597 (*hbs*4755, *six*-103-*six*)] cells were then transformed with plasmid-borne HMGB1(G35-Y109) [pHMGB1 (G35-Y109)], HMGB1(M1-E157) [pHMGB1 (M1-E157)], or *hbs* gene (pHbsu). In a second step, a plasmid-borne β recombinase [pBT333-3, (*13*)] was transferred into BG571, BG569, BG599, and BG597.

Purification of Proteins. Recombinant HMGB proteins and truncated versions of the maize HMGB1 protein were expressed in *E. coli* and purified by three-step column chromatography as decribed previously (*13, 20*). Native HMGB1 was purified from immature maize kernels, and the phosphorylation status of the protein was analyzed by acetic acid/urea/PAGE and/or MALDI-TOF mass spectrometry as described previously (*20, 21*). β and Hbsu were purified as described previously (*14*).

In Vitro Assay for β Recombination. pCB106 (*15*) and the different pBC*six* substrate plasmids that contain two directly repeated *six* sites were used as substrates for site-specific recombination assays. The reaction mixtures contained 2 μg of substrate plasmid, 50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 50 nM β recombinase, and various concentrations of Hbsu or HMGB proteins, in a total volume of 20 μL. After incubation for 1 h at 30 °C, the reaction was stopped by heating to 70 °C for 30 min. The pCB106 was digested with *Nde*I, while the pBC*six* substrate plasmids were digested with *Pvu*II and *Bam*HI. After proteinase K treatment, the DNA fragments were separated on 1.5% agarose gels in TAE buffer (for the pCB106 plasmid) or on nondenaturing 5% polyacrylamide gels in 1×TBE buffer (for pBC*six* plasmids) and stained by ethidium bromide. Quantification of the resulting DNA fragments was performed by scanning with a phosphorimager (BioRad) and analyzed using the Quantity One software (BioRad).

Analysis of β Recombination in B. subtilis. B. subtilis wt (BG571 and BG569) and *hbs*4755 (BG599 and BG597) cells bearing plasmid-borne HMGB1(G35-Y109), HMGB1(M1-E157), or *hbs* gene were transformed with the plasmid-borne β recombinase. Relative site-specific recombination frequencies were measured as the number of color-less colonies (by resolution of the *xyl*E gene, which codes for the enzyme catechol 2,3-oxygenase, in the BG571 and BG599 strains) upon spread of the colonies with pyrocatechol (0.5 M) or by PCR analysis of independent colonies. In the absence of the plasmid-borne β gene, less than 3% of the colonies undergo resolution of the intervening DNA in the wild-type and *hbs*4755 mutant background. In the *hbs*4755 strain ∼7% of the colonies undergo resolution due to the nature of the R55A mutation of the essential *hbs* gene (*19*).

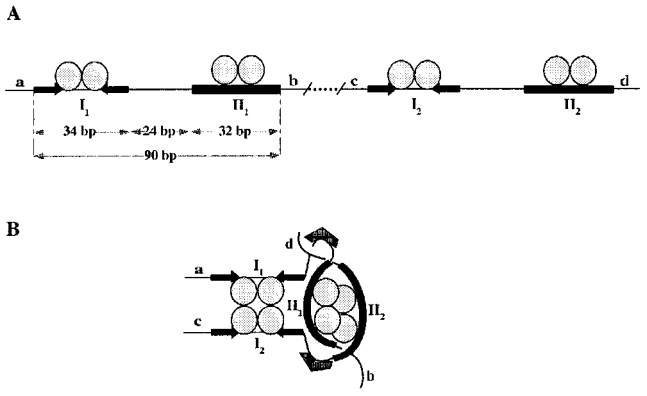

FIGURE 1: Model of the synapsis of β recombinase. (A) A β dimer bound to both arms of the inverted repeats of the 34 bp subsite I (each protomer denoted as an oval), and a protomer of a second β dimer bound to a direct repeat at the 32 bp subsite II. The two directly oriented *six* sites are shown. For clarity, the β dimers bound at subsite I are depicted as not contacting subsite II-bound β subunits, although our model proposes that the β dimers at subsites I and II synapse. The letters a, b, c, and d are shown to landmark the synaptic complex in (B), and the dotted line (b − c interval) indicates the variable distance between two *six* sites. (B) The interaction of two β dimers bound to subsite I and to subsite II in a synaptic complex upon assistance by a chromatin-associated protein (Hbsu or HMGB, indicated by an L-shaped symbol). A DNA distortion, to which HMGB binds, between subsites I and II renders a stable synaptic complex. This model is based on previously described results (*16*) and models (*18, 38*).

