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SHORT COMMUNICATION

Specific interaction of plant HMG-like proteins with cruciform DNA

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Abstract

Proteins which, on the basis of their solubility in 0.35% NaCl–2% TCA and of their electrophoretic mobility, correspond to animal HMG 1/2 family were isolated from nuclei of ungerminated pea embryos. These proteins ound with a high degree of specificity to synthetic cruciform DNA produced by annealing chemically synthesized oligonucleotides. Hence, specific binding to four-way junction DNA, previously reported for animal HMG 1 and 2 proteins proved also to be a property of plant HMG 1/2 family, in spite of their low homology to the animal ones.

Key words: *Pisum sativum*, chromosomal proteins, cruciform DNA, high mobility group proteins.

Introduction

High mobility group (HMG) proteins are a ubiquitous class of non-histone chromosomal proteins whose biological role, despite intriguing speculations, is, so far, not yet elucidated. Since there is no biological assay for HMG function, these proteins are operationally defined as nuclear proteins which can be extracted from nuclei or purified chromatin by 0.35 M NaCl and are soluble in 2% TCA. Additional characteristics can be a high content of charged amino acids and a molecular weight lower than 30 000.

The HMG 1/2 family (molecular weight of about 25 000) is the most abundant HMG family. HMG 1 and 2 extracted from mammal tissues have a tripartite structure consisting of two internal repeats of a positively charged domain of about 80 residues and a polyacidic C terminal domain. Each of the positively charged internal repeats have been called an HMG box and represent the DNA binding motif of the protein.

Bianchi and collaborators (Bianchi, 1988; Bianchi *et al.*, 1989, 1992) have shown that both the whole HMG 1 protein and HMG box 1 and 2, which have been cloned separately, interact specifically with four-way junction DNA, i.e. structures which are cross-shaped and contain angles of about 60° and 120° between their arms. Four-way junctions or 'cruciform DNA' arise *in vivo* as transient Hollyday junction during genetic recombination or from inverted repeat sequences under the effect of supercoiling. Both transcription and DNA replication, in which functions HMG proteins are generally thought to be involved, can generate branched DNA molecules either directly or through the action of induced supercoiling (Bianchi *et al.*, 1989; Ull *et al.*, 1991). The binding to four-way junctions is structure-specific and sequence-independent, since HMG boxes can bind to several four-way junction DNA of unrelated sequences, but not to linear duplex or single strand DNA of the same sequences (Bianchi *et al.*, 1992).

Studies on plant HMGs are more recent and far less numerous, but plant HMG-like proteins have been isolated and biochemically characterized from different sources (Spiker, 1984; Vincentz and Gigot, 1985; Moehs *et al.*, 1988; Grasser *et al.*, 1989, 1993; Ull *et al.*, 1991). However, overall amino acid composition, peptide mapping, partial sequence analysis and immunological studies suggest a substantial difference between plant and animal HMG proteins. For instance, in contrast to the animal proteins which contain two HMG boxes, maize HMG 1 protein (whose amino acid sequence was deduced from the correspondent cloned cDNA insert) displays one such element alone, which is more similar (42% identity) to the animal HMG box 2 than to the amino-terminal

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HMG box 1, while only a low similarity of the amino-terminal region of the maize HMG protein to HMG box 1 of the vertebrate proteins could be observed (Grasser and Feix, 1991). Despite little evidence of homology between animal and plant HMG proteins, the latter share the characteristics of vertebrate HMG proteins of being released from chromatin that has been treated with DNAase 1 and are found in chromatin fractions containing DNA complementary to mRNA (Spiker, 1988), both being properties related to structural proteins of transcriptionally poised chromatin.

Since the interaction with distorted DNA—preferentially they seem to recognize sharp angles—is the general property of HMG boxes, most probably central to their biological function, we decided to test whether plant nuclear proteins, operationally corresponding to HMG proteins, bind specifically to four-way junction DNA, like their animal counterpart. In this preliminary report we show that such proteins exist and, although their conclusive identification must be based on their amino-acidic sequence, their binding specificity to cruciform DNA could be used as an additional diagnostic criterion to assign them to the HMG class.

Materials and methods

Plant material

Pisum sativum cv. Lincoln was used for all the experiments. Seeds were treated for 10 min with NaClO 6% at 4 °C, washed in cold water, and soaked at 2–4 °C for 16 h. Embryos were manually removed and left in glycerol 30% at −10 °C until used.

Isolation of nuclei

A fraction of pure nuclei was obtained according to the procedure of Chiatante *et al.* (1990) partially modified to fit for a large quantity of starting material. The purity of nuclei was checked after staining with DAPI and direct observation at a fluorescence microscope (excitation filter BP 340–380, barrier filter LP 430) (data not shown).

