Microheterogeneous Cytosolic High-Mobility Group Proteins from Broccoli Co-Purify with and Are Phosphorylated by Casein Kinase II

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A group of low molecular weight protein substrates was found to co-purify with casein kinase II from broccoli (Brassica oleracea var italica). These substrates showed very high affinity toward casein kinase II and were efficiently phosphorylated even in the presence of an excess of exogenous substrates. The broccoli substrates were purified from cytosolic extracts as a double band of related proteins migrating at 18.7 and 20 kD. Further microheterogeneity was revealed by anion-exchange high-performance liquid chromatography and mass spectroscopy. The actual molecular masses of the three major components identified by mass spectroscopy were determined to be 12,691, 13,256, and 14,128 D. The substrates showed characteristic amino acid composition with a high content of polar amino acids, including about 20% each of acidic and basic amino acids. They were soluble in 2% trichloroacetic acid. The substrates cross-reacted with an antibody against wheat high-mobility group protein d (HMGd) but not HMGa. The isolated broccoli HMGs showed general DNA-binding activity without preference for AT-rich DNA. The presence of these HMG proteins in the cytoscopic fraction is similar to the distribution characteristics of the animal HMG-1 subgroup. On the basis of amino acid composition and DNA-binding specificity, the isolated broccoli HMGs resemble other plant HMGs homologous to the HMG-1 subgroup.

HMG proteins are abundant nonhistone components of eukaryotic chromatin sharing several biochemical characteristics. They are small (<30 kD), acid-soluble, DNA-binding proteins with both strongly acidic and basic domains. On the basis of several distinguishing properties, HMGs were classified in three major subgroups: HMG-1/2, HMG-14/17, and HMG-1/Y (reviewed in Bustin et al., 1992). The precise biological function of these proteins is not yet clear, but it is a widely accepted conjecture that by affecting the conformation and function of chromatin they could play a role in DNA replication and/or RNA transcription. Plant HMG proteins have been studied in several species. The most extensive biochemical work in wheat identified four major protein species named HMGa, HMGb, HMGc, and HMGd (Spiker, 1984). Although the general properties of these proteins clearly resemble those of their counterparts from other eukaryotes, plant HMGs also show unique features. Accordingly, no precise correspondence to the animal HMG subgroups has been established on the basis of biochemical properties alone.

Recent cloning of plant HMG genes has helped to clarify their relationships to the animal subgroups and has confirmed the uniqueness of plant HMGs. The cDNA sequence of a maize HMG protein was shown to contain the HMG box, a DNA-binding domain characteristic of the HMG-1 subgroup (Grasser and Feix, 1991). However, only one such element is present in the maize protein, whereas animal HMG-1's contain two boxes and are consequently larger. A cDNA clone homologous to the HMG-1 subgroup isolated from soybean (Laux et al., 1991) shows an AT-hook motif, which is characteristic of the HMG-1 class, but lacks a C-terminal acidic domain and instead contains an N-terminal histone-like domain.

In this laboratory we are interested in plant DNA-binding proteins that participate in control of gene expression and their possible regulation by phosphorylation. One of our candidates for the role of a nuclear regulator is CKII, a ubiquitous multifunctional protein kinase that is present in the nucleus and phosphorylates many nuclear DNA-binding proteins (Tuazon and Traugh, 1991), including plant transcription factor GBFI (Klimczak et al., 1992).

During our studies on purification of CKII from broccoli (Brassica oleracea), we observed that a group of endogenous protein substrates of 18 to 20 kD co-purified with the kinase activity and were very efficiently phosphorylated. In this paper we provide evidence that these substrates are HMG proteins. They show the characteristic biochemical properties of the HMG group and cross-react with antibodies against wheat HMGd. A unique property of these broccoli HMGs is that they are abundant in the cytosolic fraction and do not require salt extraction for solubilization.

MATERIALS AND METHODS

Plant Material and Chemicals

Broccoli (Brassica oleracea var italica) was purchased from local wholesale distributors. [γ-32P]ATP (specific activity 3000 Ci/mmol) was obtained from ICN Radiochemicals (Costa Mesa, CA). Special chemicals used in this work were purchased from the following suppliers: ammonium acetate (Acros), diethylaminomethyl cyanide (DEAM; Aldrich), diethylaminoethyl diethylammonium dihydrogen ortho-phosphate (DEAM-P, ICN Radiochemicals), N-ethylmaleimide (Worthington), N-ethyl-N′-methylmaleimide (ICN), [γ-32P]ATP (specific activity 3000 Ci/mmol) (ICN), Nα-phospho-ethanolamine (ICN), and 3′,5′-cyclic guanosine monophosphate (ICN).