RESULTS

Various Plant HMGB Proteins Facilitate β Recombination in Vitro between Closely Spaced Minimal six Sites. We have previously shown that plant HMGB proteins can facilitate in vitro β-mediated resolution using a supercoiled DNA substrate (pCB8) with two 447 bp *six* sites in direct orientation with ~2.2 kb of intervening sequence between them (*22*). Here, we have examined whether the different maize HMGB proteins facilitate β recombination between two 90 bp *six* sites (see Figure 1A) spaced by 913 bp (pCB106) as previously tested with β and Hbsu (*15*). Negative supercoiled pCB106 DNA was incubated in recombination reactions performed with purified recombinant β protein and increasing concentrations of the different HMGB proteins. To monitor the performance of the recombination, the DNA reaction products were digested with *Nde*I and separated by agarose gel electrophoresis. The restriction enzyme digest resulted in a reduced amount of the parental substrate and in the appearance of two new DNA fragments of 2.4 and 1.0 kb, if recombination has taken place (Figure 2A). From this experiment, it is evident that all five maize HMGB proteins are capable of promoting β recombination between directly oriented 90 bp *six* sites, albeit with a different efficiency (Figure 2B). While no recombination is detectable in the absence of an assisting DNA bending protein, at low HMGB protein concentrations (5−25 nM), the HMGB1 protein was the most efficient in stimulating the recombination and the reaction is saturated over 200 nM. With the exception of HMGB5, ~45% of the total DNA substrate was converted to resolution in the presence of 200 nM HMGB proteins. In the case of HMGB5, only ~25% of the total DNA substrate was converted to reaction products under these conditions, and the reaction is not saturated even at 800 nM (Figure 2B). It is likely, therefore, that the various

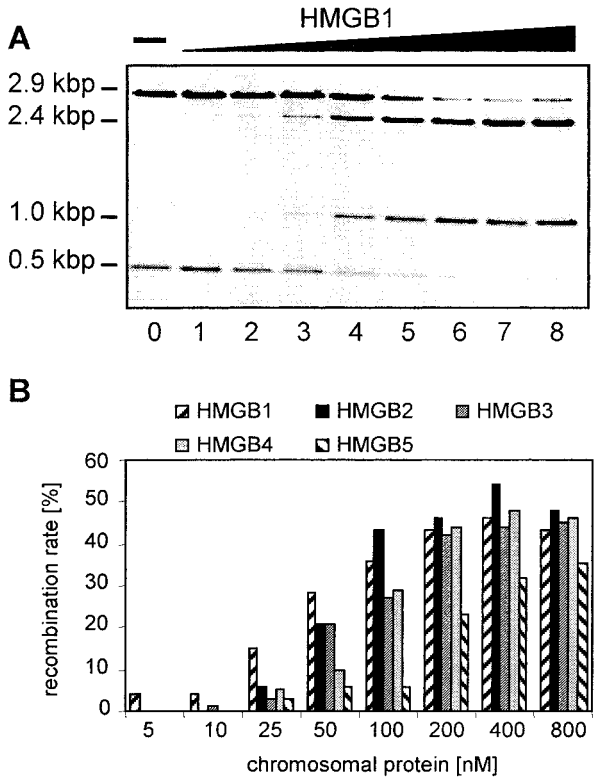

FIGURE 2: In vitro β recombination assay with the maize HMGB proteins using a DNA substrate with *six* sites separated by 913 bp. The recombination reactions were performed in the presence of a constant amount of β recombinase and various concentrations of the maize HMGB1−HMGB5 proteins. (A) Typical ethidium bromide-stained agarose gel used to separate the DNA reaction products (after digestion with *Nde*I) of a β recombination experiment performed with various concentrations of HMGB1 (0, 5, 10, 25, 50, 100, 200, 400, and 800 nM in lanes 1−9, respectively). The sizes of the obtained DNA fragments are indicated. (B) Histogram depicting the recombination rate in recombination assays obtained with the HMGB1−HMGB5 proteins at different concentrations. The recombination rate is the fraction of the input DNA substrate that was converted to the recombination reaction products. Data were quantified from DNA recombination products separated on agarose gels, and each bar of the histogram represents the mean value of three independent experiments.

HMGB proteins generate a repertoire of bends or kinks, and that the HMGB proteins, which preferentially induce a structure that matches the requirements for β synaptic complex formation, are more efficient in our assay. Alternatively, a high concentration of the DNA bending protein is required, if the protein does not induce the optimal structure of the synaptic complex. The structurally unrelated maize HMGA protein containing A/T-hook DNA binding motifs was unable to stimulate β recombination in this assay (C.S. and K.D.G., data not shown).