Isolation of HMG proteins

Putative HMG proteins were extracted from gradient-purified nuclei with 0.35 M NaCl and 2% TCA, mostly according to one of the procedures suggested by Ull *et al.* (1991).

Polyacrylamide gel electrophoresis

Electrophoresis of proteins was performed in 15% polyacrylamide gels using the Tris/glycine/SDS system, according to Schagger and von Jagow (1987). The gels were then stained with Coomassie R-250.

Construction of synthetic junction and linear DNA

Oligonucleotides were synthesized by the phosphotriester method and purified by HPLC. Duplex and four-way junction DNA molecules were obtained by annealing the appropriate oligonucleotides and purified by electrophoresis. When desired one of the strands was labelled by treating with the Klenow fragment of DNA polymerase I and (α ^{32}P) dCTP. Details of the preparation and the characteristics of the synthetic strands have been described (Bianchi, 1988, 1992).

Assay for DNA binding to cruciform DNA

For DNA binding experiments total extracts containing HMG proteins solubilized in non-denaturing medium, or single bands were used. In the latter case, the bands putatively corresponding to HMG 1 and HMG 2 proteins were visualized in SDS/polyacrylamide gels by treatment with 1 M KCl for 30 min, excised from the gel, homogenized in non-denaturating buffer at 60 °C and renatured by gel filtration on P6 resin (Bianchi *et al.*, 1989).

In band-shift experiments, total extracts or the recovered HMG 1-like and HMG 2-like proteins were added to labelled cruciform DNA (1.5 nM) in DNA binding buffer (8% Ficoll, 200 mM NaCl, 10 mM $MgCl_2$, 10 mM Hepes pH 7.9, 5 mM KCl, 1 mM EDTA, 0.5 mM DTT). After incubation for 10 min on ice, samples were applied to a 6.5 polyacrylamide gel in 0.5 M TBE buffer and run for 4 h at 10 V cm^{-1} at room temperature. The gel was then dried, autoradiographed with Kodak X-AR film and exposed for 24 h at −80 °C with intensifying screen.

In competition experiments: (a) linear double strand DNA molecules of the same sequence as the oligonucleotides used in forming the cruciform structures, and (b) unlabelled cruciform DNA, were also added to the essay mixtures. The concentrations of competitors used are indicated in Fig. 4.

Results and discussion

When studying nuclear proteins a crucial step is to start from a highly purified fraction of nuclei. The fraction of nuclei we obtained was completely satisfying, so to rule out the possibility of contamination by cytoplasmic proteins. Yet, a strong contamination by an unknown nuclear protein could not be avoided (Fig. 1). According to most authors, at least one of the two contaminating bands should correspond to histone H1 (Moehs *et al.*, 1988; Ull *et al.*, 1991). Three weaker bands of molecular weight about 28 000, 24 000, 21 000, as estimated by standard molecular weight markers, were always found. We tried to prove that these three bands were indeed correspondent to vertebrate HMG proteins, but, unfortunately, antibod-

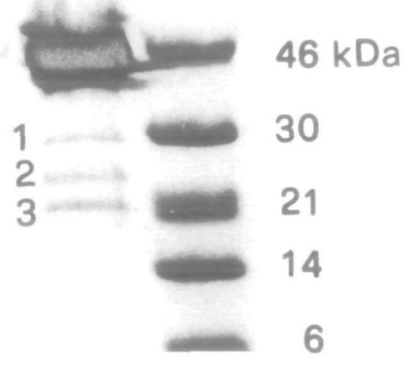

Fig. 1. SDS–PAGE of putative HMG proteins from pea embryos (1, 2, 3 in the left lane). The heavy band with lower mobility corresponds to the contaminating H1 histone. Right lane: molecular weight markers.

ies against rat HMG 1 protein reacted non-specifically with the pea proteins; on the other hand, this is consistent with the low homology of plant HMG proteins to their animal counterpart. However, though our presumed HMG proteins have not been sequenced, their specific solubility in 0.35 M NaCl and 2% TCA and their molecular weight fit well with the putative HMG proteins isolated by Ull *et al.* (1991) from the same material. We believe that band 1 and 2 might correspond to animal HMG 1 and 2.

DNA binding assays were carried out using total extract containing HMGs resuspended in non-denaturing buffer; HMG 1-like and HMG 2-like bands excised from gel slices and renatured by gel filtration on P6 resin. Each of them was added to labelled cruciform DNA (Fig. 2) under the conditions described in Materials and methods. In all three cases the binding of the proteins caused a reduction of the electrophoretic mobility of the synthetic cruciform DNA, shown by a distinct mobility shift indicative of the formation of a protein/DNA complex (Fig. 3). The protein extracted from band 2 (lane 4 in Fig. 3) seemed to be less active and gave comparable but less clear-cut results. When other unknown nuclear proteins (bands excised from gels of total nuclear proteins) were tested at random in similar binding assays experiments, no shift of labelled cruciform DNA was ever observed (data not shown).

