Differential Chromatin Association and Nucleosome Binding of the Maize HMGA, HMGB, and SSRP1 Proteins†

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ABSTRACT: In plants, chromosomal high mobility group (HMG) proteins have been identified in the HMGA family, containing A/T-hook DNA binding motifs, and in the HMGB family, containing an HMG-box DNA binding domain, that are considered architectural factors in chromatin. We have characterized the association of the HMGA protein, five different HMGB proteins, and the structure-specific recognition protein 1 (SSRP1) with maize chromatin by extraction experiments using NaCl, ethidium bromide, spermine, and distamycin A. The difference in the release of the proteins from chromatin by these reagents indicates that they are differentially associated with chromatin. This was confirmed by treatment of chromatin with micrococcal nuclease, demonstrating that the HMGA, HMGB2/3, and SSRP1 proteins are enriched in the highly nuclease-sensitive fraction of chromatin, which is likely to be transcriptionally competent. As examined by electrophoretic mobility shift analyses, the HMGA protein and the proteins containing an HMG domain (HMGB proteins and SSRP1) bind specifically to purified maize mononucleosomes that contain a histone octamer and ∼165 bp of DNA. The mode of interaction with the nucleosomes differs for HMGA and HMGB proteins. In the case of the HMGB1 protein, the full-length protein is required for specific nucleosome binding, as the individual HMG-box DNA binding domain (which is sufficient for DNA interactions) interacts nonspecifically with the nucleosomes. Collectively, these findings indicate that HMGA, the various HMGB proteins, and SSPR1 are differentially associated with plant chromatin and may act as architectural factors in different nucleoprotein structures.

In the cell nucleus, the genomic DNA of eukaryotes is assembled with chromosomal proteins, primarily the histones, into chromatin. Besides its function to compact DNA, it has become apparent that the dynamic chromatin structure plays a critical role in the regulation of DNA-dependent processes such as gene transcription. Thus, chromatin structure can control the accessibility of transcription factors to their cognate DNA sites (*2*, *3*). A variety of sequence-specific and non-sequence-specific nonhistone proteins are associated with chromatin and modulate chromatin folding.

Among these proteins are the relatively abundant chromosomal high mobility group (HMG)[1] proteins that are considered architectural factors in chromatin (*4*−*6*). The subgroup of the HMGA proteins (previously termed HMGI/Y proteins)[2] contains so-called A/T-hook DNA binding motifs that bind to the minor groove of A/T stretches in double-stranded DNA (*7*). The HMGA proteins can facilitate the action of specific transcriptional regulators by assisting the formation of "enhanceosomes", which are multiprotein complexes that control gene transcription (*8*). The subgroup of the HMGB proteins (previously termed HMG1/2 proteins)[2] contains HMG-box domain(s) as DNA binding motifs and interacts non-sequence-specifically with linear DNA but binds to certain distorted DNA structures with high affinity (*6*, *7*). Mediated by the HMG-box domain, HMGB proteins can severely bend DNA, which is a critical feature for their function as architectural factors in chromatin (*4*, *6*, *9*). Moreover, they can functionally interact with certain transcription factors, and they are involved in the regulation of gene expression (*10*−*12*). The structure-specific recognition protein 1 (SSRP1) is another much larger protein containing an HMG-box domain which has been implicated in transcription and replication (*13*−*15*).

Higher plants have a variety of HMGB proteins (13−20 kDa) that contain a central HMG box, which is flanked by a basic N-terminal domain and an acidic C-terminal domain (*9*). Whereas other eukaryotes usually contain two or three HMGB proteins, five different HMGB proteins have been identified each from maize and *Arabidopsis* (*5*). The maize HMGB1, HMGB2/3, HMGB4, and HMGB5 proteins (previously termed HMGa, HMGc1/2, HMGd, and HMGe, respectively) have relatively conserved HMG-boxes but differ substantially outside their DNA binding domain (*9*). The HMG-box protein SSRP1 (72 kDa) has been recently characterized from maize. It contains a C-terminal HMG-box domain, which is N-terminally flanked by basic and acidic regions, and the N-terminus of the protein consists of a large domain of unknown function (*15*). Depending on the species, higher plants have one or two HMGA proteins (∼20

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[1] Abbreviations: HMG, high mobility group; SSRP1, structure-specific recognition protein 1; TCA, trichloroacetic acid; EtBr, ethidium bromide; MNase, micrococcal nuclease; AUT, acetic acid−urea−Triton.

[2] The nomenclature of the HMG proteins has been revised recently (ref *1* and http://www.informatics.jax.org/mgihome/nomen/genefamilies/hmgfamily.shtml).

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kDa), which contain four copies of the DNA binding A/T-hook motif. In contrast to their animal counterparts, the plant HMGA proteins display an N-terminal domain, which has sequence similarity to the globular domain of linker histones (*5*).

Several studies addressing the in vitro DNA binding properties of the plant HMGA (*16*, *17*), HMGB (*17*, *18*), and SSRP1 proteins (*15*) have been performed, but the exact biological function(s) of these proteins is (are) still unknown. To learn more about the interactions of these proteins in the nucleus, we have studied here the association of the HMG and SSRP1 proteins with maize chromatin and analyzed their in vitro interaction with nucleosomes, as currently only limited information is available on these aspects.