To further examine the repertoire of structures produced by the different maize HMGB proteins, we have placed two directly oriented 90 bp *six* sites in a distance below the DNA persistence length; hence, the spacing of the *six* sites was reduced (distance between the b − c intervals in Figure 1) to 103 bp. From the modeled synaptic complex, we have to predict a strong topologically selected bend to allow formation of the synaptic complex and to activate strand exchange. The pBC*six*103 substrate plasmid was incubated with β recombinase and different concentrations of the various HMGB proteins, and for comparison, the Hbsu protein was

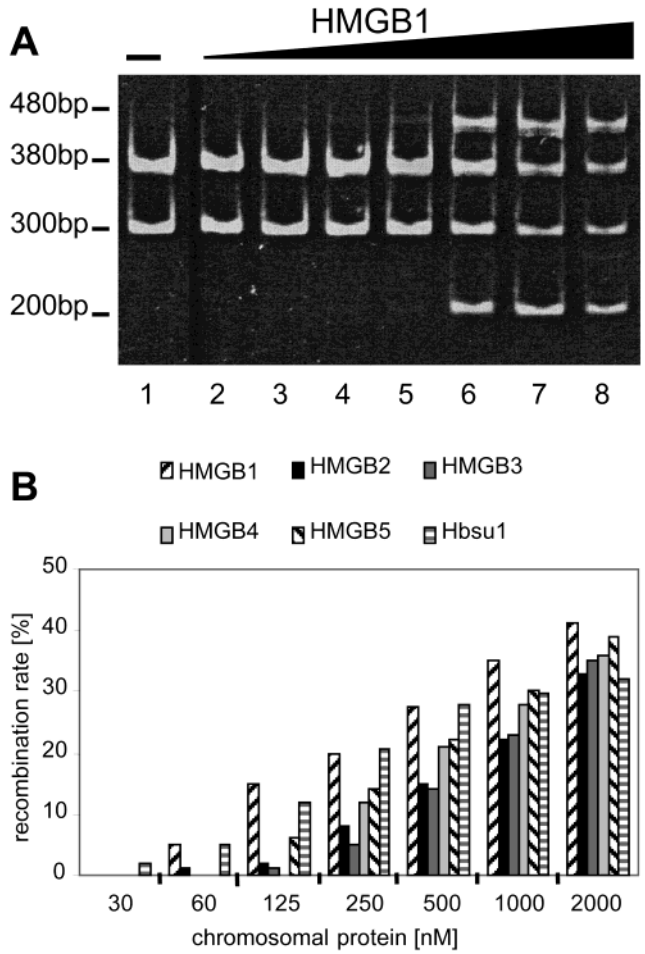

FIGURE 3: In vitro β recombination assay with the maize HMGB and the *B. subtilis* Hbsu proteins using a DNA substrate with *six* sites separated by 103 bp. The recombination reactions were performed in the presence of a constant amount of β recombinase and various concentrations of the maize HMGB1−HMGB5 and the *B. subtilis* Hbsu proteins. (A) Typical ethidium bromide-stained polyacrylamide gel used to separate the DNA reaction products (after digestion with *Bam*HI/*Pvu*II) of a β recombination experiment performed with various concentrations of HMGB1 (0, 30, 60, 125, 250, 500, 1000, and 2000 nM in lanes 1−8, respectively). The sizes of the obtained DNA fragments are indicated. (B) Histogram depicting the recombination rate in recombination assays obtained with the HMGB1−HMGB5 and Hbsu proteins at different concentrations. The recombination rate is the fraction of the input DNA substrate that was converted to the recombination reaction products. Data were quantified from DNA recombination products separated on polyacrylamide gels, and each bar of the histogram represents the mean value of three independent experiments.

also assayed. After completion of the reactions, the DNA was digested with the restriction enzymes *Pvu*II/*Bam*HI, and the DNA reaction products were separated by polyacrylamide gel electrophoresis. Two new DNA fragments of 480 and 200 bp in length are generated, if β-mediated recombination has taken place. Hbsu and all tested HMGB proteins could stimulate β recombination between the two closely spaced *six* sites (Figure 3), although in comparison to the pCB106 substrate (spacing of 913 bp between the *six* sites) ~5-fold higher concentrations of the DNA bending protein were required to yield comparable recombination rates. At low concentrations of the DNA bending proteins (30−250 nM), Hbsu and HMGB1 were more efficient in promoting the reaction. About ~23% of the total DNA substrate was converted to resolution in the presence of 250 nM HMGB1

Table 1: Summary of in Vitro β Recombination Assays Performed with Various DNA Substrates Containing Closely Spaced *six* Sites

plasmid	distance between *six* sites	minimal functional HMGB1 concentration (nM)[a]	recombination rate[b]
pCBsix106	913	5	++++
pBCsix107	107	60	+++
pBCsix103	103	60	+++
pBCsix99	99	60	+++
pBCsix88	88	60	++
pBCsix86	86	125	+
pBCsix85	85	125	+
pBCsix84	84	250	+
pBCsix73[c]	73	−	−