Abbreviations: CKI and CKII, casein kinase I and II; DEAM, diethylaminomethyl; HMG, high-mobility group; MALDI/MS, matrix-assisted laser desorption ionization mass spectrometry.
Precipitation (van der Hoeven, 1981). PEG 8000 was dissolved in 4% ammonium sulfate was added to 0.1 mM KCl in a buffer containing 20 mM K-phosphate, pH 7.0. The solution was clarified by centrifugation at 9000g for 10 min.

For cytosolic extracts, lysis was performed in 3 L (per 1 kg of broccoli) of 0.25 M Suc in buffer A and nuclei were removed by centrifugation at 700g for 10 min.

To prepare total extracts, 4 kg of broccoli heads were homogenized as 1-kg portions in 2 L of 0.4 M KCl in a buffer containing 20 mM K-phosphate, pH 7.0, 5 mM NaF, 5 mM EDTA, 50 μg/mL PMSF (buffer A). For cytosolic extracts, lysis was performed in 3 L (per 1 kg of broccoli) of 0.25 M Suc in buffer A and nuclei were removed by centrifugation at 700g for 10 min. The pellets obtained in the 20% PEG 8000 cut from total and cytosolic extracts were further clarified by PEG precipitation (van der Hoeven, 1981). PEG 8000 was dissolved in the extracts at the concentration of 4% (ammonium sulfate was added to 0.1 mM to the cytosolic extract). After stirring for 30 min, crude membranes and other amorphous material were removed by centrifugation at 9000g for 10 min to obtain the clarified extract. PEG 8000 was added to the supernatant to the final concentration of 20%, and, after stirring for 30 min, the suspension was centrifuged at 6000g for 10 min.

Enrichment of CKII and Separation from CKI

The pellets obtained in the 20% PEG 8000 cut from total and cytosolic extracts were redissolved in buffer B (50 mM Tris-HCl, pH 7.0, 5 mM NaF, 5 mM EDTA) with 200 mM KCl and freshly added PMSF (50 μg/mL final concentration) and 10 μM leupeptin (400 mL of buffer/1 kg plant material). The solution was clarified by centrifugation at 9000g for 15 min. The supernatant fraction was subjected to batch adsorption by stirring for 1 h with 100 mL of phosphocellulose suspension in buffer B. The phosphocellulose was allowed to settle, decanted, and washed two times with 400 mL of buffer B with 100 mM KCl. The batch was poured into a column (2.8 × 16 cm) and washed with 10 volumes of buffer B with 100 mM KCl. The column was eluted with 8 volumes of a linear gradient of 150 to 1000 mM KCl in buffer B with 5 mM 2-mercaptoethanol. The fractions containing casein kinase activity were pooled, supplemented with MgCl₂ to 10 mM, and loaded onto a column of hydroxylapatite (1.6 × 5 cm) equilibrated with 500 mM KCl in buffer C (50 mM Tris-HCl, pH 7.0, 5 mM 2-mercaptoethanol). The columns were washed with 10 volumes of 500 mM KCl in buffer C and 2 volumes of buffer C alone. They were eluted with a gradient of 0 to 400 mM K-phosphate, pH 7.0, in buffer C. For gel filtration, the active fractions were pooled and concentrated 5- to 10-fold using Centriprep 30 microconcentrators from Amicon. Phenyl-Sepharose chromatography was performed on a 2-mL (0.8 × 3 cm) column equilibrated with 20% saturated ammonium sulfate in buffer C and loaded with hydroxyapatite pools supplemented with ammonium sulfate to 20% saturation. The columns were washed with the equilibration buffer and eluted with 8 column volumes of a decreasing gradient of 20 to 0% ammonium sulfate and an increasing gradient of 0 to 60% ethylene glycol in buffer C, followed by 3 volumes of 60% ethylene glycol in buffer C.

HPLC

HPLC separations were performed on a J.T. Baker 4.6 × 250 mm WP DEAM column using a Spectra-Physics SP8700XR liquid control system and a Spectra-Physics SP8440 UV/VIS detector kindly provided by Dr. Andrew Binns (University of Pennsylvania, Philadelphia). A linear gradient of 50 to 500 mM NaCl in 50 mM Tris-HCl, pH 7.0, was used at the flow rate of 1 mL/min for 45 min, followed by isocratic elution at 500 mM NaCl for 30 min.