EXPERIMENTAL PROCEDURES

Release of Chromosomal Proteins from Isolated Maize Nuclei. Isolation of nuclei from typically 160 g of frozen immature maize kernels was performed essentially as described previously (*19*). The final nuclear pellet was resuspended in a total of 12 mL of resuspension buffer (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and split into six equal aliquots for extraction with either 2% (w/v) TCA or various concentrations of NaCl, ethidium bromide, spermine, and distamycin A. After addition of the extracting reagents, the samples were kept on ice for 15 min. Insoluble material was then removed by centrifugation (15000*g*, 15 min), and the proteins of the supernatant were precipitated with 25% (w/v) TCA, washed with acetone, dried, and resuspended in SDS loading buffer. Extracted proteins were identified by immunoblot analyses.

Protein Release by MNase Treatment of Chromatin. Nuclei were isolated as above and then resuspended in MNase buffer (10 mM Tris-HCl, pH 7.5, 300 mM sucrose, 0.1 mM $CaCl_2$, 0.1 mM PMSF) before the nuclei were centrifuged through a 7 mL cushion of 2 M sucrose (80000*g*, 60 min). The nuclear pellet was washed with MNase buffer and finally resuspended in the same buffer. Equal aliquots (~3.5 A_{260} units) were treated for various periods with 2 units of MNase (Sigma) at 37 °C. The reaction was stopped by chilling on ice, insoluble material was removed by centrifugation (15000*g*, 10 min), and the proteins of the supernatant were precipitated with 25% (w/v) TCA, washed with acetone, dried, and resuspended in SDS loading buffer. Released proteins were identified by immunoblot analyses.

Production of the HMGA Antiserum and Immunoblot Analysis. Purified recombinant maize HMGA protein was used to raise an antiserum in Balb/c mice, which was used for immunoblots as described previously (*20*), except that the ECL system (Amersham Pharmacia Biotech) was used for detection of antibody binding. The chemiluminescent exposed X-ray films were scanned and analyzed using ImageQuant software (Molecular Dynamics).

Isolation of Mononucleosomes. Chromatin was typically prepared from 50 g of 10-day-old frozen maize seedlings. Seedlings were homogenized with a pestle and mortar in liquid nitrogen, and the resulting homogenate was mixed in a blender (Kenwood) with 500 mL of nuclei isolation buffer. The suspension was centrifuged (15000*g*, 10 min), and the pellet was resuspended in buffer 2 [0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM mercaptoethanol, 0.1 mM PMSF] and centrifuged again. The pellet was washed with MNase buffer, centrifuged (38000*g*, 40 min), and then resuspended in MNase buffer. The chromatin (~9 A_{260} units) was digested with 200 units of MNase for 15 min at 37 °C. The reaction was stopped by addition of EDTA to a final concentration of 2 mM and chilling on ice before the samples were centrifuged (15000*g*, 15 min). The supernatant was subjected to preparative gel electrophoresis in 3.5% polyacrylamide/0.5% agarose gels in nucleoprotein electrophoresis buffer (6.4 mM Tris base, 3.2 mM sodium acetate, 0.32 mM EDTA, pH 8.5) at 4 °C (*21*). The band containing the mononucleosomes was electroeluted into 0.5 × TBE buffer supplemented with 0.5 mM PMSF. Purified nucleosomal DNA was checked by PAGE in 0.5 × TBE buffer, while nucleosomal proteins were examined by SDS−PAGE, AUT−PAGE [18% polyacrylamide, 7 M urea, 5% (v/v) acetic acid, 0.5% (v/v) Triton X-100; run in 5% (v/v) acetic acid], and immunoblot analyses.

Expression and Purification of Recombinant Proteins. The coding region of the maize HMGA protein was amplified by PCR using the primers 5′-AAGGATCCATGGCCAC-CGACGAAGCCACCA and 5′-AATTAAGCTTAAGC-CGCGGCCGTCTCGCT and the cDNA clone (*22*) as a template. The obtained PCR fragment was digested with *Bam*HI and *Hin*dIII and cloned into the expression vector pQE9cm (*23*) giving plasmid pQE9cm-HMGA which was checked by DNA sequencing. The protein was expressed in *Escherichia coli* and purified by Ni−NTA−agarose chromatography and Resource S FPLC as described previously for the HMGc protein (*23*). Full-length HMGB proteins and the truncated HMGB1 proteins were expressed in *E. coli* and purified by three-step column chromatography as described previously (*23*, *24*). SSRP1(K532-D639) was expressed in *E. coli* and purified by three-step column chromatography as described previously (*15*). All the recombinant proteins used in this study contain an N-terminal 6×His tag to facilitate purification.

Protein Binding to Nucleosomes. Purified recombinant HMG and SSRP1 proteins were incubated at different protein:nucleosome ratios for 10 min in a total of 5 μL of a buffer containing 10 mM sodium phosphate, pH 7.0, 1 mM DTT, 1 mM EDTA, and 0.5 mM PMSF at room temperature. Complex formation was analyzed by electrophoresis in 0.8% agarose gels in 0.5 × TBE at 4 °C. The gels were stained with SYBR Gold (Molecular Probes) and analyzed using a Typhoon 8600 phosphorimager (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Differential Release of the HMG and SSRP1 Proteins from Chromatin by NaCl, Ethidium Bromide, Spermine, and Distamycin A. To study the association of the maize HMGA, HMGB, and SSRP1 proteins with chromatin, we analyzed the extractability of the chromosomal proteins from maize nuclei by NaCl, ethidium bromide, spermine, and distamycin A. Association of the proteins with chromatin of different accessibility was analyzed by measuring the release of the HMG and SSRP1 proteins upon limited nuclease treatment of the chromatin. Furthermore, we have examined the binding of the chromosomal proteins to purified maize mononucleosomes.