[a] In vitro β recombination assays performed in the presence of HMGB1. The minimal concentration of HMGB1 is given that resulted in the formation of DNA recombination products, detectable by ethidium bromide staining of the polyacrylamide gels. [b] The recombination rate is the fraction of the input pBC*six* plasmid that was converted to recombination reaction products relative to pCB*six*106. [c] No recombination products could be detected with this substrate plasmid.

or Hbsu, whereas ~2-fold more of HMGB4 and HMGB5 and ~4-fold more of the closely related HMGB2/3 proteins were required to obtain a comparable recombination rate. Interestingly, HMGB5 is more efficient than HMGB2/3 and HMGB4 in promoting the reaction of the DNA substrate with the *six* sites spaced by 103 bp, which is different when the spacing between the six sites is 913 bp (Figure 2).

The Efficiency of the β Recombination Reaction Depends on the Distance between the six Sites. Since the β recombination reaction worked also with a spacing of 103 bp between the two directly oriented *six* sites (Figure 3), we further reduced the distance between the *six* sites to examine whether the distance between the *six* sites and/or their relative helical orientation on the DNA influence the reaction. Since it could be expected that the reaction (due to the inherent stiffness of DNA) is inhibited, when the recombination sites were very closely spaced, we wanted to determine the limits of the reaction in terms of the spacing between two directly oriented 90 bp *six* sites (see Figure 1). For this purpose, several substrate plasmids were constructed in which the spacing of the *six* sites varied in the range between 107 and 73 bp (Table 1). The various substrate plasmids were analyzed in recombination reactions as described before, using different concentrations of the HMGB1 protein. The gels used to analyze the performance of the reaction were quantified, and from these data, the lowest HMGB1 concentration was determined that yielded detectable ethidium bromide stained DNA bands corresponding to the recombination products. Furthermore, the recombination rate relative to the efficiency of the reaction using the pCB106 substrate plasmid was deduced. As summarized in Table 1, it can be inferred that HMGB1 helps the β protein to promote recombination, if the distance between the *six* sites was in the range between 107 and 84 bp. However, the closer the *six* sites were spaced on the substrate plasmid, the higher was the required concentration of HMGB1 to promote the reaction. In parallel with that, the recombination rate decreased when the *six* sites became more closely spaced. At a distance of 73 bp between the *six* sites, no recombination products could be detected, even in the presence of 2 μM HMGB1, suggesting that a spacing of the *six* sites of 73 bp is no longer compatible with β recombination. When the

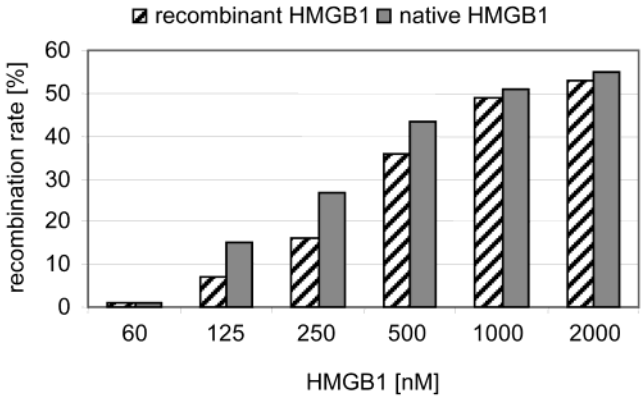

FIGURE 4: In vitro β recombination assay with recombinant (nonphosphorylated) and native (phosphorylated) HMGB1 protein using a DNA substrate with *six* sites separated by 103 bp. The recombination reactions were performed in the presence of a constant amount of β recombinase and various concentrations of recombinant maize HMGB1 or HMGB1 purified from immature maize kernels. The histogram depicts the recombination rate in recombination assays obtained with HMGB1 at different concentrations. The recombination rate is the fraction of the input DNA substrate that was converted to the recombination reaction products. Data were quantified from DNA recombination products separated on polyacrylamide gels, and each bar of the histogram represents the mean value of three independent experiments.