Amino Acid Analysis

Protein samples were hydrolyzed for 1 h at 160°C in 6 M HCl and the hydrolyzates were derivatized with phenylisothiocyanate and separated by C₁₈ reverse-phase HPLC as described by Ebert (1986). This work was also performed by the Protein Microchemistry Core Facility of the Wistar Institute.

Electrophoretic Mobility Shift Assay

DNA protein binding was performed in 20 μL of a solution containing 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 1 mM EDTA, 25 μg/mL poly(dI:dC) (or other competitor as indicated), 0.3 mg/mL BSA, and 8 fmol (10,000 cpm) of radioactively labeled oligonucleotide probe. The probe was a dimer of the AT-rich −566 to −533 fragment of pea rbcS-3.6 promoter (the sequence of this fragment was published by Datta and Cashmore [1989] in figure 2 therein). The protocols used for cloning, preparation, and labeling of the probe were described by Schindler and Cashmore (1990). After 30 min of incubation at room temperature, the samples were separated in a 4% polyacrylamide gel in 25 mM Tris, 190 mM Gly, pH 8.3. The gels were dried and autoradiographed.
Other Methods

Protein kinase assay was performed as previously described by Klimczak and Cashmore (1993) using 1 mg/mL partially dephosphorylated casein or other substrates as indicated. For analysis of phosphorylated products, proteins were precipitated by 25% TCA. Protein electrophoresis, immunoblotting, and gel filtration through Sephacryl S-200 were performed as described previously (Klimczak and Cashmore, 1993). Protein gels were stained for 15 min with Coomassie brilliant blue R-250 (0.005% dye in 10% acetic acid, 10% isopropanol) and destained overnight and for two 1.5-h changes of 10% acetic acid, 5% methanol.

RESULTS

Partial Purification of CKII from Total Extracts

Our initial purification procedure for CKII used total extracts of broccoli to obtain all the enzyme activity regardless of its cytosolic/nuclear distribution. Total extracts were prepared in a hypotonic buffer containing 0.4 M KCl to accomplish simultaneous lysis of nuclei and extraction of chromatin. The crude extract contained large amounts of amorphous pigmented protein material that interfered with subsequent chromatography. This material was removed together with lysed organelles and membranes by PEG 8000 precipitation at 4% (van der Hoeven, 1981). Protein pellets collected in the 4 to 20% PEG cut contained over 95% of casein kinase activity.

The PEG pellet was processed to enrich CKII and to remove major contaminating protein kinases, in particular CKI. Since high affinity for phosphocellulose is one of the defining properties of CKII, phosphocellulose chromatography was used to obtain a highly enriched preparation from which contaminating CKI was subsequently removed by chromatography on hydroxylapatite. The hydroxylapatite step was equivalent to separation of CKII and CKI on DEAE-cellulose by using the basic character (isoelectric point > 9.0) of CKI. Separation of basic proteins on hydroxylapatite offered the advantage of not requiring prior dialysis: acidic and neutral proteins (such as CKII) can bind to hydroxylapatite in the presence of moderate concentrations of monovalent salts (e.g. 0.5 M KCl present in the phosphocellulose eluate), whereas basic proteins (such as CKI) are recovered in the flow-through (Gorbunoff, 1985). CKII activity was then eluted from hydroxylapatite with a gradient of K-phosphate.

Endogenous Substrates in CKII Preparations

CKII peak fractions from hydroxylapatite were subjected to gel filtration on a Sephacryl S-200 column. Two peaks of protein kinase activity were observed in the eluate using a routine filter paper assay (Fig. 1A). The molecular sizes of the peaks measured in Stokes radius were 46 and 28 Å, which was equivalent to 150 and 48 kD native molecular mass, assuming a globular shape of the proteins. These peaks appeared to correspond to the oligomeric and monomeric forms of CKII-like activities from maize seedlings (Dobrowolska et al., 1987) named CKIIA and CKIIB, respectively.

When phosphorylated protein products from these activity peaks were analyzed on SDS-PAGE, significant levels of phosphorylation of endogenous substrates were observed (Fig. 1B), despite about a 10- to 20-fold excess of exogenous casein (at 0.5 mg/mL). In particular, the 28-Å peak of protein kinase activity represented predominant phosphorylation of an endogenous protein doublet of about 18 kD, with almost complete exclusion of casein phosphorylation. This doublet was at this stage the major protein component of the preparation and constituted about 90% of protein (data not shown, identical to Fig. 2, lane 3).