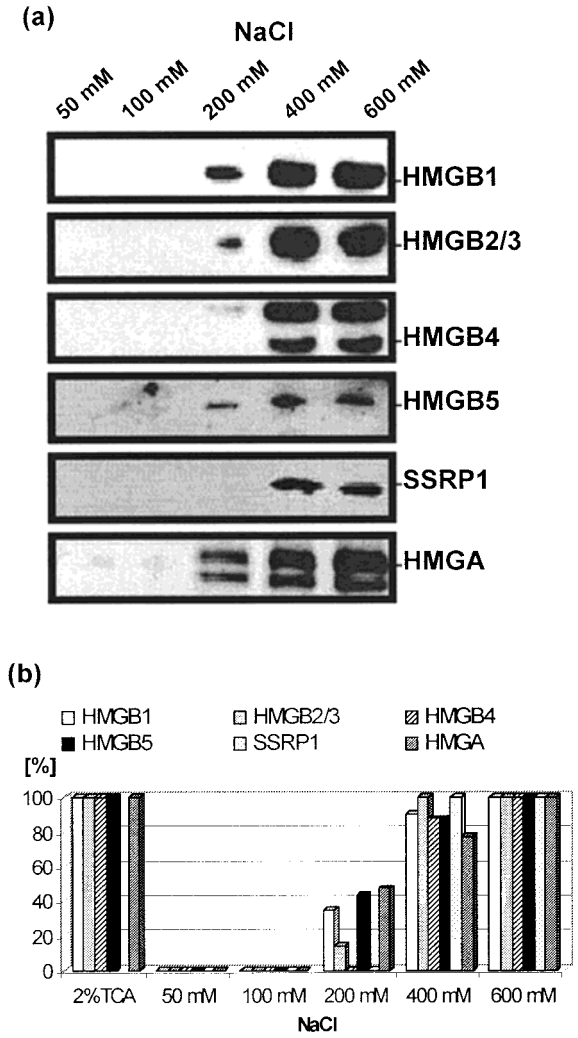

FIGURE 1: Release of HMG and SSRP1 proteins from chromatin by NaCl. (a) Immunoblot analysis of NaCl-extracted proteins from nuclei. Nuclei were extracted with the indicated concentrations of NaCl, and the released proteins separated by SDS−PAGE were probed with antisera raised against the recombinant HMG and SSRP1 proteins. (b) Quantification of NaCl-released proteins by immunoblot analyses. The bars (as indicated for the individual proteins) represent the mean value from two independent experiments of the protein released by a certain NaCl concentration (given in percent of total). The total amount of protein was determined by 2% TCA extraction for the HMG proteins or 600 mM NaCl extraction for SSRP1 and was considered 100%.

Initially, we tested the association of the proteins with chromatin by extracting the HMG and SSRP1 proteins from nuclei with NaCl. Nuclei prepared from immature maize kernels were extracted with increasing concentrations of NaCl. The released proteins were examined by SDS−PAGE and immunoblot analyses (Figure 1a) using antisera raised against recombinant HMGB1, HMGB3, HMGB4, and HMGB5 (*18*, *20*), SSRP1 (*15*), and HMGA (this work). The HMGB1 and HMGB4 antisera displayed some cross-reactivity, and the HMGB3 antiserum recognized the HMGB2 protein equally well. Due to their comigration in SDS−PAGE (*23*), we could not discriminate between HMGB2 and HMGB3 (~90% amino acid sequence identity) in the extraction experiments. The HMGA antiserum detected, in addition to the HMGA band, a second band of slightly higher electrophoretic mobility which could be a second HMGA protein or an alternatively modified form of HMGA (*25*). As a control, the nuclei were also extracted using 2% trichloroacetic acid (TCA), since HMG proteins can be isolated completely using 2% TCA. Quantification of the immunoblots (Figure 1b) revealed no striking differences in the extractability of the proteins by NaCl. At 50 and 100 mM NaCl there were no proteins released; release started at a concentration of 200 mM (except HMGB4), and all proteins were released (almost) completely using 400 mM NaCl. In comparison to the plant HMGB proteins, the vertebrate HMGB proteins seem to be bound less tightly to chromatin, as significant proportions of the proteins are released from chromatin and nucleosomes by ≤140 mM NaCl (*26*, *27*). Furthermore, chromatin fractionation and immunofluorescence demonstrated that vertebrate HMGB1 is not stably bound to chromatin (*28*).