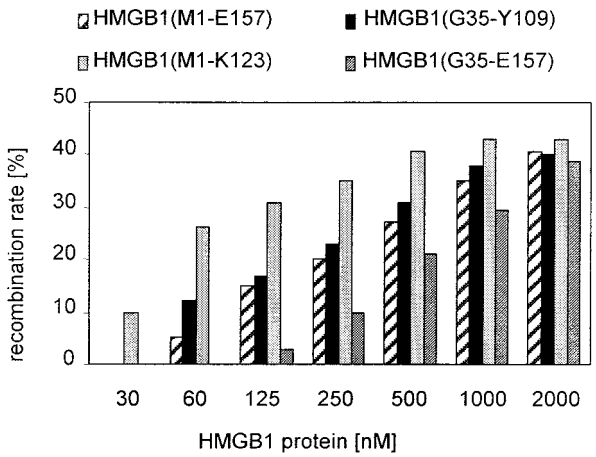

FIGURE 5: In vitro β recombination assay with full-length and truncated versions of the HMGB1 protein using a DNA substrate with *six* sites separated by 103 bp. The recombination reactions were performed in the presence of a constant amount of β recombinase and various concentrations of the maize HMGB1 derivatives (M1-E157, M1-K123, G35-Y109, G35-E157). The histogram depicts the recombination rate in recombination assays obtained with the HMGB1 derivatives at different concentrations. The recombination rate is the fraction of the input DNA substrate that was converted to the recombination reaction products. Data were quantified from DNA recombination products separated on polyacrylamide gels, and each bar of the histogram represents the mean value of three independent experiments.

recombination rates obtained with recombination substrates having a spacing of the *six* sites of 107, 103, and 99 bp (4 bp difference each) were compared, no differences in the efficiency of the β recombination reaction were observed, indicating that in the presence of HMGB1 the relative helical orientation of the *six* sites on the DNA has no detectable influence on the reaction. On the other hand, lower recombination rates were obtained, when the spacing of the *six* sites was reduced from 88 to 84 bp, but this observation may be due to the fact that in this range of spacing between the *six* sites the reaction is driven to its limits. Thus, using substrates containing a short length of intervening DNA ($\leq$107 bp), the efficiency of the β recombination is clearly dependent on the distance between the *six* sites, whereas the helical orientation of the *six* sites is apparently not a critical parameter.

Phosphorylation Enhances the Activity of HMGB1 in β Recombination. Some plant HMGB proteins are phosphorylated in vivo and in vitro by protein kinase CK2 (*21*). We have examined whether the phosphorylation of HMGB1 influences the efficiency of β-catalyzed site-specific recombination. Native (phosphorylated) HMGB1 purified from immature maize kernels and recombinant (nonphosphorylated) HMGB1 purified using the same protocol were examined for their ability to facilitate β recombination. As analyzed by acetic acid/urea/PAGE and MALDI-TOF mass spectrometry, the native HMGB1 occurred as a mixture of single-, triple- and tetra-phosphorylated variants, whereas the non- and double-phosphorylated forms or other types of posttranslational modifications could not be detected [(*21*) and data not shown]. At low protein concentrations (125−250 nM), phosphorylated HMGB1 was ~2-fold more efficient in promoting β recombination than the nonphosphorylated protein, when the recombination was tested with a plasmid substrate containing two directly oriented *six* sites spaced below the DNA persistence length (pBC*six*103) (Figure 4). Similarly, in vitro phosphorylation of HMGB1 by recombinant CK2 (*21*) enhanced the activity of HMGB1 in the recombination reaction using the pCB8 DNA substrate in which the *six* sites are spaced by 2.2 kb (G.L. and J.C.A., data not shown). Since the HMGB1 proteins compared in these experiments differed only in their phosphorylation status, the phosphorylation of HMGB proteins may result in a different efficacy in promoting β recombination. The finding of an increased efficiency of phosphorylated HMGB1 (compared to nonphosphorylated HMGB1) in β recombination is in contrast to the observed reduced DNA binding of HMGB1 upon phosphorylation (*21*).

The Individual HMG-box Domain Is Sufficient To Promote β Recombination. To examine the contribution of the various domains of the plant HMGB proteins in the promotion of β recombination, we have selected the HMGB1 protein, which displays the highest efficiency in stimulating this reaction. Full-length HMGB1 and various truncated derivatives of HMGB1 were tested for their ability to facilitate the reaction. Using the in vitro β recombination assay with the pBC*six*103 substrate, full-length HMGB1(M1-E157) was compared to the individual HMG-box domain HMGB1(G35-Y109), HMGB1(M1-K123), and HMGB1(G35-E157). The protein HMGB1(M1-K123), comprising the entire basic part of the protein including the HMG-box domain, but lacking the acidic C-terminal tail, was the most effective in promoting the reaction, as it facilitates the recombination at 30 nM (Figure 5). HMGB1(G35-E157), which lacks the basic N-terminal domain, displayed the lowest activity in this assay, requiring ~8-fold higher protein concentrations to obtain recombination rates comparable to HMGB1(M1-K123). The full-length protein and the individual HMG-box domain HMGB1(G35-Y109) stimulated the reaction to intermediate levels. Hence, the HMG-box domain is sufficient for promoting β recombination, and it represents the most important element of the HMGB1 protein in this