Purification of the 18- to 20-kD Substrates from the Cytosolic Fraction

We compared the distribution of CKII activity in total broccoli extracts to extracts fractionated into cytosolic and nuclear components. Since we found that cytosolic extracts contained most of the monomeric but little of the oligomeric CKII activity (Fig. 1A; L.J. Klimczak, unpublished data), the cytosolic fraction was used in purification protocols focused on the monomeric activity.

The cytosolic extract was prepared with no salt added, nuclei were removed by low-speed centrifugation (700g), and a PEG 8000 precipitation step was performed at low salt (0.1 M ammonium sulfate), followed by medium-speed centrifugation (10,000g). Since nuclei and other particulate com-
protein and other minor contaminants constituted only a minor fraction of protein in the hydroxylapatite pool.

The separated substrates became efficiently phosphorylated again when they were recombined with the phenyl-Sepharose-adsorbed monomeric CKII fraction, as well as the oligomeric CKII form isolated from nuclei or total extracts (Fig. 2B). These two activities phosphorylated the substrates with equal efficiency. Both the oligomeric and monomeric protein kinase fractions isolated from the cytosolic and total extracts showed the essential features of CKII activity: utilization of GTP as a phosphate donor, inhibition by low concentrations of heparin, and preferential phosphorylation of acidic protein substrates. The majority of their quantitative characteristics were identical to each other and to those described previously for partially purified CKII activity from isolated broccoli nuclei (Klimczak et al., 1992; see table II therein). The most notable difference was the level of stimulation of casein phosphorylation by polylysine, which was 10- to 20-fold for the oligomeric and 3- to 5-fold for the monomeric activity. (Additional properties of these forms will be presented in detail elsewhere [L.J. Klimczak, unpublished data]).

**Heterogeneity of the Endogenous 18- to 20-kD CKII Substrates**

The purified phenyl-Sepharose flow-through fraction contained a predominant protein doublet migrating on SDS-PAGE at 18.7 and 20 kD, as well as additional minor bands in this molecular mass range. It appeared that the two major bands were related, since no separation was accomplished on several other chromatographic columns (Reactive Blue-agarose, BioRex 70, DEAE-cellulose; data not shown). To further evaluate the chromatographic behavior of these proteins at higher resolution but still under native conditions, the fraction was separated by anion-exchange (DEAM) HPLC. A complex pattern of elution was observed: the proteins eluted as several peaks in about 40 fractions, with two major overlapping peaks in the center (Fig. 3A). However, this procedure did not result in separation of the two major bands; both eluted throughout the entire elution spectrum (Fig. 3B).

It appears that the two bands represent two related populations of microheterogeneous proteins. This conclusion was further supported by protein microsequencing work on isolated Glu-C peptide fragments: a 5-kD fragment was common to both bands but yielded multiple protein sequences (data not shown).

**Substrates Co-Purifying with CKII Are HMG Proteins**

The very efficient phosphorylation of the substrates made it particularly interesting to determine their identity because only a few CKII substrates are known in plants. The proteins showed quite distinctive chromatographic behavior: lack of adsorption to phenyl-Sepharose at a high concentration of ammonium sulfate indicated highly hydrophilic character, and strong adsorption to both cation and anion exchangers indicated the presence of both basic and acidic domains. Amino acid analysis of the purified preparation confirmed a high content of Lys (19.8%), Glu/Gln (13.3%), and Asp/Asn components (such as chromatin released from broken nuclei) were removed in this two-step centrifugation/clarification procedure, it should result in a reduced level of contamination with nuclear proteins. After such cytosolic extract was purified for CKII activity, the amount of protein kinase activity in the 46-Å peak was indeed substantially reduced (Fig. 1A), but activity in the 28-Å peak was not affected. Again, the 18- to 20-kD substrates became co-enriched with CKII activity, as in total extracts, and represented over 90% of protein in the hydroxylapatite fraction (Fig. 2, lane 3). The two major bands were optimally resolved in 15% PAGE, so this concentration was used in further experiments.