We used a similar approach to measure the release of the chromosomal proteins from nuclei by ethidium bromide, spermine, and distamycin A. The HMG and SSRP1 proteins were released by ethidium bromide to very different extents (Figure 2a). While SSRP1 was extracted completely at the lowest EtBr concentration (1 mM), the HMGB2/3 and HMGB4 proteins were efficiently released only at higher concentrations. The HMGB5 and HMGA proteins were extracted to lower extents, and the HMGB1 protein was not released at all. Comparable to our results with HMGB2/3 and HMGB4, it was reported that two wheat HMG proteins were selectively extracted by EtBr, while other HMG proteins were not detected in the released fraction (*29*). An interesting observation is that, in comparison to the other tested HMG-box proteins (the HMGB proteins), the SSRP1 protein is extracted much more efficiently by EtBr. Despite having the same structural DNA binding motif, the chromatin association of these proteins differs substantially. By increasing the EtBr concentration in the extraction experiments, the efficiency for the release of a certain protein appears to have an optimal value (Figure 2a and data not shown). This suggests that the effect of EtBr on chromatin could be related to the relaxation of supercoiled DNA. Therefore, the various proteins could be adapted to recognize certain superhelical states of the chromosomal DNA, but other rather unclarified effects of EtBr on the chromatin structure (*30*) might contribute to the release of the proteins.

When the nuclei were extracted with increasing concentrations of spermine, again the chromosomal proteins were released differently (Figure 2b). Whereas HMGB1 and SSRP1 were detected in the released fraction starting with a spermine concentration of 5 mM, the HMGA protein was released to a significantly lower extent and the HMGB2/3, HMGB4, and HMGB5 proteins were not extracted at all. In line with our results, a protein similar to HMGB1 was released selectively by spermine from rice chromatin (*31*). Thus, the alteration of the chromatin structure by spermine (*32*) destabilizes primarily the association of HMGB1 and SSRP1 with chromatin.

By extraction of the nuclei with distamycin A, selectively the HMGA protein was released (Figure 2c). This could be explained by the fact that distamycin A binds DNA by inserting into the minor groove of A/T-rich sequences. Therefore, it competes for binding with proteins which have similar binding sites. Since plant HMGA proteins have a markedly higher affinity for A/T stretches in DNA than the

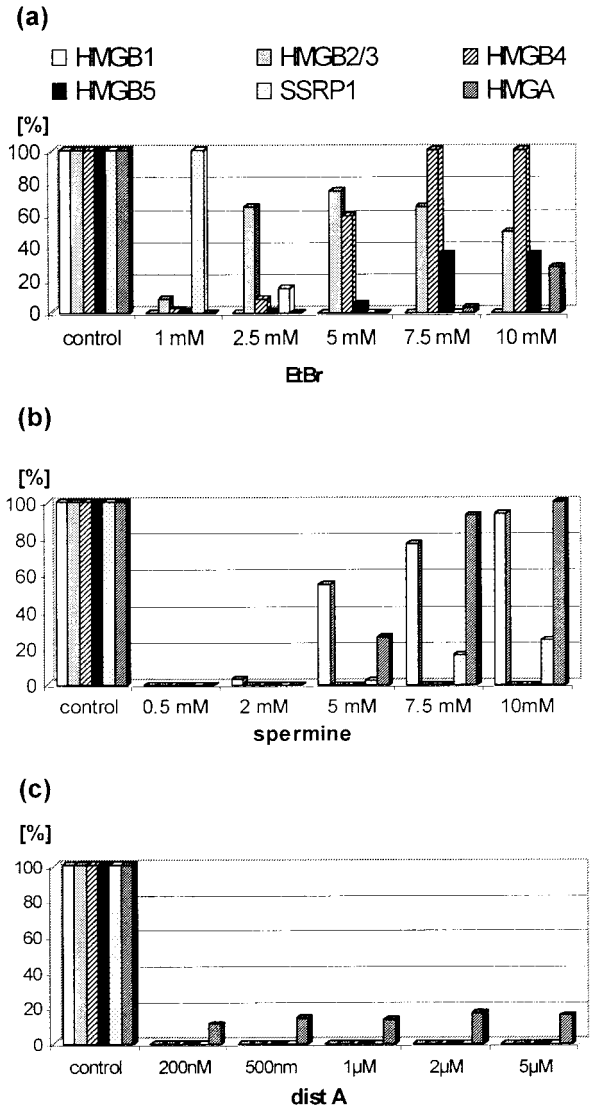

FIGURE 2: Release of HMG and SSRP1 proteins from nuclei by EtBr, spermine, and distamycin A. (a) Quantification of EtBr-released proteins by immunoblot analyses. The bars (indicated for the individual proteins) represent the mean value from two independent experiments of the protein released by a certain EtBr concentration (given in percent of total). (b) Quantification of spermine-released proteins by immunoblot analyses. The bars represent the mean value from two independent experiments of the protein released by a certain spermine concentration (given in percent of total). (c) Quantification of distamycin A-released proteins by immunoblot analyses. The bars represent the mean value from two independent experiments of the protein released by a certain distamycin A (dist A) concentration (given in percent of total). The total amount of protein in these experiments was determined by 2% TCA extraction for the HMG proteins or 600 mM NaCl extraction for SSRP1 and was considered 100%.

HMGB proteins in vitro (*17*, *33*), our finding indicates that the HMGA proteins interact with A/T-rich DNA also in the chromatin context. The fact that ~15% of the HMGA is selectively extracted by distamycin A from maize chromatin, whereas the pea HMGA protein is completely released from naked DNA by distamycin A (*17*), suggests that other factors such as protein/protein interactions might contribute to the chromatin association of HMGA.