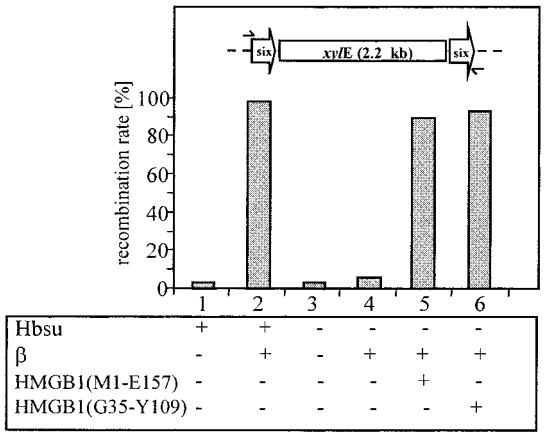

FIGURE 6: β-Mediated resolution between two *six* sites separated by 2.2 kb in the presence of Hbsu, full-length HMGB1, and the individual HMG-box domain in *B. subtilis*. The efficiency of β-catalyzed resolution between two directly oriented *six* sites separated by 2.2 kb in a single copy in the chromosome of *B. subtilis hbs*4755 cells in the presence of different plasmid-derived chromatin-associated proteins was quantified by PCR analysis. The 90 bp *six* sites are denoted as open arrows, the primers used in the PCR reaction as small half-arrows, and the chromosomal DNA as a broken line. The + and − symbols indicate the presence or absence of a given plasmid-borne gene.

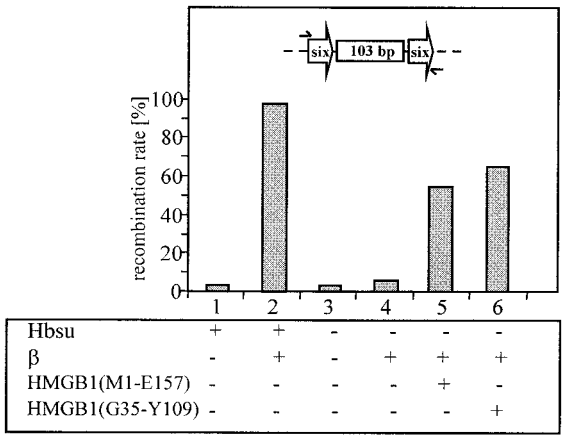

FIGURE 7: β-Mediated resolution between two *six* sites separated by 103 bp in the presence of Hbsu, full-length HMGB1, and the individual HMG-box domain in *B. subtilis*. The efficiency of β-catalyzed resolution between two directly oriented *six* sites separated by 103 bp in a single copy in the chromosome of *B. subtilis hbs*4755 cells in the presence of different plasmid-derived chromatin-associated proteins was quantified by PCR analysis. The 90 bp *six* sites and their orientation are denoted as open arrows. The primers used in the PCR reaction are indicated as small half-arrows, and the chromosomal DNA as a broken line. The + and − symbols indicate the presence or absence of a given plasmid-borne gene.

reaction. The activity of the HMG-box domain is modulated by the flanking basic and acidic domains, similarly as previously demonstrated for other DNA interactions (*13*).

HMGB1 and Its Individual HMG-box Domain Can Promote β Recombination in Vivo. After having established that plant HMGB proteins can stimulate β recombination in vitro using the minimal 90 bp *six* sites with variable spacing (from 913 to 84 bp), we have examined whether the HMGB proteins can facilitate the reaction also in vivo in *B. subtilis* cells. The nucleotide sequence analysis of the SPβ-free *B. subtilis* genome revealed the presence of only one gene coding for a sequence-independent chromatin-associated Hbsu protein, whereas genes coding for sequence-specific architectural proteins such as *E. coli* IHF, Fis, H-NS, StpA, CRP, or Hha cannot be predicted (*23*). To discriminate the action of the endogenous Hbsu protein from the HMGB protein expressed from a plasmid-borne *HMGB* gene, the SPβ-free and the *hbs* isogenic *B. subtilis* mutant (*hbs*4755, BG405) strain was employed. In the *hbs*4755 strain, β-mediated recombination dropped more than 13-fold due to the low activity of the mutant (Arg55Ala) *hbs*4755 gene [(*19*), Figures 6 and 7).