Because of the predominant phosphorylation of these proteins, even in the presence of a large excess of exogenous substrates, we entertained the idea that the 28-Å peak may represent autophosphorylation of the 18- to 20-kD proteins. However, when the hydroxylapatite fraction was loaded onto phenyl-Sepharose in the presence of 20% saturated ammonium sulfate, the protein substrates were found in the flowthrough in the presence of only low amounts of protein kinase activity. The bulk of protein kinase activity was recovered from the column by elution with ethylene glycol (L.J. Klimczak, unpublished data). At this stage, the activity eluted with ethylene glycol showed characteristic properties of CKII, including efficient phosphorylation of casein (data not shown). This result explains that the predominant phosphorylation of these 18- to 20-kD substrates was due to their high affinity for the kinase and competitive inhibition of exogenous substrates, but not autophosphorylation. The phenyl-Sepharose step contributed to purification of the 18- to 20-kD substrates by removing protein kinase activity, but it did not result in any major changes in protein composition pattern (compare lanes 3 and 4, Fig. 2A).
Broccoli High-Mobility Group Proteins Co-Purifying with Casein Kinase II

Figure 3. Separation of purified substrates by anion-exchange HPLC and their binding to an AT-rich DNA probe. A, Elution profile of separation of phenyl-Sepharose flow-through pool detected as A280. B, Coomassie blue-stained polyacrylamide gel of fractions 87 to 116 pooled pairwise. C, Mobility shift assay with the same fractions, as described in “Materials and Methods.”

(15.9%), as well as Ser (8.9%) and Ala (15%). Such unusual amino acid composition is very characteristic of HMG proteins and corresponds well with the amino acid composition of various plant HMGs, both determined from isolated proteins and deduced from the cDNA sequences (Table I). Since acid solubility is a characteristic feature of HMG proteins, we incubated the broccoli preparation in 2% TCA and, following centrifugation, found all the proteins in the supernatant (data not shown). Consequently, the isolated CKII substrates are acid soluble.

To confirm the hypothesis that these proteins are indeed HMGs, we performed western blot analysis using antibodies raised against wheat HMGa and HMGd proteins. As shown in Figure 4, no cross-reaction was observed against the anti-HMGa antibody, but both major bands cross-reacted with antibodies raised against wheat HMGd fraction. Interestingly, this antibody also recognized two protein bands of very similar mobilities in the control preparation of wheat HMGs, the upper of them being HMGc. It appears that the broccoli proteins represent homologs of wheat HMGc and HMGd and that these two groups may be interrelated.

Molecular Masses of the Protein Substrates

We were interested in determining the molecular masses of the proteins more exactly to compare them with the known ranges of HMG subgroups and the predicted molecular masses of cloned plant HMGs. Because their unusual amino acid composition is known to cause aberrant migration in SDS-PAGE, we used MALDI/MS. The MALDI/MS spectrum of the highest peak eluting in DEAM HPLC (fractions 102-104 in Fig. 3A) revealed three major components of 12,691, 13,256, and 14,128 D (Fig. 5). This experiment revealed yet further heterogeneity and suggested that most likely the lower of the two bands that visibly separated in SDS-PAGE may be composed of two species.

DNA-Binding Properties of Isolated Broccoli HMGs

We investigated whether the broccoli HMGs possessed DNA-binding activity, and in particular, whether such binding would show any preference for AT-rich DNA. For this purpose, we performed a mobility shift assay using an AT-rich fragment of the rbcS-3.6 pea promoter (Datta and Cashmore, 1989) in the presence of various competitor DNAs. When identical volumes (4 μL/assay) of fractions eluted from DEAM HPLC were used in the binding assay, formation of several retarded protein-DNA complexes was observed throughout the elution profile.

The amount of probe retardation increased in positive correlation with the amount of protein in the fractions tested, mirroring the shape of the HPLC elution pattern (compare Fig. 3, A, B, and C). The fractions corresponding to the protein peak also formed the slowest-migrating complexes, whereas shoulder fractions showed a discrete complex of higher mobility (Fig. 3C).

Such multiple complexes do not necessarily correspond to separation of various DNA-binding proteins, since formation of a series of slower-migrating complexes with increasing amounts of protein is characteristic of multiple HMG molecules binding to single DNA targets (Pedersen et al., 1991). To confirm that this is indeed the case, we performed the mobility shift assay with increasing amounts of protein from individual HPLC fractions. When low amounts of protein (<1 μg) were added, only a high-mobility retarded band was observed (Fig. 6A, lane 2), but multiple lower-mobility bands were formed with higher amounts of protein (Fig. 6A, lanes 3 and 4).

To determine possible binding preference for AT-rich sequences, we investigated the effect of increasing amounts of AT-specific and nonspecific competitors on the formation of retarded protein-DNA complexes. When increasing amounts of poly(dA-dT) and poly(dl-dC) were used as the respective competitors, formation of the retarded protein-DNA complexes was abolished. This effect was again gradual: slow-migrating complexes disappeared first at low concentrations of competitor DNA and were followed by decreasing retar-
Table I. Amino acid composition of broccoli HMCs compared to those from other plants

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* Amino acid determination, this work.  
* From cDNA sequence, modified after Grasser et al. (1991).  
* Calculated from cDNA sequence retrieved from GenBank, Laux (1991).  