Differential Release of the HMG and SSRP1 Proteins by MNase Treatment of Chromatin. Differential sensitivity of chromatin to nuclease digestion is a classical method used to discriminate between transcriptionally competent chromatin and bulk chromatin. The transcribed chromatin generally occurs in a less compacted form and is more readily digested by nucleases (*34*). To examine the association of the chromosomal proteins with transcriptionally active chromatin vs bulk chromatin, micrococcal nuclease (MNase) digestion experiments were performed. Nuclei were treated with MNase for various periods to obtain chromatin that was digested to different extents, as analyzed by agarose gel electrophoresis of the DNA purified from the individual digestions (Figure 3a). Special care was taken to get both chromatin that was only very mildly digested (most of the chromatin still undigested; 15 s, 30 s digest) and chromatin that was almost completely digested (6 min digest). The chromosomal proteins released by MNase treatment were analyzed as described above by immunoblot analyses (Figure 3b). Quantification of the immunoblots revealed that the chromosomal proteins were differentially released by MNase treatment (Figure 3c). Whereas the majority of the HMGB2/3, SSRP1, and HMGA proteins were detectable in the fraction of released proteins from a very limited chromatin digest (15 s), the release of HMGB1 required a much stronger digestion. The HMGB4 and HMGB5 were only significantly released by an almost complete MNase digest of the chromatin. This result indicates that the HMGB2/3, SSRP1, and HMGA proteins are enriched in the highly MNase-sensitive chromatin fraction, which is likely to be transcriptionally active, while the HMGB4 and HMGB5 proteins occur rather in bulk chromatin. Nevertheless, the HMGB4 and HMGB5 proteins could occupy certain sites in chromatin, as these two proteins are the least abundant of the five tested HMGB proteins of maize (*20*). An association of wheat and barley HMG proteins with nuclease-sensitive chromatin has been reported previously (*35*, *36*), but in these studies, apparently only a single nuclease digestion condition was analyzed, which makes a detailed interpretation of these results more difficult. In line with our results, the HMGB proteins of the dipteran insect *Chironomus*, termed cHMG1a and cHMG1b, are distinctly distributed in chromosomes (*37*). The cHMG1a protein is uniformly distributed in the chromosome, whereas the cHMG1b protein is specifically localized in chromosomal puffs of interphase giant chromosomes, indicating a role in transcription.

Binding of HMG and SSRP1 Proteins to Mononucleosomes. In the second part of our study, the interaction of the chromosomal proteins with nucleosomes was examined. Mononucleosomes were produced by MNase treatment of chromatin isolated from maize seedlings and purified by preparative polyacrylamide/agarose gel electrophoresis (*21*). The obtained nucleosomes were first checked by native agarose gel electrophoresis, demonstrating that the mononucleosomes are essentially pure (Figure 4a). From an analysis of the purified nucleosomal DNA by polyacrylamide gel electrophoresis it was evident that the nucleosome particles contained DNA of 160−170 bp (Figure 4b), which means that the nucleosomes contain, in addition to the nucleosomal core DNA, internucleosomal linker DNA. Examination of the nucleosomal proteins by SDS−PAGE (Figure 4c) and acetic acid−urea−Triton (AUT) PAGE (Figure 4d) revealed that the nucleosomes contain, as expected, the core histones H2A/B, H3, and H4, while no linker histones could be detected. The observed maize core