The cruciform-binding activity of pea HMG-like proteins is highly specific for DNA structure rather than for DNA sequence. This was shown in competition experiments where HMG 1-like protein (extracted and renatured from band 1) readily bound to the synthetic cruciform DNA, but did not bind to control linear DNA of the same sequence (Fig. 4A). Binding of HMG 1 was not even competed by a 1000-fold higher concentration of linear DNA, but was competed, as expected, by cold four-way junction DNA (Fig. 4B). Quite similar results have been previously reported for animal HMG proteins (Bianchi *et al.*, 1989; Bianchi, 1991).

There is increasing evidence that the consensus DNA structure for HMG 1 box binding is a local deformation of the DNA helix at the site of interaction. The interaction with distorted DNA, a general property of HMG boxes, must be crucial for their biological function. Deformations along the DNA molecule can be required for DNA transcription, replication, repair, and packaging, so that HMG may have a generalized role in DNA bending, looping, folding and wrapping, as proposed by Ferrari *et al.* (1992).

Moreover, evidence is emerging that HMG 1-related proteins can induce bends in linear DNA containing the central dinucleotides CA or TG (Landsmann and Bustin, 1993). So these proteins deform the DNA but also recognize bent DNA; as a matter of fact, the preferential binding to DNA in an already bent configuration might reflect a decreased energy requirement in the association step.

The present report, showing that HMG proteins from plants, like the animal ones, bind with high specificity to cruciform DNA is in agreement with the recent works of Kotani *et al.* (1993) reporting similar properties for an

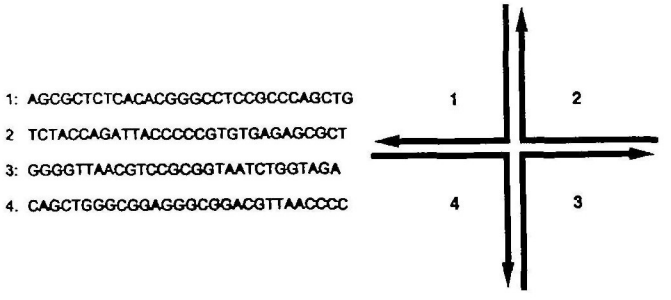

Fig. 2. Design of the synthetic cruciform DNA used.

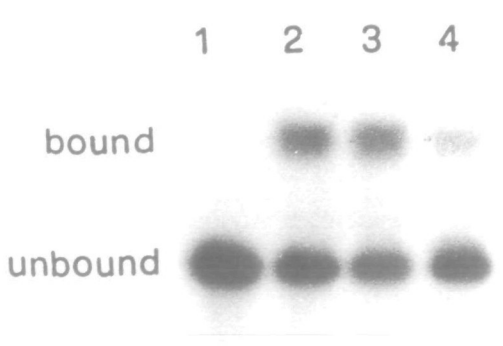

Fig. 3. Cruciform DNA-binding activity as revealed by gel-retardation assay. Reaction mixtures contained ^{32}P-labelled cruciform DNA (1.5 nM) alone (lane 1), or added to total extract containing HMGs (lane 2), protein 1 (lane 3), protein 2 (lane 4) recovered from gel slice and renatured. The shift is evident for total HMGs (lane 2) and for HMG 1-like band (lane 3), less marked for HMG 2-like band (lane 4)

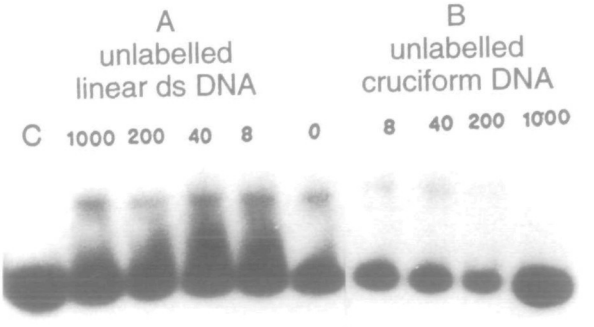

Fig. 4. Gel retardation assay to verify the binding specifity of HMG-like proteins to cruciform DNA. ^{32}P-labelled cruciform DNA was incubated in the presence of pea HMG 1-like protein, and of nanomolar amounts (1000, 200, 40, 8, 0) of unlabelled unspecific linear duplexes (A), and specific competitor (unlabelled cruciform DNA) (B) containing the same sequences. Extreme left lane: C = control (labelled cruciform DNA alone).

HMG-like protein from *Ustilago maydis* and of Griess *et al.* (1993) about the interaction of maize HMG proteins with the rDNA external spacer region exhibiting DNA curvature. These findings, as a whole, add further evidence to the evolutionary conservation of the properties of HMG box all over the eukaryotic kingdoms, implying that it constitutes an essential module for the correct structure and/or function of DNA.

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