Previously it has been shown that purified monomeric plasmid DNA, having no homology with the host chromosome, cannot transform competent *B. subtilis* cells, although dimers or higher-order oligomers are active in transformation (*24*). Furthermore, the actively transforming plasmid forms, when isolated from *B. subtilis* cells, account for about 3−5% of the total plasmid DNA (*24*, *25*). When isolated from *B. subtilis* cells, plasmids bearing the β recombination system are composed almost exclusively (>99%) of monomers, because dimers or higher oligomers generated by homologous recombination are resolved by the β recombinase (*19*, *26*). We took advantage of this characteristic of the *B. subtilis* DNA uptake mechanism to address whether the plasmid-borne HMGB1(G35-Y109), HMGB1(M1-E157), or *hbs* genes can support in vivo β protein-mediated site-specific recombination.

pBT233-3, containing an active β recombination system, was transferred into wild-type BG571 and BG569 strains (containing a single chromosomal copy of *six*-*xyl*E-*six* and *six*-103-*six*, respectively) and the *hbs*4755 isogenic derivative BG599 and BG567 competent cells bearing pHMGB1(G35-Y109), pHMGB1(M1-E157), or pHbsu, and the frequency of pBT233-3 transformation was scored. As control, the plasmid DNA was linearized, ligated under conditions that favor plasmid oligomerization (1−2 μg/mL), and used to transform the same strains.

When wt (BG571 and BG569) or *hbs4755* (BG599 and BG597) competent cells bearing pHMGB1(G35-Y109), pHMGB1(M1-E157), or pHbsu were transformed with pBT233-3 DNA, about the same transformation frequency was observed (after correction for the competent state of the cells). However, only in vitro oligomerized pBT233-3 DNA (linearized and ligated) was able to transform plasmid-free *hbs4755* competent cells. It is likely, therefore, that HMGB1-(G35-Y109) and HMGB1(M1-E157) facilitate β-mediated recombination with similar efficiency than Hbsu does. This is consistent with the observation that after overnight growth >95% of the nontransformed colonies are yellow after spraying with pyrocatechol, but <10% of the transformed colonies are yellow. Accordingly, in ~90% of the transformed colonies there is no degradation of pyrocatechol because the *xyl*E gene (which encodes an enzyme that renders a yellow product upon degradation of catechol) has been deleted by β recombination.

B. subtilis wt (BG571) and *hbs*4755 (BG569) containing a single chromosomal copy of the *six*-*xyl*E-*six* cassette, bearing pHMGB1(G35-Y109), pHMGB1(M1-E157), or pHbsu and pBT233-3, were grown overnight. The relative frequency of DNA resolution was analyzed by PCR using chromosomal DNA of the different strains as template. β-Mediated recombination could only be observed when both β protein and a functional DNA bending protein were

present. The DNA bending protein can be either the endogenous Hbsu of wt *B. subtilis* cells or the plasmid-borne Hbsu and HMGB1 proteins in the Hbsu-defective strain. When the relative recombination frequencies of DNA isolated from *hbs*4755 containing a single chromosomal copy of *six*-*xyl*E-*six* were compared, no striking differences in the efficiency of the β recombination reaction were observed (Figure 6).

We then addressed the relative recombination frequency of DNA isolated from wt (BG599) and *hbs*4755 (BG597) containing a single chromosomal copy of the *six*-103-*six* cassette. *B. subtilis hbs*4755 cells bearing the plasmid-borne *hbs* gene are the most efficient in facilitating β-mediated recombination (Figure 7). The relative efficiencies of full-length HMGB1(M1-E157) and the individual HMG-box domain HMGB1(G35-Y109) are similar (∼60% of the recombination rate of Hbsu).

DISCUSSION

The results reported here demonstrate that the maize HMGB proteins are necessary and sufficient as essential cofactors for the β protein to catalyze in vitro site-specific recombination between two directly oriented minimal 90 bp *six* sites spaced by 84−913 bp. In addition, the HMGB1 protein is shown to promote synapsis of two *six* sites spaced either by 2.2 kb or by 103 bp, and to activate the β recombinase catalytic activity in vivo. Therefore, the maize HMGB proteins have the ability to alter the global architecture of the DNA substrate to assist the four dimers of the β recombinase to form a functional synaptic complex between two directly oriented *six* sites. Both in vitro and in *B. subtilis*, the HMG-box DNA binding domain of maize HMGB1 is sufficient to promote β recombination (Figures 5−7). In line with this finding, the HMG-box domain of the structure-specific recognition protein SSRP1 can also facilitate in vitro β recombination (*27*), although outside the DNA binding domain SSPR1 and HMGB proteins are structurally different.