Figure 4. Immunological cross-reaction of broccoli CKII substrates with antisera against wheat HMGa (A) and HMGd (B). Western blots shown in both panels contained positive control of wheat HMGs in lanes 1 (the positions of migration of wheat HMGs are indicated by a, b, c, and d) and broccoli phenyl-Sepharose flow-through pools in lanes 2, and were probed in A with a 1:1000 dilution of the antiserum against wheat HMGa and in B with a 1:100 dilution of the antiserum against wheat HMGd. The positions of prestained molecular mass markers are given in kD.

DISCUSSION

Our work on identification of the endogenous substrates in plant CKII preparations brings together several lines of investigation from different laboratories. Dobrowolska et al. (1987) observed during purification of maize seedling CKII a double band of very efficient endogenous substrates of 16.5 and 19 kD. That study was the first to demonstrate in plants the co-existence of oligomeric and monomeric forms of CKII-like activities; it was also the monomeric fraction that contained these substrates. HMGs are very persistent contaminants in the preparations of this unique CKII-like activity, and by competing with other substrates they seriously interfere with its characterization. The identification and the procedures for separating these overpowering substrates described in this paper will help in the study of monomeric CKII-like activity. The existence of this abundant activity in plants will facilitate the study of CKII in vivo. 

Identification of the fast-migrating discrete band (Fig. 6B). Since identical levels of competition were observed for the specific and nonspecific competitors, we conclude that the broccoli HMGs possess general DNA-binding activity with no preference for AT-rich sequences.

No differences were observed in DNA-binding activity between untreated HMGs and HMGs phosphorylated by either the monomeric or oligomeric form of CKII (data not shown).

No differences were observed in DNA-binding activity between untreated HMGs and HMGs phosphorylated by either the monomeric or oligomeric form of CKII (data not shown).
animal and yeast systems almost exclusively in the oligomeric \(\alpha_2\beta_2\) form (Tuazon and Traugh, 1991). Further studies of the monomeric activity should contribute not only to the understanding of the role of CKII in plants, but also to the understanding of the evolution of CKII functions among eukaryotes. Especially intriguing are the biochemical differences observed between the monomeric CKII-like activity isolated from plant material and the recombinant plant CKII \(\alpha\) subunit (catalytic monomer) expressed in bacteria (Boldyreff et al., 1993; L.J. Klimczak, unpublished data), which suggest that either separate genes or extensive posttranslational modifications are involved.

Plant HMGs were explicitly studied in several laboratories as substrates for phosphorylation and with regard to their DNA-binding properties. Phosphorylation of two plant HMGs isolated from maize nuclei was observed by Crasser et al. (1989) and a double band of about 20 kD was shown to bind to a radiolabeled fragment of a promoter (Crasser et al., 1990). Jacobsen et al. (1990) found two proteins of 21 and 23 kD in leaf nuclear extracts from soybean that preferentially bound to AT-rich motifs in soybean promoters. Pedersen et al. (1991) extensively characterized the DNA-binding properties of wheat HMGs and their preference for AT-rich tracts. Using purified preparations of HMG subgroups, they showed that AT-specific binding was observed with HMGa or HMGb but not with HMGc or HMGd. Czarnecka et al. (1992) identified in soybean nuclear extracts HMG proteins of more distinct molecular masses (23 and 32 kD) by binding in southwestern blots to AT-rich sequences.

The general consensus that emerges from those studies and ours is that a particular class of HMGs from various plant species may be purified as two bands closely migrating at about 20 kD and that these proteins are very efficiently phosphorylated by CKII. Their positions of migration in SDS-PAGE provide a recognizing feature but are not adequate as determinations of molecular mass.

strated by Spiker (1984), the molecular masses derived for plant HMGs from calibration of SDS-PAGE gels with conventional protein standards represent overestimates. We confirm this by direct determination of molecular masses of broccoli HMGs by MALDI/MS.