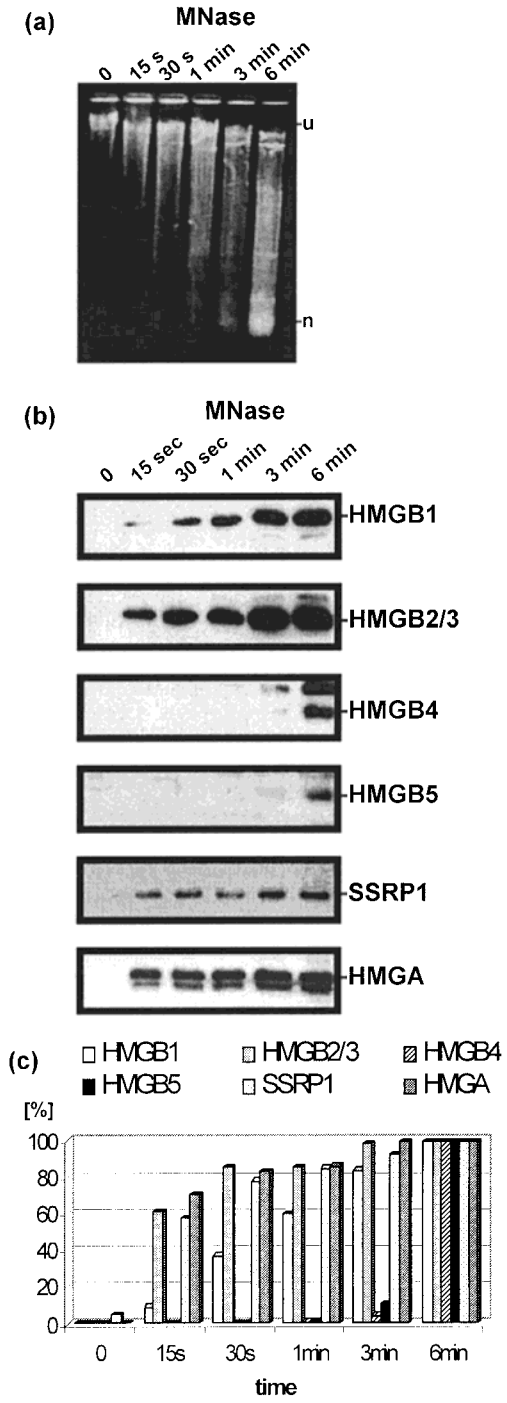

FIGURE 3: Release of HMG and SSRP1 proteins from chromatin by MNase treatment. (a) Electrophoretic analysis of the DNA purified from MNase-treated chromatin. Maize chromatin was treated for the indicated periods with MNase. The DNA of the different samples was purified and analyzed by 1% agarose gel electrophoresis and EtBr staining. The electrophoretic migration positions of the undigested chromosomal DNA (u) and of the chromosomal DNA corresponding to the size of mononucleosomes (n) are indicated. (b) Immunoblot analysis of the proteins released by MNase treatment. Chromatin was digested for the indicated periods with MNase, and the released proteins were probed with antisera against the HMG and SSRP1 proteins. (c) Quantification of the proteins released by MNase treatment using immunoblot analyses. The bars (as indicated for the individual proteins) represent the mean value from two independent experiments of the protein released by a certain MNase treatment (given in percent of total). The amount of protein released after 6 min of MNase treatment (almost complete digestion of the chromatin) was considered 100%.

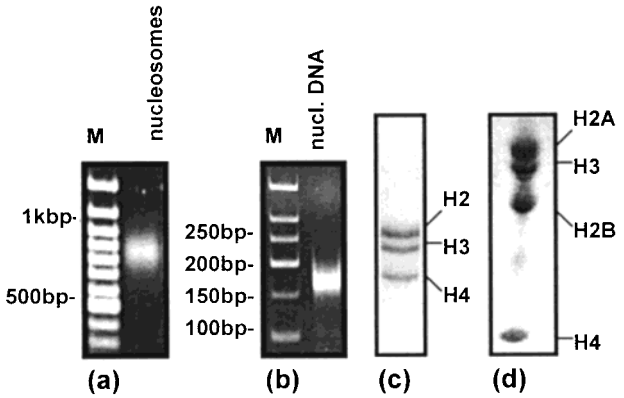

FIGURE 4: Characterization of isolated maize mononucleosomes. (a) Electrophoresis of the nucleosomes in a 0.8% agarose gel along a DNA size marker stained with ethidum bromide. (b) Electrophoresis of the DNA purified from the nucleosomes in a 5% polyacrylamide gel along a DNA size marker stained with ethidium bromide. (c) SDS−PAGE analysis in a 18% polyacrylamide gel and Coomassie staining of the proteins isolated from the nucleosomes. (d) AUT−PAGE analysis in a 18% polyacrylamide gel and Coomassie staining of the proteins isolated from the nucleosomes. The electrophoretic migration positions of the maize core histones are indicated.

histone pattern matches that published by other researchers (*38*, *39*). The nucleosomes were also checked for their HMG protein content by immunoblots using various HMG antisera (data not shown), demonstrating that, in addition to the linker histones, the HMG proteins have been removed during purification of the nucleosomes.

To test the interaction of the HMGA, HMGB, and SSRP1-(K532−D639) proteins with the purified mononucleosomes, increasing amounts of the purified recombinant chromosomal proteins were incubated with the nucleosomes, and the formation of complexes was examined by electrophoretic mobility shift analysis using native agarose gel electrophoresis (*40*). The analyzed chromosomal proteins interacted differently with the nucleosomes. They displayed various affinities for the nucleosomes and formed complexes that migrated differently in the gel (Figure 5). The HMGB5, SSRP1, and HMGA proteins showed the highest affinity for the nucleosomes, as complex formation can be observed even at the lowest protein input ratio (0.5:1 protein:nucleosome molar ratio), while the affinity of the HMGB1, HMGB2/3, and HMGB4 proteins was somewhat lower. The binding of the chromosomal HMG-box proteins (HMGB proteins, SSRP1) to the nucleosome leads only to a minor retardation of the complex (compared to the migration of unbound nucleosomes) during gel electrophoresis. HMGA forms at low input ratios also a specific complex that has only a slightly reduced mobility, whereas at higher input ratios (3:1, 4:1) the complexes are more strongly retarded, which is clearly different from the HMGB proteins. It has been shown that up to four molecules of mammalian HMGA proteins can bind to nucleosome core particles (*41*). In another study (*42*), it has been reported that wheat HMG proteins interact with trimmed mononucleosomes (core histones and ~146 bp of DNA). The wheat HMGA protein (termed HMGa) bound to the nucleosome core particles specifically, while the HMGB proteins formed at increasing protein input rather nonspecific complexes with a gradually reduced electrophoretic mobility in polyacrylamide gels. In