Using 90 bp *six* sites, we show that depending on the DNA substrate (differing in the spacing of the *six* recombination sites), the various maize HMGB proteins have significantly different activities in β-mediated DNA recombination in vitro (Figures 2 and 3). The HMGB1 protein is most efficient in facilitating β recombination independent of whether the *six* sites are spaced by 913 or 103 bp, and HMGB1 displays an activity comparable to that of the natural promoting factor Hbsu. Interestingly, the phosphorylation of HMGB1 further increased its efficiency in β-mediated resolution (Figure 4), although the affinity for DNA is reduced upon phosphorylation (*21*), suggesting that the phosphorylated protein forms a slightly different structure, which is more favorable for the recombination reaction to occur. The HMGB5 protein has only a relatively low activity in stimulating the reaction (in the lower concentration range of 50−200 nM), when the *six* sites are spaced by 913 bp requiring ∼4-fold higher protein input to achieve an efficiency comparable to that of HMGB2/3 and HMGB4. When the *six* sites are spaced by 103 bp, however, the HMGB5 protein is more efficient than the HMGB2/3 and HMGB4 proteins (in the lower concentration range of 125−250 nM), and only about half the amount of HMGB5 is required to obtain similar recombination rates (Figures 2 and 3). Despite the conservation of the global fold of the HMG-box domains, the DNA interactions of domains of different proteins are variable (*10, 11*). Moreover, the properties of HMG-box domains are markedly modulated by basic and acidic domains flanking the domains within the HMGB proteins (*10, 12*). Thus, subtle differences in both the HMG-box domains of the various maize HMGB proteins and their variable basic and acidic domains, flanking the HMG-box domain, may be responsible for the different activity of the various plant HMGB proteins in mediating β recombination. The differential ability of the HMGB proteins to facilitate the formation of the β synaptic complex with substrate plasmids having differentially spaced *six* recombination sites suggests that depending on the specific requirements of the nucleoprotein structure to be formed, various HMGB proteins (and their posttranslationally modified versions) may be differentially adapted to fulfill the structural requirements for assisting the assembly of the final complex. Since the HMGB proteins presumably are involved as architectural factors in different DNA-dependent processes such as transcription, recombination, and replication (*3, 9, 10, 12*), it can be expected that they are able to act as assistant proteins in a wide variety of biological contexts in the plant cell nucleus. In line with this possibility, the maize HMGB proteins are differentially associated with plant chromatin (*28*).

In some of the nucleoprotein structures, physical interactions between the sequence-specific regulatory proteins and the assisting non-sequence-specific DNA bending proteins are required to recruit the architectural bending factor to its site of action (*3, 9, 10, 12*). Thus, HMGB proteins have been found to interact functionally with various transcription factors, stimulating the binding of these factors to their cognate DNA recognition sequences (*29*−*32*). Similarly, in the mammalian V(D)J recombination, the recombination protein RAG1 recruits HMGB proteins to the recombination complex by direct protein/protein interactions with HMGB1 or HMGB2. In this specific nucleoprotein structure, mammalian HMGB proteins stimulate the efficiency of the recombination reaction (*33*−*37*). In other structures, however, the architectural proteins are recruited without physical interaction with the sequence-specific factors, for instance, by structural trapping through a transiently formed DNA structure, which is specifically bound and stabilized by the chromosomal bending proteins (*3, 10, 12*). Like the Hin and lambda Int recombination, the β recombination is an example for the latter case, as HMGB proteins can efficiently replace the structurally unrelated HU/Hbsu proteins, indicating that direct protein/protein contacts are dispensable (*6*−*8*). In agreement with this notion, we were unable to detect β/HMGB complexes in chemical protein cross-linking experiments (C.S. and K.D.G., data not shown).

The synaptic complexes required for resolution by different serine recombinases are topologically equivalent [(*18, 38*); Figure 1]. We envisage that Hbsu or HMGB1 have to distort the DNA structure remarkably to assist the two β dimers bound to each of the directly oriented *six* sites separated by only 84 bp, to modify their relative orientation for forming an active parallel synaptic complex. The examination of DNA substrates with various distances between the recombination sites revealed that the efficiency of the reaction in vitro decreases as the *six* sites become more closely spaced

(Table 1). In parallel with that, the stimulation of β recombination requires increasing concentrations of HMGB1, probably reflecting the increasing difficulty of folding the DNA into the appropriate structure compatible with the formation of a functional synaptic complex. However, in the presence of HMGB1, the β-mediated reaction seems to be independent of the helical orientation of the two *six* sites on the DNA. As shown in DNA circularization experiments in the presence of DNA ligase, the mammalian HMGB2 protein can increase the torsional flexibility of the double helix, overcoming the torsional rigidity of DNA (*39*). Unlike the Hin-mediated recombination in which the correct helical position of the binding site of the sequence-specific DNA-bending Fis protein on the DNA is critical for the appropriate Hin-Fis contacts (*40, 41*), in the β-promoted recombination reaction performed in the presence of HMGB1, the relative helical position of the two *six* sites on the DNA is not essential for productive synapsis. In summary, our experiments have demonstrated that because of its strict dependence on an assistant sequence-independent DNA bending protein, the β recombination is an excellent tool to study the function of architectural chromosomal proteins in the formation of a specific nucleoprotein structure, both in vitro and in vivo.

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