With regard to the specificity of DNA binding, our data are in full agreement with the work of Pedersen et al. (1991): the broccoli HMGs cross-react with wheat anti-HMGd antibody and, as the HMGd subgroup, possess only nonspecific DNA-binding activity with no preferential binding to AT-rich sequences. Therefore, we have designated the two major bands of broccoli HMG visible in SDS-PAGE HMGc (20 kD) and HMGd (18.7 kD), with the understanding that they still represent microheterogeneous populations. We note, however, that the co-migrating broccoli and wheat HMGs do not have to be most closely related: the upper broccoli HMG band (HMGc) cross-reacts slightly more strongly with the anti-wheat HMGd antibody than the lower broccoli band (HMGd), which co-migrates with wheat HMGd. The immunological cross-reaction patterns between HMGs from different plant extracts appear to be more complex than what would be needed to indicate a simple correspondence of co-migrating bands, as already shown by Moehs et al. (1988) for wheat and Arabidopsis (see figure 6 therein).

Interestingly, the properties of the broccoli HMGc and HMGd proteins resemble very much those of maize HMGs described by Grasser et al. (1990). Although the two major HMGs described in that work are later referred to as HMGa and HMGb (Grasser et al., 1991), no data about their equivalence (for example, immunological cross-reaction) to the eponymous wheat HMGs has been published so far. Wheat HMGa stands out among both animal HMGs (see table I in Spiker, 1984) and plant HMGs (Table I) for its unusually low

![Figure 5](image)

**Figure 5.** MALDI/MS spectrum of the phenyl-Sepharose flow-through pool. The abscissa axis shows mass-to-charge ratio in D, and the ordinate axis shows relative signal intensity.

![Figure 6](image)

**Figure 6.** DNA-binding properties of broccoli HMGs. Mobility shift assay was performed with a radiolabeled AT-rich DNA probe. A, Effect of increasing amounts of protein (HPLC peak fraction 104) in the presence of 0.25 \(\mu\)g of poly(dl-dC) as competitor. Lane 1, 0.1 \(\mu\)g; lane 2, 0.25 \(\mu\)g; lane 3, 0.5 \(\mu\)g; lane 4, 1 \(\mu\)g. B, Effects of increasing amounts of competitors. Lanes 1 to 4, Poly(dA-dT), lanes 5 to 8, poly(dl-dC). All lanes show the binding of a constant amount of protein (1 \(\mu\)g, same as in A, lane 4). Lanes 1 and 5, 0.05 \(\mu\)g; lanes 2 and 6, 0.1 \(\mu\)g; lanes 3 and 7, 0.5 \(\mu\)g; lanes 4 and 8, 1 \(\mu\)g of
content of Asx + Glx, and among plant HMGs for its much higher content of Pro (these are at least 2-fold differences in molar percentage, much higher than the level of variation observed commonly). This composition resembles that of a soybean SB16A cDNA clone (Laux et al., 1991), which lacks the C-terminal acidic domain but contains the Pro-rich AT-hook repeats characteristic of the HMG-I class (Reeves and Nissen, 1990). Thus, wheat HMGa appears to be the most likely candidate for an HMG-I homolog.

This difference in amino acid composition (low Asx + Glx, high Pro) also sets wheat HMGa apart from the cloned maize HMG homolog (Grasser and Feix, 1991), which was isolated using an antibody to the preparation of maize HMGs described by Grasser et al. (1990). That maize HMG gene is homologous to animal HMG-1/2 class, which does not show preference for AT-rich DNA. Although partially purified preparations containing maize HMGa were originally observed to bind to AT-rich DNA probes (Grasser et al., 1990), this binding was competed out by low amounts of both poly(dA-dT) and poly(dI-dC) (0.5 μg DNA/μg HMG, in contrast to 12.5 μg used by Pedersen et al. [1991]) and appears to represent DNA binding not specific for AT-rich DNA (for further discussion of that work see Pedersen et al. [1991]), as does that of broccoli HMGc and HMGd. In addition, the amino acid composition predicted from the cDNA sequence of the maize clone matches closely that of broccoli HMGc and HMGd (Table I). Therefore, we tentatively conclude that plant equivalents of animal HMG-1 subgroup could include, in addition to maize HMGa, wheat and broccoli HMGc/ HMGd.

We postulate that yet other HMG proteins should be present in maize and broccoli, which would correspond to wheat HMGa by preferential binding to AT-rich DNA and by the distinct amino acid composition, and that they would be homologous to the HMG-I subgroup and likely would be encoded by a gene such as SB16 (Laux et al., 1991).