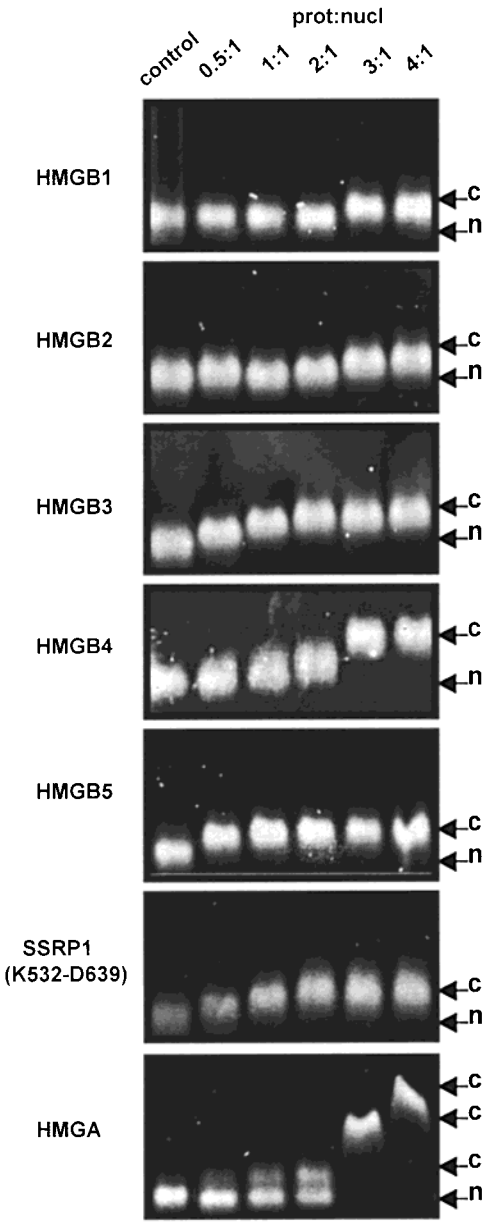

FIGURE 5: Binding of the HMG and SSRP1 proteins to mononucleosomes. The truncated derivative of SSRP1, residues K532−D639 (containing the HMG domain and the basic domain involved in DNA binding), was used in our experiments, since full-length SSRP1 has a strong tendency to aggregate in gel shift experiments (*15*). The recombinant HMG and SSRP1 proteins were incubated at the indicated molar protein:nucleosome ratios with nucleosomes. Formation of complexes was examined by electrophoresis of the samples in 0.8% agarose gels. The gels were stained with the SYBR Gold reagent and analyzed using a phosphorimager. The migration positions of the unbound nucleosome (n) and the complex(es) (c) formed with the added protein are indicated.

our experiments, the HMGB proteins form specific complexes with the nucleosomes (containing also the linker DNA), which indicates that the linker DNA is a critical feature for the nucleosome binding of plant HMGB proteins, whereas the linker DNA seems to be dispensable for specific nucleosome interactions of HMGA (*42*). The requirement of the linker DNA for nucleosome binding of HMGB proteins was also reported for vertebrate HMGB proteins which contain in contrast to plant HMGB proteins two HMG-box domains (*43*, *44*). Studies using reconstituted nucleosomes which contain a specific DNA sequence revealed that chicken and *Xenopus* HMGB proteins bind the linker DNA immediately flanking the nucleosome core (*40*, *45*).

To identify the protein domain(s) that is (are) critical for the binding of the HMGB proteins to mononucleosomes, we used truncated recombinant proteins of the maize HMGB1 protein (*24*) for further electrophoretic mobility shift experiments. In comparison to the full-length protein, HMGB1-(M1−E157), HMGB1(G35−E157) lacking the N-terminal basic domain, HMGB1(M1−K123) lacking the acidic C-terminal domain, and HMGB1(G35−Y109) representing the individual HMG-box domain were used in this experiment. Incubation of increasing amounts of these four HMGB1 protein derivatives with nucleosomes followed by gel shift analysis (Figure 6a) demonstrated that HMGB1(G35−E157), which also has a reduced DNA binding activity (*24*), does not bind to the nucleosome at all. HMGB1(M1−K123) interacts with the nucleosome with an affinity similar to that of the full-length protein, while the individual HMG-box domain forms a complex with the nucleosome even at a lower protein input ratio (starting from a 1:1 input ratio). To further examine the specificity of the complexes formed with the nucleosomes, we repeated this experiment at higher protein input ratios (Figure 6b). At input ratios of 10:1 and 25:1, there is still no nucleosome binding of HMGB1(G35−E157), while the full-length protein forms the same specific complex as observed at lower input ratios. Therefore, the nucleosome seems to have a limited number of specific binding site(s) for plant HMGB proteins, due to the nucleosome structure most likely one or two sites. Accordingly, the binding of one or two molecules of vertebrate HMGB proteins to nucleosomes has been reported (*40*, *44*, *45*). HMGB1(M1−K123) and HMGB1(G35−Y109) interact with the nucleosome nonspecifically, as they form complexes that have gradually reduced electrophoretic mobility at increasing protein input. Interestingly, the full-length HMGB1 protein is required to bind nucleosomes specifically. Thus, both the basic N-terminal domain and the acidic C-terminal domain appear to be involved in specific nucleosome interactions of HMGB1, whereas structure-specific DNA binding (and other DNA interactions) reside within the HMG-box domain (*24*).

Possible Function of the HMG and SSRP1 Proteins in Plant Chromatin. The HMGA, HMGB, and SSRP1 proteins are implicated in the regulation of transcription but may also be involved in other processes such as recombination and replication (*4*, *6*, *46*). A number of plant HMG and SSRP1 proteins have been identified which display structural similarities as well as dissimilarities to their animal counterparts (*5*). As shown here using chromatin extraction with distamycin A, HMGA appears to be the only protein among the tested proteins which is bound to A/T-rich DNA in the chromatin context, reflecting its in vitro DNA interactions (*16*, *17*, *33*), although, in addition to DNA binding, other interactions (e.g., with other proteins) seem to be involved in the chromatin association. This might also be true for the various maize HMGB proteins which bind similarly to naked DNA (*18*) but are differentially released from chromatin by the reagents used in this study and bind nucleosomes with different affinities. Therefore, protein/protein interactions with histones and other chromosomal proteins are likely to be important determinants of the chromatin association of

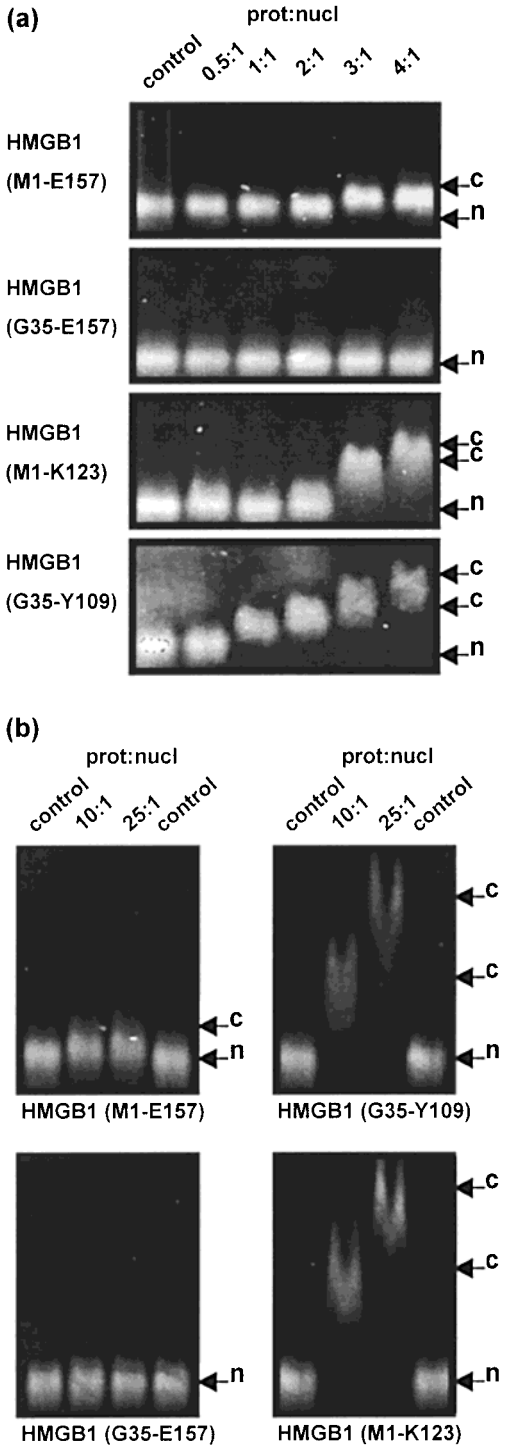

FIGURE 6: Binding of full-length and truncated derivatives of the HMGB1 protein to mononucleosomes. Nucleosome binding was analyzed (a) at low and (b) at high molar protein:nucleosome input ratios. The indicated derivatives of the HMGB1 protein were incubated with the nucleosomes. Formation of complexes was examined by electrophoresis of the samples in 0.8% agarose gels. The gels were stained with the SYBR Gold reagent and analyzed using a phosphorimager. The migration positions of the unbound nucleosome (n) and the complex(es) (c) formed with the added protein are indicated.

the HMGA and HMGB proteins. According to the selective release of the proteins by limited MNase treatment of the chromatin, the HMGA, HMGB2/3, and SSRP1 are associated with the highly nuclease-sensitive fraction of chromatin, which is commonly considered transcriptionally competent. An involvement of the plant HMGA and HMGB proteins in transcription is also implied by their stimulatory effect on the DNA binding of certain transcription factors (*47*−*49*). Furthermore, mammalian HMGA and HMGB proteins contribute to the regulation of transcriptional activation (*4*). In yeast, the HMGB proteins play an important regulatory role, repressing as well as potentiating the expression of various genes (*11*, *12*, *50*). Mammalian SSRP1 has also been reported to act in the initiation and elongation of transcription (*14*, *51*).

The HMGA and HMGB proteins are considered versatile chromatin-associated proteins which function as architectural factors facilitating the assembly of specific nucleoprotein structures (*4*−*6*). Accordingly, primarily the HMGB proteins have been implicated to act, in addition to transcription, in other DNA-dependent processes such as recombination, replication, and DNA repair. In contrast to other eukaryotes, higher plants have a variety of HMGB proteins which differ in their chromatin association and nucleosome binding (this work), in their expression in the plant (*20*), and in their potential to facilitate the formation of specific nucleoprotein structures in vitro (*18*). They have the ability to generate, depending on the biological context, a variety of nucleoprotein complexes. Because of their non-sequence-specific DNA binding, the HMGA and HMGB proteins are targeted to their site of action by interactions with sequence-specific regulators such as transcription factors and recombination proteins (*4*, *6*, *9*). Therefore, depending on the regulatory proteins with which they interact, the various plant HMGA and HMGB proteins may be adapted to act in different nucleoprotein structures in vivo, enhancing the stability and specificity of these complexes.

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