An interesting feature of the isolated broccoli HMGs is the fact that they do not require salt extraction for solubilization but are present in the cytosolic fraction. Although HMGs were traditionally defined as a fraction extractable from chromatin by 0.35 M NaCl, it became apparent that significant amounts are also present in the cytosol. In particular, the HMG-1/2 subgroup was shown to be distributed between the nucleus and the cytoplasm (Bustin and Neihart, 1979), and the distribution was dependent on the stage of the cell cycle: during the S phase they accumulated in the nucleus and during the rest of the cycle they migrated to the cytoplasm (Einck and Bustin, 1985; Bustin et al., 1992). This distribution was also affected by development (Mosevitsky et al., 1989). Thus, the presence of broccoli HMGc and HMGd in the soluble fraction is yet another similarity to the HMG-1 group. Since cells at various stages of the cell cycle are processed in the biochemical preparation of broccoli, the isolated soluble HMGs may originate from that particular stage at which they are present in the cytoplasm. It is still possible, though, that they may also constitute a fraction that is loosely associated with the structure of chromatin (for instance, nucleoplasm) or that they become dissociated during homogenization through interaction with some released plant compounds. The matter of the intracellular localization of this particular class of HMGs, as well as of plant HMGs in general, needs to be addressed in separate studies.

Jacobsen et al. (1990) claimed that two proteins (LAT) of 21 and 23 kD identified in leaf nuclear extracts from soybean possess a functional relationship to the HMG-I group. These studies were performed using AT-rich DNA probes, but again in the presence of low amounts (0.4 μg) of nonspecific competitor. Although no competition data were shown, the authors stated that 10-fold higher excess of poly(dl-dC) than of poly(dA-dT) was necessary to out-compete the binding of the HMG-like proteins (Jacobsen et al., 1990). On the other hand, Czarnecka et al. (1992) identified two AT-binding HMG proteins in nuclear extracts from soybean: two single bands of 23 and 32 kD. Considering the co-purification of the LAT proteins as a characteristic doublet migrating at about 20 kD (Jacobsen et al., 1990), it will be very interesting to clarify their relationship to broccoli HMGc and HMGd; for this purpose, it may be necessary to study their AT specificity in more detail.

In addition to low molecular mass proteins of the HMG type, plant nuclear extracts contain distinct non-HMG DNA-binding proteins with preferences for AT-rich regions (referred to as AT-binding proteins; Czarnecka et al., 1992). The complexes formed by such proteins with the target DNA probes migrate slower than those formed by HMCs. Consequently, in unfractionated nuclear extracts two major retarded complexes of both higher and lower mobility are formed. Such complexes were described as b1 and b2 in tobacco (Schindler and Cashmore, 1990), LAT1/NAT1 and NAT2 in soybean (Jacobsen et al., 1990), BC1 and BC2 in pea (Pedersen et al., 1991), and H and L again in soybean (Czarnecka et al., 1992), respectively. We have observed analogous low-mobility complexes only when using nuclear extracts from broccoli, and, similar to results in those other studies, they were formed only by the non-HMG fraction (L.J. Klimczak, unpublished data). Since those non-HMG factors and their relationship to bona fide HMGs were discussed extensively recently (Czarnecka et al., 1992), we will not reiterate their characteristics here.

HMGs are isolated as phosphoproteins and their phosphorylation has been studied both in vitro and in vivo (for an extensive list of citations, see Ferranti et al. [1992]). Most of the phosphorylation studies focused on the HMG-I subclass, which was found to be phosphorylated by p34cdc2 and CKII (Nissen et al., 1991; Ferranti et al., 1992). The precise role of phosphorylation of this subclass is not yet clear, nor is the role of less-studied phosphorylation of HMG-1, which is less efficiently labeled in vivo (Kimura et al., 1985; Elton and Reeves, 1986). Our data on phosphorylation of broccoli HMGc and HMGd by CKII do not show any effects on DNA-binding activity, similar to the observations made for maize HMGa (Grasser et al., 1990), but more research is required to address its functional relevance.

Further work on plant HMGs, including the broccoli HMGs, is still required to resolve some ambiguities. First, HMG proteins isolated from plant material have to be more extensively cross-identified between different species (using, for instance, the Spiker [1984] classification as a reference point) and unequivocally assigned to cloned cDNAs, either those already available or others to be isolated in less random
and more protein sequence-directed approaches. Higher-resolution fractionation methods have to be used to achieve separation and classification of microheterogeneous forms, similar to those established for animal HMGs (Giancotti et al., 1991). Second, the DNA-binding properties of the proteins encoded by the isolated clones will have to be compared with those of plant HMGs by expressing the protein products of these clones. Individual plant HMG proteins expressed in bacteria will also provide useful material for the in-depth study of the effects of phosphorylation.

As the complexity of plant HMGs becomes unraveled, it can be expected that their unique properties will offer interesting insights into how structural components of chromatin affect gene expression.

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LITERATURE CITED

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